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OLD HERBORN UNIVERSITY SEMINAR ON PROBIOTICS: BACTERIA AND BACTERIAL FRAGMENTS AS IMMUNOMODULATORY AGENTS

MINUTES AND OVERVIEW OF THE DISCUSSIONS

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Charles L. Bevins:

Four potential roles for human defensins can be recognised:

1. Protection of the stem cells located just distal of the Paneth cells in the crypts of Lieberkühn in the small intestines
2. Microbes ingested with food and water may be confronted with a hostile environment created by defensins
3. Defensins may shape the intestinal microflora as they have a broad spectrum antimicrobial activity while some bacteria are more susceptible than others
4. The colon is more heavily colonised than the small intestines, which may be partly due to activity of defensins.

In addition to antimicrobial activity, defensins may have a signal function to the adrenals where they act as 'corticostatins' as they down regulate corticosteroid production. Furthermore, defensins are chemo-attractants to neutrophils and finally inside the crypts defensins would stimulate Paneth cells.

- Paneth cells are already during pregnancy present in the gut of the human foetus and thus at birth. Activity, however, is lower than at birth. In mice Paneth cell activity is turned on much later after birth namely at the time of weaning to become of adult type at six weeks of age.
- It is not yet known which bacteria in particular stimulate Paneth cells in particular; perhaps *Bacteroides* species are good candidates.
- Breast milk may have a stimulatory effect on Paneth cell function; however, this is still uncertain.
- Many human cancer cell types are sensitive to defensins, which may physiologically originate from neutrophils.
- Paneth cells develop/differentiate from crypt stem cells which to this end migrate downward and have a lifespan of about four weeks.
- Defensins are produced as double molecules which are later clipped by a trypsin which is simultaneously produced by Paneth cells.

Note: *Shigella* species seem to be able to shut down Paneth cell activity (by blocking or enzymatic breakdown).

Necrotising enterocolitis has been described in patients with a Paneth cell deficiency.

In human neutrophils, quite a number of defensins have been found but not in the mouse. Deficiencies but also

the opposite excess of defensins have been described in plants. Deficiencies exist also in man but the genome is not yet determined; in knock out mice, perhaps more than one genome is involved in Paneth cell activity.

Germain Trugnan:

The glycosylation is being studied *in vitro* and *in vivo* as one of the targets of resident bacteria in human intestinal cells. Glycosylation may play several roles in the gut such as modulating interactions with pathogens, changing cell proliferation and differentiation. Glycosylation changes may represent the mechanism by which resident bacteria from the gut may influence gut functions through a remote control process. The works presented demonstrated that *Bacteroides* species release soluble factors that specifically modulate galactosylation, *in vitro*. *In vivo* studies confirmed that a soluble factor of *Bacteroides* modulates galactosylation but also shown that sialylation and fucosylation of proteins and lipids were modified at the surface of the host cell. Small molecules of *Bacteroides* have been isolated and shown to carry the glycosylation modifying activity. In vitro data shown that increasing the surface galactosylation by the soluble factor of *Bacteroides* cause the cells to resist rotavirus infection.

Note: The signalling molecules can pass the mucus layer.

Lars Å. Hanson:

The human new-born has a small immune system. Therefore, it is amazing that it can manage the influx of bacteria from the outside world upon birth and keep it under control. In newborns, Th1 precursor cells function normally, there is a deficiency in neutrophil and monocyte function. This implies that although the new-born has to a certain extend a deficient innate immune system and a marginally functioning adaptive immune system, yet the new-born can control the microbial attack from the outside world. However the help from the mother via transplacental IgG antibodies and milk secretory IgA antibodies provides important support.

In the mother, microbes that get in contact with Peyer's patches in the gut, (may reach mesenteric lymph nodes and spleen and) stimulate specific secretory IgA production. Lymphocytes producing such secretory IgA also migrate to (reaches) the mammary glands via (with) the blood stream. Once in the mammary glands they produce the milk antibodies against the mother's intestinal flora. However, quite a number of neonates are raised successfully without breastfeeding, although their risk of repeated even lethal infections is considerably higher. How babies (manage) expand their immune system to take over the role of breastfeeding is still not fully known.

Important monitoring factors during pregnancy are the cytokines stemming from the mother's immune response to the paternal structures of the foetus. The mother's milk is also full of full of signals. This includes among else cytokines which affect (stimulate) insulin production, fatty acid metabolism, the central nerves system and of course lymphocytes in the neonate.

Mycobacteria (and endotoxines) are microbial stimulants, which play a crucial role as stimuli of the development of the immune system as has been found in

experimental animals.

In mothers suffering from *Myasthenia gravis* and in *Lupus erythematosis*, evidence has been found that their babies produce the same auto-antibodies. This is possible due to transplacental passage of anti-idiotypic antibodies which relate to the disease and which turn on a secondary response in the neonates.

Presumably, defensins are not playing an essential role in the shaping of the microflora since baby mice do not have defensins in the gut and yet develop their microflora much more gradually than occurs in man.

Tolerance in human babies develops almost directly after birth. This could be an important factor in the acceptance of the developing intestinal microflora. This capacity to tolerance is to avoid immunological reactivity to food proteins (either the cow's milk proteins in the milk-drinking mother's own milk or in case the neonate is given formula). It is safer to provide host defence against the microbes.

Note: Microbial signals coming from the skin and the gut, may reach the nervus vagus along peripheral receptors and thus reach the brain area to influence various brain functions.

Bengt Björkstén:

In several studies, differences in the composition of the intestinal microflora have been described between allergic and non-allergic individuals. Consistent in these studies was differences between and numbers of *Clostridium* and *Bifidobacteria* species. It is unknown whether this is the cause or an epi-phenomenon.

In infants with skin allergies, improvement has been claimed upon treatment with "Lactobacillus GG". However, the study was small and the infants were only studied for one month. The immunological implications of this kind of treatment are at present being studied.

Administration of antibiotics early in life and even during pregnancy, may play a role in development of allergy. Furthermore, the composition of the maternal vaginal microflora seems to be related to bronchial obstruction in four-year old children.

Prematurity as well as low birth weight is inversely related to development of atopy. High birth weight on the other hand may enhance the risk for atopy.

Breastfeeding appears to beneficially limit "infant wheezing" but not respiratory tract allergies like 'asthma'. At this point, there may be confusion about the term asthma. For example until some ten years ago, in Germany asthma was considered non-existing because a different definition of asthma was used.

Fatty acids in the diet would enhance cytokine production in the gut and therewith influence the risk for development of an allergy. However, this is till uncertain and certainly not a major factor. The intestinal microflora may modify long chain fatty acids, which may play a role in allergy.

In a recent study published in the Lancet, the possibility to prevent allergy was assessed in infants who were followed over the first two years of life. In the breast-fed group, the mother was treated with lactobacillus GG during and after birth; in the bottle-fed group lactobacillus GG was administered to the child (it was then no longer given to the mother). The authors claimed that this treatment in both groups would be comparable. In the groups with 'Lactobacillus GG' treatment, a 50% reduction

in atopy was found. It is unlikely that, if the conclusion is correct and both treatment groups were comparable, that the outcome would be confined to "Lactobacillus GG". It is considered likely that *L. reuteri* and *L. plantarum* would have a similar effect as "Lactobacillus GG". *L. plantarum* would adhere better to mucosal cells than *L. reuteri*.

For proper study designs, all children with an atopic disease should be sampled for a year and be followed up for two years. This is however very expensive in a double blind placebo controlled study design. In Cologne, Joseph Beuth is working on a computer model and is of the opinion that such an "expert model" would make an expensive study, as described above, unnecessary.

Rudolf Kunze:

In a pilot study the effect of auto-vaccines, (made from killed *E. coli* isolates from the patients included in the study) was studied. Blood samples were taken at 1 and 3 weeks after subcutaneous auto-vaccine administration in inflammatory bowel disease inflammatory bowel disease (IBD) patients. In the blood samples the concentration of IFN-α and GM-CSF (granulocyte/monocyte colony stimulating factor) were determined. It was found that both were down-regulated by auto-vaccine treatment. The question arose whether auto-*E. coli* would work better than a vaccine prepared from any *E. coli* strain and it was suggested that a non-vaccinated control group would be of help in subsequent studies.

Graham A.W. Rook:

Advocates of the "hygiene hypothesis" have suggested in the past that the striking increase in the incidence of allergies in the clean rich countries might be due to lifestyle changes leading to insufficient activation of Th1 (T helper 1) cells. In the absence of sufficient Th1 cell activity, there might be an increase in the activity of Th2 (T helper 2 cells) that mediate allergies. However this view of the hygiene hypothesis must now be changed. There is a simultaneous rise in the incidence of diseases mediated by Th1 cells, such as autoimmune type 1 diabetes and multiple sclerosis. Similarly there is also a rise in inflammatory bowel diseases (IBD: ulcerative colitis and Crohn's disease). This simultaneous rise in allergies (Th2), autoimmunity (Th1) and IBD (mixed Th pattern) implies that the problem lies *not* in Th1/Th2 balance, but in the balance of regulatory T cells (such as TR1 and Th3) to effector cells (Th1 and Th2). It is also possible that changes in the pattern of disorders of mood and behaviour (such as autism, depression, chronic fatigue syndrome) are also linked to the changes in cytokine balance that result from this disturbed ratio of regulatory to effector T cells. Recent epidemiological studies show links between some of these disorders and allergies or IBD, and cytokines such as Interferon-α and IL-1 and IL-2 have profound effects on mood.

It was suggested that a decreased exposure to mycobacteria in the modern lifestyle might be one factor because these organisms have been shown to evoke not only Th1 effector cells, but also, more importantly, IL-10-secreting regulatory T cells that can treat allergic manifestations. Thus mycobacteria from the environment, and probably other genera such as *Lactobacilli* found in the

bowel flora, may play a crucial role as "regulatory cell adjuvants", that are an evolutionarily determined necessity.

Kurt Zimmermann:

The central question discussed in view of the proposed health claims proposed for probiotics was whether live or dead bacteria were equally effective in modulating some important immune functions associated with improvement of diseases. Up to now, among functional foods, the community of probiotic bacteria has become increasingly important under scientific as well as under commercial aspects in the past decade.

It is generally accepted from the actual definition of probiotics, that these products should contain predominantly living microorganisms. Among the numerous strains marketed as probiotics, some „dominant" probiotic strains have emerged such as *Lactobacillus rhamnosus* GG, *Lactobacillus johnsonii* La1 and *Bifidobacterium lactis* Bb12, for which a substantial amount of literature claimed several important health effects. The majority of "health promoting effects" include, as yet mostly still unproven functions such as a "general improvement of well being". Also clear medically relevant effects have been reported in several recent publications like beneficial effects of probiotics in inflammatory bowel disease, allergy, cancer or prevention of intestinal tract diarrhoea as well as general GI infection. However, these probiotic-mediated effects in these serious medical indications remain to be proven with well-controlled clinical studies and the immunological mechanisms underlying the sometimes-observed clinical efficacy await further clarification

Assuming that a considerably important part in health promoting effects may result from interactions of the bacteria with the human immune system, it is still not yet clear which molecular mechanism(s) may underlie these interactions particular in view of the viability of the bacterial strains. It was discussed that interacting with different subtypes of TLRs, LPS or Lipoteichoic acids from Gram-negative and Gram-positive bacteria may induce different immunomodulatory functions. However, with regard to preventing infections in the GI tract, live bacteria may considered to be more effective than dead ones. But the different bacterial cell wall compounds, characteristic for Gram-negative and Gram-positive bacteria, may be in some kind as effective as live microorganisms in terms of cytokine induction.

This was reported during the meeting by experimental data to occur on the cytokine modulating capacity of a heat-inactivated bacterial preparation named Pro-Symbioflor® which contains a mixture of Gram-negative and Gram-positive human non-pathogenic strains, *E. coli* and *Enterococcus faecalis*, respectively. This preparation could induce a predominant TH_1-immune response in an *in vitro* model in blood. It was tentatively concluded that use of this probiotic preparation to counteract the overactivation of TH_2-lymphocytes seen in atopic allergy.

Note: The capacity of Pro-Symbioflor® to upregulate IL-12, IFN-γ and the immunoregulatory cytokine IL-10 *in vitro* contrasts some recent literature findings that Gram-negative and Gram-positive bacteria would induce preferentially cytokines like IL-12 and IL-10. Furthermore, reports dealing with the cytokine inducing capacity dependent on bacterial growth phase and their heat treatment obviously cannot be applied to Pro-Symbioflor®. This

makes likely, that important qualitative and quantitative differences exist among medical-probiotic strains of bacteria.

Henrich H. Paradies:

Autovaccines prepared from *E. coli* (named AutoColiVaccines in the contributions by *Schmolz* and *Ottendorfer*) can loose their kDO side chains (two or three units as determined by MALDI-TOF-MS and determination of the number of carboxyl groups), depending on initial concentration, decrease of pH (pH 5.6) and subsequent incubation at 70°C (5 minutes). The remaining material consists of free Lipid-A analogues of which 80% (w/w) is surprisingly unphosphorylated and 20% (w/w) is phosphorylated at C-1 of one of the glucosamine disaccharide. Pure free Lipid-A analogues aggregate into colloidal supermolecular structures depending entirely on particle number density rather than on absolute mass at extremely low ionic strength at ambient temperature as determined by light scattering methods and small-angle neutron scattering measurements (SANS). The formed colloidal crystals can grow to sizes as large as 1-5 μm, revealing highly ordered structures, showing either a bcc lattice with a = 41.5 nm at low number particle density, or a fcc lattice with a = 51.7 nm (SANS) at moderate number particle density, or e.g. inter-twinned ropes (TEM and HRTEM), which show elastic and extremely contractile properties as found by AFM and MFM (Magnetic Force Microscopy). These colloidal assemblies are extremely sensitive towards Ca-ions (nM, yielding compact structures) and Mg-ions (μM, giving rise to expanded and monolayer structures), which influence the elastic and contractile properties of LPS and free Lipid-A in Gram-negative bacteria like an ion-sensitive pump (osmometer) due to influencing the osmotic and thermodynamic properties (compressibility) of this dynamic system.

Note: Autovaccines obtained from patients suffering from various chronic diseases have been found to have different chemical structures for the free Lipid-A as well as for the covalent attachment of the number of the kDO units, and their sugar components within the kDO units, whereas the glucosamine disaccharide units are conserved and not altered at all. However, the number of acyl chains bound to the disaccharide moiety and partly the chain lengths can be significantly altered, e.g. shortened from C_{14} to C_{12}. The significant changes in the TNF-α, IL-10, IL-5, IL-12 and IFN-γ activities, which is upward or downward regulated in a concentration dependent manner in the presence of free Lipid-A including the influence of the kDO units.

Manfred W. Schmolz:

Five different AutoColiVaccines (ACV) were prepared from the stools of 5 individuals. When these ACV were tested in whole blood cultures of these subjects, the response of the leukocytes, measured by cytokine syntheses, depended strongly upon which ACV was applied to the cultures. The leukocytes responded to the ACV of other individuals with a much more homogenous cytokine pattern compared to the ACV of the donor from which they were taken. Generally the response followed a Th1-type mediator pattern, although when leukocytes saw the "autologous" ACV, there was large

variability. The leukocytes of some donors even refused to secrete TNF-α, which is one of the major cytokines released upon challenge with Lipid-A like molecules such as the ACV.

The question arose as to which mechanisms could modulate individual responses to a molecule that, when given to leukocytes from other donors elicited mediator productions exactly as could be expected when testing Lipid-A or LPS. Future experiments will have to elucidate if this is the result of an antigen-specific mechanism (such as antibodies neutralising Lipid-A in the cultures, Lipid-A specific regulatory T cells) or some up to now unknown signalling through Toll-like receptors and their adjunct co-regulatory proteins.

Doris Ottendorfer:

The discussion concerned immunoregulatory function of AutoColi-Vaccines (ACV) developed in Herborn. Potential receptors on T-lymphocytes and macrophages such as Toll-like receptors, scavenger receptors or glycolipid specific receptors (such as the CD1 antigens), that possibly were involved in responses to ACV, were discussed. All receptors were found able to influence the secretory and functional activities of different cell types of the innate and adaptive immune response. Several possible mechanisms were discussed by which the ACV could affect the cytokine response of monocytes, B-lymphocytes or T-cell subpopulations found *in vitro*. Assuming that different intracellular signalling pathways might be initiated upon the ligation of the different receptors, the recently shown immunoregulatory actions of ACV in the whole blood culture model appear quite extensive. It was speculated that the internalisation properties of the ACV might be altered by its physical state. The way by which different-sized "superstructures" of the ACV interact with leukocyte membranes could determine the observed cytokine response profile, particularly due to their dynamic and elastic mode of solution behaviour e.g. formation of ordered compact structures and specific monolayers in the presence of cytoskeletons, actin cells and adhesins by influencing the rheologic properties of the entire process. The author speculated that possibly these effects might be of value for treatment of chronic inflammatory diseases.

It was considered important in this respect that, if a rabbit is injected i.v. with an ACV preparation, which resembled in structure the core complex of the natural Lipid-A molecule of Gram-negative bacteria, no fever occurred in response. Presumably pure LPS would caused IL-1 release and therewith fever. It was proposed that upon i.v. injection of ACV an increase in IL-10 may contribute to this activity after administration of ACV This assumption is based on *in vitro* observations in the whole blood culture model which revealed a clear induction of IL-10 release by B-lymphocytes and monocytes.

Note: Intra-dermal injection of ACV does not stimulate general antibody production in human subjects, as antibodies to various antigens tested do not rise in titre in the blood. This is possibly due to the site of administration: the skin and its cellular composition i.e. dendritic cells, keratinocytes, 'skin-homing' T-cells and Langerhans cells. These cell types may play a role in the initiation and regulation of an immune response to intra-dermal applied bacterial components like those present in ACV.

Joseph Beuth:

Propionibacterium avidum and *P. agnes* as well as defined coagulase-negative staphylococci (CNS) were chosen from 200 bacterial strains because of their immunologic activity. The highly active strain (*P. acnes*) caused fibrosis, which was obviously an unwanted side effect so that *P. avidum* was selected for further studies. In mice the effect of different doses on the thymus was studied as well as its effect on tumours and infections (e.g. induced by *E. coli*). In colorectal cancer patients as well as in patients with malignant melanoma or breast cancer, *P. avidum* was i.v. injected with an apparent success. However, the study had to be discontinued because approval for the study was stopped.

In mice injection of whole LTA molecules caused side effects (e.g. wasting-like syndrome). An active peptide was extracted from the whole bacterium *P. avidum*. These peptide-induced effects on T-cells, cytotoxic T-cells, NK-cells and monocytes, however, were less pronounced than the whole cell vaccine.

From CNS, three peptides were isolated which were shown to stimulate B- respectively T-cells to proliferation and were therefore considered for further evaluation. These proliferation-inducing peptides may be used for induction of stem cell proliferation.

P. avidum peptide was further purified meanwhile. A heat-killed vaccine (whole bacterium, *P. avidum*) can be applied orally in capsules.
The cytokines induced are not toxic but stimulate cell proliferation (e.g. of T-cells) presumably due to the low size (molecular weight) of the peptide.