Old Herborn University Seminar Monograph 22:
Biological consequences of host-microbe interactions.
Editors: Peter J. Heidt, Lars Å Hanson, Helena Tlaskalová, and Volker Rusch.
Old Herborn University Foundation, Herborn-Dill, Germany: 135-141 (2009).

BIOLOGICAL CONSEQUENCES OF HOST-MICROBE INTERACTIONS: SUMMARY OF THE SEMINAR

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INTRODUCTION: MICROBIOTA AND PROBIOTICS

Each organism lives in continuous interaction with its environment. This interaction is of great importance but at the same time it could be life-threatening. The largest interface between the organism and its environment is represented by surfaces covered by epithelial cells. Mucosae represent in humans about 300 m^2 while skin covers approximately 2 m^2 of the human body. Starting from first hours after the delivery from the sterile uterine environment the interaction of the macroorganism with microorganisms begins. Physiologically occurring interaction with bacteria leads to colonization of epithelial surfaces and this co-existence is usually beneficial for the host. A complex, open ecosystem formed by resident bacteria and transiently present microbes interacting with the macroorganism is founded. The number of bacteria colonizing mucosal and skin surfaces exceeds the number of cells forming human body. However, under some conditions the interaction with "endogenous" microbes can be harmful for the host. Beneficial or harmful effects of commensal bacteria and their components in pathogenetic mechanisms of various complex and multigenic diseases have been recently recognized. Animal models of human diseases reared in defined gnotobiotic conditions (e.g. germfree animals) are helping to elucidate the aetiology of these frequently occurring disorders. An improved understanding of commensal bacteria – host interactions employing germfree animal models with selective colonization strategies combined with modern molecular biological techniques analysing the microbiota composition is bringing new insights into the mechanisms of several inflammatory autoimmune and neoplastic diseases.

Increased interest in an influence of intestinal microflora in human and animal health resulted in attempts of improving optimally its composition by using probiotics (most frequently bacteria of lactic fermentation). Probiotics, live microorganisms acting beneficially on host health were shown to influence favourably development and the stability to microflora, inhibit colonization by pathogens, influence the mucosal barrier by their trophic effect on intestinal epithelium and stimulate innate and adaptive components on the immune system. Orally administered probiotic bacteria are expected to survive passage through stomach and colonize intestinal mucosal surfaces even if it were only for a short period. It was shown that colonization of newborns with probiotic *E. coli* 083 stimulated

local and systemic humoral and cellular immune responses and reduced the number of pathogens. Moreover it was shown that repeated oral application of a non-pathogenic *E. coli* strain in the early postnatal period prevented the incidence of allergies as confirmed by a long-term (10 and 20 years) study. Understanding the regulation of mucosal immune responses to commensal microflora may be the key for the targeted manipulation of microflora composition and successful intervention in a wide range of chronic diseases.

There are more than 4000 hits on the Internet concerning the various outcomes of using probiotics. Numerous strains of microbes have been tested, including recombinant bacteria. Very variable results have been reached. There are claims of success i.e. against rotavirus infections, reducing diarrhoea by one day in infants. The effect was better in rats. It was stated on one hand that critical reviews of the literature only left orchitis as a condition, which can be efficiently treated by probiotics. It was claimed that the extensive literature on other conditions did not give support for proven effects against other conditions. This was debated by others who claimed that several other diseases can be treated with probiotics, like irritable bowel disease and atopic dermatitis. Rotavirus infections can be shortened with probiotics. It was mentioned that just for Lactobacilli more than 20 strains have been tested.

More probiotic effects have been noted in mice models than in man. The effects of probiotics on tumours were studied. It was found that germfree animals get adenomas, whereas conventional animals get carcinomas. Microbes and microbial enzymes were said to be without significance for carcinogenesis, e.g. β-glucuronidase. This is also true for nitrosamine. An animal model, with the animals carrying a tumour, was mentioned in which Bifidobacteria were given together with an anti-tumour drug. The tumour took up the microbes and the drug followed the bacteria; as a consequence the tumour contained substantial amounts of the anti-tumour drug.

BREASTFEEDING

Human milk is said to contain some 100.000 components. Of these we have knowledge of about just a few, and also that information remains incomplete. Breastfeeding should start immediately after birth. Actually, if the baby directly after delivery is put onto the mother's abdomen, it will start crawling up towards her breast and massage it to start the milk to flow. Thus breastfeeding is initiated. Within an hour just about all babies have performed this task, which also adds to the bonding between mother and offspring. But in many societies the baby is taken away from the mother and in some cultures it may instead be given various fluids and foods, which may be heavily contaminated, especially in poor communities, bringing a high risk of infections in early life, contributing to high infant mortality. Also it may derange the early bacterial colonization so that the neonate not only will be colonized with the mother's microbes, but also those from the hospital staff and others. This may expose the neonate to microbes of higher virulence and microbes to which the mother is not providing protection via her transplacentally transferred IgG antibodies.

A recent study from Ghana showed that infant mortality was 12% higher if the baby was not breastfed until one

day after delivery and 20% higher if it was not breastfed until day 2-3, compared to breastfeeding within one hour.

The milk secretory IgA (SIgA) antibodies may be specifically protective since they, thanks to the enteromammaric link, are directed against the intestinal microflora of the mother.

Normally this is the microflora the offspring is exposed to during normal delivery. The microbial exposure starts already in the birth canal. However, Caesarean section is becoming more and more common and impairs the normal contamination of the neonate with the mother's microflora. From South America up to 30-40% of deliveries are now reported to be section deliveries. It has not been properly studied what this means for the early microbial colonization of neonates. It has recently been made likely that the M cells covering the Peyer's patches have receptors binding SIgA. This might in the newborn result in milk SIgA antibodies, which are taken up together with the microbes against which they are directed. Protection would be provided, but also enhancement of mucosal immune responses in the infant.

Human milk has numerous additional defence components, e.g. lactoferrin, which on one hand can kill microbes and on the other turn off proinflammatory host responses, presumably because they are costly to the host by causing loss of appetite, tiredness etc, all symptoms most undesirable in early life. The blocking of inflammation occurs because the large amounts of milk lactoferrin is taken up by leucocytes and block their NFκB. Thus, they cannot produce proinflammatory cytokines.The major milk protein, α-lactalbumin, has recently been shown to have the capacity together with adequate co-factors to kill tumour cells. Human milk contains about 5-7% carbohydrates. They are not taken up by the infant, but remain in the gut where they function by being analogues to the structures used as receptors by the microorganisms, which try to invade the host.

The potential capacity of the major human milk protein α-lactalbumin to take a shape that makes it capable of killing tumour cells was also discussed. The structure with this capacity appears as HAMLET (*H*uman *A*lpha Lactalbumin *M*ade *L*ethal to *T*umour Cells) after being exposed to the low pH in the stomach together with exposure to oleic acid.

There are some suggestions that breastfeeding may enhance certain vaccine responses, e.g. against *Haemophilus influenzae* type b. The mechanism is unknown.

A very impressive study of the effect on IQ by breastfeeding showed a gain of 11 points, presumably indicating uptake of structures from milk favourable for brain development.

The question was brought up of when gut closure, preventing uptake of large molecules, takes place. It was indicated that in man this occurs before birth, in contrast to what is seen in certain other species, like mice and rats. On the other hand there are suggestions of uptake both of cells and large proteins later. There are several studies showing uptake in various species. In man one might consider that these studies should be repeated in the light of the fact that the receptors on the M cells covering the Peyer's patches are capable of uptake of SIgA, but not other immunoglobulins. On the other hand this special mechanism also results in an uptake of the SIgA-bound antigens. The great majority of the milk SIgA remains in the gut, preventing microbes from entering the gut mucosa. Other species have different mecha-

nisms for their mucosal defence and protection of the offspring via the milk.

Banks for human milk were discussed. They are no longer used in USA, because of the risk of giving one mother's milk to the baby of another mother. The risk of infection was regarded as a major problem, especially the risk of transfer of HIV-virus. CMV can be transferred to infants via the milk also from their own mothers, but symptoms do not follow. In Europe human milk banks remain in use and are regarded as safe. There is limited information about how much biological function of milk proteins are lost by heating. This varies of course with what form and extent of heating which is used and this seems not to have been carefully studied.

Human milk has been tried for treatment of inflammatory diseases, but there are no controlled studies. Commercial cow's milk comes in 80% from pregnant cows and contains much oestrogen, which might be carcinogenic. The potential risk of this oestrogen content of the milk fat, e.g. for causing breast cancer, has not been investigated. The finding of pesticides in human milk is a problem, which has not been properly taken care of.

By now there are 3 major evidence based studies of the short and long term effects of breastfeeding, one from the WHO, one from Holland and one from the US Dept of Health. They conclude that breastfeeding provides significant protection against the acute conditions sudden infant death syndrome (SIDS), necrotizing enterocolitis (NEC), acute gastroenteritis, pneumonia and otitis media. Long-term therapeutic, preventive, or reducing effects are noted on blood pressure, obesity, cardiovascular disease, acute lymphatic and myelogenous leukaemia, diabetes type 1 and 2, coeliac disease, serum cholesterol, asthma and eczema, if there is heredity. Furthermore, breastfeeding mothers experience less risk for breast cancer and rheumatoid arthritis.

VAGINAL MICROBIOTA OF THE FEMALE GENITAL TRACT

As an example of luminal habitation was discussed the female genital tract microbiota. The microbiota of the vagina is in contrast to those of other sites of the body regulated by oestrogen. As shown by Gram-staining of wet smears it appears as if lactobacilli may not be firmly attached to the epithelial cells, but rather are located in the mucus. When imbalance of the microbiota appears (bacterial vaginosis, BV), characterized by high numbers of anaerobic bacteria (at least 1000-fold increase), *Gardnerella*-like bacteria are firmly attached to the cells. This induces increased levels of IL-1 and sometimes IL-8 without signs of inflammation, showing that possibly the total number of bacteria or the increased number of attached bacteria to cell surfaces is triggering a higher level of "host-awareness". In fact, we have found a correlation between the number of CFU in the vagina and cervical IL-1 in asymptomatic women.

Also, there is a commensal symbiosis between various bacterial species. For example it has been shown that *P. revotella bivia*, a typical BV-associated species may enhance the growth of both *G. vaginalis* and *Peptostreptococcus anaerobius* by producing ammonia and amino acids, respectively (*in vitro* experiments). We have observed that there are significant correlations between certain bacteria such as *G. vaginalis* and *Atopobium vaginae*, *F. nucleatum* and *S. anginosus* and may

imply that some accumulated metabolites favours such symbiosis.

As an example of influence on the luminal habitation Dr. Mattsby-Baltzer briefly presented their investigation on the prevalence of *Lactobacillus*-dominated biota (LBD) in healthy fertile women of three age cohorts and relation to contraceptive methods. The study was performed on 313 women scheduled for cervical screening. It was found that women in the age cohort 20-29 years had a LDB in approximately 90%. The frequency was reduced with age to ca 70% at 30-39 and 50% at 40-49 years of age. The use of oral contraceptives resulted in almost no difference between the three age groups, a high level of LBD was present in all (83-90%). The use of copper intra-uterine device (IUD) disturbed the LBD, since the frequency decreased to 40% in the age group 30-39 years of age (no IUD users in the 20-29 year group). The frequency among women using hormone-releasing IUDs was 50% in the age group 30-39 years and appeared to increase to 70% in the age group 40-49. The most deviating LDB frequencies were thus observed between non-users and IUD-users in the 30-39 age group, or oral contraceptive-users and hormone-releasing IUD-users in the 40-49 age groups. The two latter ones were increased with respect to LDB.

It was concluded that decreasing oestrogen levels with age, contributes to a lower frequency of LBD. However, use of IUDs and oral contraceptives strongly influences the vaginal microbiota.

MICROBIOTA IN ALLERGY AND AUTOIMMUNITY

It is generally accepted that gut microflora has an important impact on mechanisms of immune regulation. It is believed that the microflora is involved in induction and maintenance of physiological tolerance, preventing hyper-responsiveness leading to allergies and food enteropathies. The literary data on studies in germfree animals show diverse results on the significance of the microflora for tolerance induction. Data presented in the seminar show that mucosal tolerance induction can be equally induced in experimental model of pollen allergy, in mouse with or without the presence of commensal bacterial flora: Oral as well as intranasal tolerization led to suppression of allergen specific serum antibodies as well as cytokine production by splenocytes in both germfree and conventional animals. We therefore concluded that the absence of the microflora does not influence the ability to mount Th2 responses nor to establish tolerance towards the aeroallergen Bet v 1.

We propose that dysfunction of the immune system associated with the gut and other mucosal surfaces is a prerequisite for impairment of physiologically developing regulatory mechanisms. The balance in intestinal mucosa may be disturbed by pathogenic microorganisms and toxins attacking the mucosae, by qualitative or quantitative changes in the composition of mucosal microbiota, or by inadequately functioning components of the innate or adaptive immune system occurring in cases of dysregulated mechanisms of mucosal immunity, or in immunodeficiencies. An expression of pathologically increased immunological activity may induce inflammatory processes of a different character, depending on that type and mediators of inflammation. Thus, numerous chronic diseases may occur as a result of disturbances of mu-

cosal barrier function or of changes in mechanisms regulating mucosal immunity. The main characteristics of chronic, "idiopathic", inflammatory, and autoimmune diseases are tissue destruction and functional impairment as a consequence of immunologically mediated mechanisms that are principally the same as those functioning against dangerous (pathogenic) infections. One of the most attractive explanations for inflammatory and autoimmune phenomena has centred on various infections as natural event capable of initiating the process in genetically predisposed individuals. We propose that not only pathogenic microorganisms but also components of normal microflora could participate in the triggering and development of inflammatory and autoimmune processes.

We used colitis, induced by dextran sulfate sodium (DSS) feeding of mice, to study the immunological factors involved in the pathogenic mechanisms of chemically triggered intestinal inflammation.

These experimental models were used also to analyze the role of commensal bacteria and innate immunity in the development of intestinal inflammation. Using the DSS-induced model of intestinal inflammation, we have shown that, as in conventionally reared, immunocompetent Balb/c mice, mice with severe combined immunodeficiency (SCID) developed profound inflammatory changes in colonic mucosa. Balb/c and SCID mice reared in germfree conditions developed only minor signs of mucosal inflammation. Interestingly, conventionally reared SCID mice, lacking T and B cells, developed intestinal inflammation similar to the inflammation that developed in immunocompetent Balb/c mice. This finding suggests that under physiological conditions, innate immunity components are able to regulate (keep in balance) the interaction of macroorganisms with commensal bacteria, and, after chemically induced breakdown of mucosal barrier, commensal bacteria could induce severe forms of intestinal inflammation in the absence of components of adaptive immunity (T and B cells).

Experiments performed in gnotobiotic models suggest that the composition of gut microbiota plays a decisive role in the pathogenetic mechanism of intestinal inflammation. Exogenous application of commensal organisms (probiotics) exerting beneficial effects on host health has recently been shown to have protective and therapeutic effects on diarrhoeal disease, including IBD, and to reduce the risk of infections and allergies. Oral application of probiotic bacteria is associated with an alleviation of intestinal inflammation and normalization of increased intestinal permeability, together with promotion of intestinal barrier functions.

Components of commensal bacteria were tested in experimentally (DSS) induced intestinal inflammation to study the effects of their oral application. DSS colitis in immunocompetent Balb/c mice was mitigated by oral administration of the lysate and fractions of some *Bacteroides* strains. Similarly oral application of heat shock proteins alleviated intestinal inflammation induced by DSS treatment. This protective effect was accompanied by stabilization of intestinal microflora composition and decrease of proinflamatory cytokine production.

A not yet solved crucial question is why we see increased prevalence of autoimmune and allergic diseases during the last 30 years. Many hypotheses have been proposed to explain the increasing tendency in occurrence of inflammatory and neoplastic diseases: Less exposure to microbes, decreased

diversity of the microflora, changes in food intake and food quality, etc. The skewing of the immune system noted in early life is considered of aetiological significance. The argumentation about autoimmunity indicated that autoimmune diseases can be mediated via TH1 as well as TH2 mechanisms. The crucial role of T-regulatory cells and the role of microflora in their development and differentiation were pointed out. Since infections can induce autoimmunity, at least certain autoimmune diseases may be contagious. The example was mentioned about the discovery of the Gm factor. It was found that infections with cross-reacting herpes viruses gave rise to the Gm factor, which had found its clinical use as a test of autoimmune disease in the form of rheumatoid arthritis.

VACCINATION PROGRAM AND SAFETY/REGULATIONS

A rotavirus vaccine is now produced in Austria and further vaccines are being developed. For instance in Belgium a vaccine is being worked on, containing Lactococci producing IL-10. There are many other vaccines being considered for development, now in a broader way than previously. Thus probiotic effects are sought, and new adjuvants are tested.

Existing vaccines are being improved and new areas are considered like effects on the microflora. The role of undernutrition of the host is better analysed and existing vaccines are improved.

The control of drugs and biologicals is fundamental for our safety. It is a complex issue.

Bacteria, which have grown for 4 hours compared to 40 hours, may be quite different. Sthe same is true for live compared to dead bacteria. Thus determining safety of a bacterial product becomes complicated. Vaccines come on the market easier than probiotic products. The presence of gross contaminants is a major issue.

The requirements in US for regulation of vaccines and probiotics were presented in rather strict details. The corresponding rules for biologicals like probiotics seemed to be more lax. The same is true for the regulatory demands within the European Union. The rules are continuously being adjusted and it is expected that the rules within EU will become similar to those now in use in USA. Lobbying by large companies is common. Clinical trials seem to be simpler approved in the EU than in the USA.