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18th OLD HERBORN UNIVERSITY SEMINAR: SUMMARY AND OVERVIEW OF THE DISCUSSIONS

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FROM FRIENDS TO FOES: SPECIFIC EXAMPLES

Helicobacter pylori is the first named species of the Helicobacter/Wolinella family. This genus consists of more than 20 species and about 10 candidate species. With a few exceptions all these organisms are micro-aerophilic "mucinophiles". *Helicobacter pylori* is the prototype for a number of bile-sensitive organisms able to colonise the stomach of most animals, including dolphins and whales. The link between gastritis and peptic ulcer disease and the presence of this spiral shaped organism on the surface of gastric mucosa was discovered in 1982 by Warren & Marshall. *Helicobacter pylori* is a common widespread organism with a worldwide spread of 30-90%. It has a higher prevalence in developing countries with an increase up to 60 years of age. *Helicobacter pylori* is found almost entirely in humans, and oral-oral transmission between humans in crowded institutions, parents and children seems to be common.

Although *Helicobacter pylori* is strongly associated with the development of pathological conditions of the stomach, most of the infected patients never develop *Helicobacter* related diseases. This indicates that some of the bacterial strains might be more pathogenic than others or that certain persons are more susceptible to develop *Helicobacter pylori* related disease. *Helicobacter pylori* has several ways to persist in colonising the mucus of human stomach. One of these mechanisms to facilitate persistence is to increase diversity. Within a large population a small proportion of cells arise that have heightened mutation rates. Most strains of *Helicobacter pylori* are considered to be such a hypermutator phenotype; this favours the emergence of selective variants. A good example is the point mutation that leads to high-level resistance to commonly used antibiotics such as the macrolide clarithromycin.

The ability to cause life-long infections depends on the low toxicity/biological activity of the lipopolysaccharide and several other unique factors like host protein binding and protein shedding. Other factors that are supposed to play a role in evading the host defences are the location in the gastric lumen, beyond the reach of most host immune recognition and effector mechanisms. TLR stimulation triggers pro-inflammatory signalling through NF-κB activation, and *Helicobacter pylori* has evolved to minimise such stimulation, the TLR5 is not stimulated by *Helicobacter pylori* flagella. The highly methylated DNA of *Helicobacter pylori* is barely recognised by the TLR9, which recognises largely unmethylated DNA of most bacteria. *Helicobacter pylori* LPS is anergic compared with that of other Gram-negative enteric bacteria. This is caused by lipid A modifications, nevertheless it stimulates macrophage TLR4, but it does not stimulate gastric epithelial TLR4. Although the cloaking abilities of this CagA positive strains do stimulate NF-κB activation in epithelial cells, apparently through recognition by NOD, an intracellular pathogen-recognition

molecule that detects soluble components of bacterial peptidoglycans, the resultant NF-κB induced pro-inflammatory cytokine expression is an important and continuing stimulus to inflammatory cell infiltration and thus to pathogenesis. Another way to evade the host response is by mimicry of the gastric epithelial fucosylated antigens, and by antigenic variation of surface proteins including a critical pilus molecule, CagY. Other mechanisms that are proposed in the improvement of *Helicobacter pylori* survival in the stomach are superoxide dismutase, catalase and phospholipases. It is unknown if extracellular and immunoglobulin proteases play a role in the survival of *Helicobacter pylori*. The pathogenesis of *Helicobacter pylori* has been linked to the expression of two proteins, the CagA (cytotoxin associated protein A) and the VacA (vacuolating cytotoxin). No homologues for the CagA gene have been found suggesting that it reflects a human gastric specific gene of Helicobacter pylori. The CagA protein contains tyrosine phosphorylation sites that are recognised by the host cell Src kinase. Once phosphorylated, CagA interacts with the SHP-2, a tyrosine phosphatase, which affects spreading, migration, and adhesion of epithelial cells. The helicobacter CagA protein interacts with several major signal-transduction pathways present in epithelial cells. The Cag apparatus promotes anti-apoptotic pathways, which may aid persistence by slowing turnover of the epithelial cells to which they are attached.

VacA is a high molecular weight multimeric pore-forming protein that causes massive vacuolation in epithelial cell lines, which lead to egress of anions and urea. This is important for *Helicobacter pylori* since urea hydrolysis catalysed by *Helicobacter pylori* urease protects against gastric acidity. VacA also induces loosening of epithelial tight junctions, potentially allowing nutrients to cross the mucosal barrier, which can favour the organism in the gastric lumen. VacA also blocks phagosome maturation in macrophages, selectively inhibits antigen presentation in T-cells, blocks T-cell proliferation and downregulates Th1 effects by interacting with calcineurin to block signalling. Peptic ulcer disease is considered to be associated with CagA+ strains; there is also strong evidence that CagA+ strains are associated with the development of atrophic gastritis and gastric cancer.

Another important factor in the severity of pathogenesis is the heterogeneity in immune response among human populations. This leads to the presence of cytokine polymorphism.

Polymorphisms that increase the IL-1β response to *Helicobacter pylori* are associated with an increased risk of developing gastric atrophy, hypochlorhydria and adenocarcinoma. Polymorphisms in TNF-α and IL-10 genes have a similar, butt less pronounced association.

Thus *Helicobacter pylori* has several ways of adaptation in order to facilitate its persistence on the human gastric mucus layer. It does this so by mutation, evasion of the immune response, the presence of proteins that can interact with several major signal-transduction pathways and which have a direct influence of the epithelial tissue. But this host-pathogen relation seems to be pathogenic for the host, as indicated by the extensively studied relation between the presence of certain *Helicobacter pylori* strains in the stomach and gastric ulcer disease and gastric carcinoma.

Another example of chronic disease related to infection, given by dr. Ljung, is the growing evidence that an infectious agent plays a role in atherosclerosis. In 1921 the hypothesis that infections could lead to atherosclerosis was proposed by Ophus. This hypothesis was based on pathologic specimens of blood vessel, which showed macrophage

infiltrates and foam cells. But this theory was overshadowed by the common conception that atherosclerosis was caused by factors such as smoking, hypercholesterolaemia, diabetes and hypertension. It was in the late 70's that the hypothesis of infection related pathogenesis of atherosclerosis re-emerged. The progress in diagnostic techniques contributed to the acceptance of the concept that microorganisms could be involved in the multi-factorial process of atherogenesis. In this regard, sero-epidemiological evidence of varying designs as well as experimental evidence has focused primarily on three pathogens: CMV, *Helicobacter pylori* and *Chlamydophila pneumoniae*. Anti-*Chlamydophila pneumoniae* antibodies are unusual in children younger than 5 years and can be found in about 50% of individuals by the age of 20. The prevalence of antibodies continues to increase with age, reaching a peak seropositivity of 80% in men and 70% in women by age of 65 years. There are several pathogenic mechanisms by which microbial infection could directly or indirectly induce atherogenesis, thrombosis and plaque rupture. Chlamydia LPS has been shown to enhance LDL uptake en to down-regulate cholesterol efflux in monocytes or macrophages. LPS can induce not only LDL oxidation but also transforms human mononuclear phagocytes into foam cells, which is a key atherogenic event. Chlamydial LPS can directly alter atheroma cell function by inducing the production of TNF-α, IL-1β and IL-6 which lead to the to the propagation of the inflammatory response with the atherosclerotic tissue.

Another important substance in atherogenesis by *Chlamydophila pneumoniae* is HSP60. This 60 kDa protein is considered to have a low biological activity based on structure-to-function studies. The HSPs form a group of highly preserved proteins among species. This can lead to significant cross-reactivity and immunopathology from anti-body response to HSP60. Human and Chlamydial HSP60 activate human peripheral blood mononuclear cells and monocyte-derived macrophages by a CD14-dependent mechanism. Signalling through this pathway resembles LPS mediated cell activation and LPS and HSP60 may share signal transduction machinery to activate a cell. Thus HSP60 may contribute to atherogenesis by antigenic stimulation and subsequent cross-reactivity to self-proteins, as well as direct modulation of atheroma cell function. HSP60 also induces inflammatory cytokines such as TNF-α by endothelial cells, macrophages and smooth muscle cells. LPS and HSP60 both induce the formation of metalloproteinases which can destabilise the atheromatous plaque. All these events contribute to the development of cardiovascular disease.

Further evidence of the causal relationship of bacterial infections and chronic disease states can be found in inflammatory bowel disease. It is evident that there is a life-long counteraction between the enteric bacterial flora and the host immune system. This relationship starts immediately after birth. Nevertheless this overwhelming chronic immune stimulation does not lead to illness in healthy people; in fact it is believed that this leads to a better development of the intestinal immune system. Inflammatory bowel disease results in the chronic inflammation of the intestine. The current working hypothesis is that inflammatory bowel disease is due to a dysregulated mucosal immune (CD4+ T-cell) response to enteric bacterial antigens, in a genetically susceptible host. This postulates that bacterial flora drives the disease, and since the lower intestine harbours the largest concentration and diversity of resident microbial antigens in the body this should be an overwhelming chronic stimulation. So the question arises how the commensal

bacteria of the gut are involved in the pathogenesis of Crohn's disease. It is suggested that inhibitory cytokines produced by mucosal cells after antigen specific recognition may play a role in maintaining hyporesponsiveness to gut bacterial antigens and may be responsible for the lamina propria phenotype of activated yet hyporesponsive cell. In fact, data from several animal models suggest that there are 2 functionally different populations of CD4+ cells. One is capable of inhibiting disease and the other is able to mediate disease. Dr. Elson and his team showed in an animal model with C3H/HeJBir mouse that the inflammation in the intestine is due to unrestrained effector T-cell responses to the enteric bacteria, and that a small subset of antigens out of the thousands of bacterial proteins is responsible for an inflammatory response (CD4+). The possible antigen proposed by dr. Elson is the Flax-flagellin. This structure was found after sequence homologies of Cbir proteins cloned from caecal bacteria. These flagella show a significant positive colitis score in animal models that have Cbir1-reactive CD4+ T-cells. They also showed that the colitis was driven by bacterial specific T-cells and that effector T-cells reactive to commensal bacterial antigens are pathogenic unless their activity is properly regulated. Dr. Elson proposed 2 subsets of regulatory cells. The first one is the TR1 cell which inhibit both IL-12 and IFN-γ production in cultures of bacterial-reactive TH1 cells in animal models. Another subset of regulatory cells termed T helper 3 which are characterised by their high TGF-ß production but lower levels of IL-4 and IL-10. Mice deficient in TGF-ß develop spontaneous inflammation. So these regulatory mechanisms form a working frame for the possible role of bacteria in inflammatory bowel disease.

Even in the field of psychiatry relations between bacteria and chronic disease can be found. The role of bacteria in chronic psychiatric disease was presented by dr. S. Rosseneu. She discussed the possible relationship of abnormal aerobic gut flora and the presence of autism in children. Autism is a life-long developmental disorder which affects 1 in 500 children. The diagnosis is conferred after extensive evaluation according to the DSM IV criteria. Usually the child is 2 to 5 years old. The symptoms appear after some time of normal development. They gradually suffer a loss of newly acquired skills (language, eye to eye contact, and sociability). The cause for this disease is yet unknown but it is well accepted that there are multiple causes for this disorder. Studies with monozygotic twins show a 60% concordance in suggesting that there is a genetic basis for autism. The need to understand the cause(s) of autism and the underlying pathogenesis has become more acute since the number of diagnosed cases has risen markedly in recent years. There is growing evidence that there is a relationship between intestinal pathology and autism. In several studies with children suffering from autism and gastrointestinal symptoms showed significant more ileal and colonic lymphoid nodular hyperplasia. An active acute inflammation and chronic inflammation could also be seen in respectively 8% and 88% of the patients. Another study showed altered function of the upper gastrointestinal tract in children with autism. The results of these different studies suggest a widespread gastrointestinal pathology in patients with autism. Earlier studies have shown that children with autism and celiac disease have worsened symptoms after gluten consumption. Another piece of evidence is provided by the measurement of TDC (transcephalic direct current) in children with autism and celiac disease. After taking gliadin the TDC showed a significant inhibition of frontal voltage. In two separate studies children

with autism showed an improvement in social skill, cognitive function and communication after eradication of gluten and cow's milk in their diet. Recent research has also shown that there is a difference in microflora between normal children and children with late-onset autism. Rosseneu and her team showed that there is a significant difference in the amount of aerobic Gram-negative bacilli between autistic children and normal children. Overgrowth was determined as $\geq 10^5$ CFU AGNB per ml of saliva and/or g of faeces. Rosseneu showed that in a population of children with autistic spectrum disorder with 95% regressive or late-onset autism, 95% of GI symptoms and 72% with gluten, dairy-free diet have an abnormal amount of AGNB. About 61% of the subjects have abnormal AGNB in overgrowth, 95% have *E. coli* overgrowth, and 55% have *S. aureus* in overgrowth and *Candida spp.* showed no difference. The consequences of AGNB overgrowth are AGNB translocation and/or endotoxin absorption; liver macrophages (Kupffer cells) detoxify endotoxin and release cytokines which leads to inflammation of the intestine and eventually to systemic effects and influence distant organs. She also found differences in glycocalyx expression in patients with ulcerative colitis and patients with autism but not Crohn's disease, decreased colonic sialylation and α-1,2 fucosylation in UC colon, decreased α-1,4 fucosylation in both ileum and colon in autism. The question remains whether this is the cause or result of this abnormality. Rosseneu attempted to answer this question by trying to reduce the abnormal carrier state to a normal carrier state. This was realised by administering polymyxin E and tobramycin for three months to patients with autism and GI symptoms. Improvement was measured by using the global behavioural scale. This therapy led to decrease of abdominal pain score, decrease of laxative intake, improvement in overall behaviour/cognition and overgrowth concentrations were significantly reduced. Another important fact is that the improvements were transient, after stopping the antibiotic treatment the bacterial overgrowth rose to the pre-treatment levels and symptoms gradually reappeared. This suggests that there is a relationship between the state of the enteric flora and the pathogenesis of autistic disease.

EVOLUTIONARY PERSPECTIVE

Over the past centuries, disease has been separated into three categories: Infectious disease, genetic disease and disease caused by too much or too little of some non-infectious environmental constituent. These three categories offer a conceptual framework for understanding diseases, but they pose a danger of canalising thinking. They have for example, contributed to the rejection of infectious causation when evidence in favour of non-infectious causes has been acquired but evidence against infectious cause is lacking. This tendency to dismiss infectious causation has occurred in spite of the recognition that for infectious diseases host genetic and non-infectious environmental influences are of importance.

In the 1970's and 1980's medical texts typically attributed peptic ulcer to gastric acidity, stress, smoking, alcohol consumption and genetic predisposition. Infectious causes were not mentioned, even though evidence of infectious causation had been accumulated from the 19th century: A spiral bacterium was associated with ulcers at the end of the 19th century, ulcers had been experimentally transmitted in laboratory animals during

the second decade of the 20th century, and peptic ulcers had been successfully treated with antibiotics in New York City hospitals during the late 1940s. In spite of the accumulated evidence, several attributes of ulcers made infectious causation cryptic: The loose correlation between infection and ulcers, the internal site of infection, and variable delays between onset of infection and the onset of overt disease. The net effect is a chain of transmission that is so cryptic that the transmission of *Helicobacter pylori* is still not totally resolved today. The same crypticity can be seen in the relationship between an infectious cause of atherosclerosis, which was proposed over a century ago. Despite the growing evidence of *Chlamydophila pneumoniae* as the leading suspect in the infectious cause of atherosclerosis it is only until recently that medical doctors are accepting the possible role of *Chlamydophila pneumoniae* in the formation of atherosclerosis besides the role of cholesterol, high fat diets, stress, smoking and genetic predisposition in the formation of atherosclerosis.

Because genetics can alter the course of infection, we expect to find that genetic determinants of disease may sometimes be best-explained genetic influences on infection. The epsilon 4 allele of the human apolipoprotein E gene has been identified as a genetic risk factor for atherosclerosis, stroke, and Alzheimer disease. It also appears to increase susceptibility to *Chlamydophila pneumoniae* infection. The genetic risk imposed by the epsilon-4 allele may therefore result from a genetic vulnerability to infection rather than a direct influence of epsilon-4 on disease progression. In other words the evolutionary maintenance of "bad" alleles is problematic and not desirable in an evolutionary sense. A resolution to this problem is to make the host more vulnerable to infectious causes in order to eliminate the persistence of the supposed "bad" allele in the evolutionary path.

The high allelic variability of genes that are involved in resistance pathogens (e.g. HLA variability) suggests that this situation may be common.

Examples as given above generate a growing sense that more chronic diseases will prove to be caused by pathogens that may be familiar causes of acute infection, identified but not yet associated with disease, or not yet identified. The key problem is how to facilitate recognition of infectious causation among these diseases. One step toward resolution of this problem involves increased awareness of the sources of crypticity that can likely be encountered in ascribing infectious causation. One source of crypticity is the increasing difficulty in obtaining suitable animal models. Few mammals live as long as humans, it is therefore difficult to find experimental animals that can be infected by an organism thought to cause long-delayed chronic disease and that is able to survive long enough to demonstrate the same chronic disease found in humans. Nevertheless the evolutionary relationship between chronic disease and bacterial infection provides a challenging concept and this provides an interesting working frame for further research.

TARGETS FOR MANIPULATION OF HOST DEFENCES AGAINST CHRONIC DISEASES

Mast cells

Until recently mast cells were considered as primarily harmful which was based on the facts that mast cells play a key role as effector cells of allergic and potential lethal anaphylactic reactions and that their contribution was limited to the elimination of parasites. However

there is growing evidence that the mast cells could be involved in other processes. Recently, mast cells have been shown to exert beneficial functions such as in tissue repair and in acquired and innate immune responses against foreign molecules and infectious agents. Mast cells fulfil all basic prerequisites to be classified as Antigen Presenting Cell. These basic functional prerequisites are phagocytosis, antigen uptake, expression of adhesion molecules, attraction and activation of lymphocytes, amplification via cytokine production and endothelial activation. To further clarify this dr. Maurer presented data from his own research with animal models using Kit+/+ and Kit w/Kitw-v mice, of which the latter is deficient in mast cells. He showed that mice deficient in mast cells which develop bacterial sepsis in a caecal ligation and puncture model of acute peritonitis have significantly larger chance of dying in an early stage of disease. In other words mast cells protect from mortality in septic peritonitis. In another example with the same type of mice he showed that skin lesions after infection with *Pseudomonas aeruginosa* are increased in the absence of mast cells and that mast cells control the skin lesion size. He also showed in this model that neutrophil recruitment in *Pseudomonas aeruginosa* infections in the skin is mast cell dependent. To show the role of mast cell in parasitic infections dr. Maurer conducted the same experiment with *Leishmanias major*. These results show similar effects of mast cells on the lesion size. The mice deficient in mast cells show larger skin lesions, exhibit markedly increased parasite numbers, lower IFN-γ levels, higher IL-4 levels after injection with *Leishmanias major*, impaired recruitment of dendritic cells, macrophages and CD8+ T-cells. These data show strong support that mast cells are key players in innate host responses. Skin mast cells protect from bacteria and parasites and bridge innate and adaptive immunity.

Infectious diseases may cause frank infections in the host, like pneumoniae and/or septicaemia, but infectious agents may also cause more chronic diseases due to the failure of the host to eliminate the pathogen or by a breakdown in normal truce. Infectious agents have been for a long time connected with arthritis. Microorganisms can be involved in the aetiology of arthritis in three different ways: There are septic forms of arthritis in which microorganisms are directly involved in the arthritis and reactive arthritis and post-infectious arthritis. Reactive arthritis mostly is triggered by specific microorganisms in for instance causing infections in the GI tract or genito-urinary tract, the joint symptoms appear 1 to 3 week after the infection. Moreover a role for microorganisms in the development of idiopathic arthritis and autoimmune arthritis (RA and AS) has been supposed.

Several mechanisms may play a role in the development of arthritis after mucosal infection. This may be the result of mimicry of bacterial substances with human antigens, as has been supposed for *Campylobacter*. But also the way the intestinal immune system reacts with intestinal microorganisms may play a role in the aetiology of autoimmune arthritis

Although there is a long history of the relationship of mucosal infections and rheumatism, real aetiological agents have not been found. The theory is that within a certain genetic condition commensal microorganisms may trigger an immune response leading to inflammatory joint disorders. So, arthritis maybe the result of an immunological response to microorganisms. On the other hand different syndromes in which joints may be involved may be caused by different mechanisms. So in AS and RA different structures and microorganisms eliciting different immunological responses may be involved.

Especially attention has been focused on the role of Gram-positive microorganisms in the aetiology. Gram-negatives although important in reactive arthritis do not seem to play a major role in autoimmune arthritis.

Special attention has been paid to the involvement of peptidoglycans in the immune response. Several mechanisms have been suggested; peptidoglycans may have cross reactivity with joint tissue and it also may activate an autoimmune response. In the past a role for EBV has been suggested. Although EBV may trigger B-lymphocytes and stimulate antibody synthesis, there is no evidence for a role for EBV in the pathogenesis of RA.

There are some studies that show those microbial products – peptidoglycans and bacterial DNA – to react with lymphocytes in the joint, which can be traced in joint fluid.

In conclusion, several microorganisms may be involved in several diseases and several mechanisms may trigger an inflammation of the joints.

Microbial immunomodulation against cancer

Different factors are involved in the aetiology of malignant diseases. Toxins and metabolites produced by microorganisms are thought to play a role in the aetiology of cancer. Examples of such microorganisms are *Helicobacter pylori* (gastric carcinoma), *hepatitis B virus* (hepatocellulair carcinoma), *HPV* (cervix carcinoma) and *EBV* (Burkitt lymphoma, B-cell lymphoma), and *Schistosomiasis haemotobium* (urinary bladder cancer). So, although microorganisms may play a role in the aetiology of malignant diseases, they may also play a therapeutic role in cancer treatment. Already at the end of the 19th century the therapeutic potential of microorganisms in cancer treatment has been supposed.

Microorganisms may have anti-tumour capacity by different mechanisms: Toxicity for the cancer cells by microbial toxins, immuno-activation and inflammation including fever induction. Although several microorganisms have been used in cancer therapy - *Corynebacterium parvum, Newcastle Disease Virus, Streptococcus pyogenes* - the best-documented results have been reached with BCG in bladder carcinoma and *Propionobacterium avidum* in colon carcinoma.

P. avidum possesses anti-tumour capacity by its immunomodulating properties. It proliferates and activates the RES-cells and thymocytes. It activates granulocytes, macrophages, lymphocytes and NK-cells. In mice it reduces significantly the number of metastases in experimental cancer models. In human experiments lyophilised *P. avidum* has been shown to enhance immunity and increase survival in patients with colorectal cancer

Beside their anti-tumour capacity, microorganisms may also play a complementary role in cancer treatment. Probiotics are not an alternative for standard treatment but may play a role in optimisation of the standard therapy.

Possible prophylactic/therapeutic approaches to controlling infection-induced chronic diseases

For new approaches to treat infections, there are different organisations involved, such as the pharmaceutical industries (of which there are only 5 major companies involved), the biotech industry which comprise about 3000 small companies whit very small breakthroughs, regulatory agencies, academics and professional societies. To achieve a major breakthrough in agents against infectious microorganisms it is important that the above-mentioned institutions collaborate. In the USA this is already the case, in Europe this collaboration is planned for the near future.

The ongoing struggle to battle infectious diseases requires continuous de-

velopment of antimicrobial agents. The development of newer vaccines against opportunistic pathogens is promising. Research in the field of immunomodulators provided many new compounds and clinical trials but so far this has not lead to any significant breakthrough. Another area which is of great interest nowadays is the field of probiotics. As stated before it is of great importance that the collaboration between the different industries, regulatory agencies and academic institutions is realised to develop a better understanding and the development of therapeutic or prophylactic measures against the possible effect of chronic infections.