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# OLD HERBORN UNIVERSITY SEMINAR ON NEW ANTIMICROBIAL STRATEGIES: REVIEW OF THE DISCUSSION

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## INTRODUCTION

This year, the program of the meeting covered - unlike previous meetings - a wide scope in the field of interactions between the intestinal microflora and the host organism. The program ranged from the evolution of the immune system to development of a model in large animals and a mathematical model of all interactions involved in physiologic defence to microorganisms. This deviation of the standard procedure was due to the fact, that it was the 10th OHUS-meeting. The second lustrum of OHUS should reveal its intimate relation with the much younger International Study Group on New Antimicrobial Strategies (ISGNAS).

## DISCUSSIONS

The discussions in the meeting were held in the order of the program and are summarised hereafter likewise.

Because the first and the last subject, respectively the evolution of the immune system and the uses and development of computer models were to most discussants new and unfamiliar; e.g. subjects with foreign terminology, these items are summarised more extensively than most of the others. For further clarification, a short reference list is added to the first item.

### Evolution of the Immune System

To understand the evolution of immunity requires an appreciation of the biological context in which it occurs. The following discussion illustrates some key points emerging during the phylogeny of immune reactions. We must be aware that some animals have deviated of the main evolutionary pathway, leading to more complex immune mechanisms. Specific instances of these defence strategies include the maximal shortening of life span, rapid reproduction or the rapid rate of change of generations. This is typical for some arthropod species. The immune vectors of these animals are represented by only limited numbers of phagocytes recognising invading pathogens mainly on the basis of more or less specific lectin receptors, and various humoral substances with relative non-specific activi-

ties. The development of hierarchised and mutually co-operating immune machinery is in these animals constricted by their simple morpho-functional endowment.

The relatively long-living or more complex animals, on the other hand, possess a rather well developed defence system. This is well documented in annelids and especially in some species of the oligochates. Worms do have plenty of coelomic free-wandering cell types which bear the co-operative immune functions (phagocytosis, production of humoral defence factors). Within this animal phylum, the first immune structures have developed as derivatives of the digestive tract, probably owing to a tight contact with antigens in the food. A typhlosole within the annelid gut can be considered as an example of such a primordial immune structure. This organ is found in the evolutionary sequence annelida - acrania - agnata - chondrichthyes (spiral valve) in which it ceases and the spleen develops (*Síma* and *Slipka*, 1995).

The central immune organ, the thymus, appeared as a derivative of the anterior part of the digestive tube (in the dorsal parts of each brachial cleft) in chondrichthyans, 400 million years ago. The other important immune organs, the peripheral lymph nodes, developed in amphibians 350 million years ago.

The second central immune organ regulating humoral immune response, the Bursa of Fabricius, appeared in birds 150 million years ago. The palatine tonsil, a derivative of the brachial region, which, in lower vertebrates, gave origin to the thymus, is considered to be an evolutionary novelty of mammals (with the exception of rodents). Its strategic position forms an antenna of the mammalian adaptive immune system. The palatine tonsil represents an organ of which the immuno-functional importance is not yet clearly understood.

Presently, it is clear that the development of immune structures and organs, and even the differentiation of immunocytes is under control of specific homebox genes. These homeotic genes have been identified in all animal phyla determining their fundamental body plans (*Holland* and *Garcia-Fernandez*, 1996). Genetic manipulation of these genes results in changes in body pattern (malformations) and in the development of organs. The PAX-6 gene for example controls the development of the eye. Other homebox genes are involved in lymphopoiesis, leucopoiesis and haematopoiesis in mammals. Several homebox genes regulate B-cell development during antibody formation (Oct-2) or they are expressed in T-cell neoplasias (HOX 11 which is also responsible for the genesis of the spleen). This subject is more comprehensively reviewed by *Lawrence* and *Largman* (1992); *Roberts* et al. (1994); and *Corcoran* and *Karvelas* (1994).

**Intestinal Immunity**

A recent finding has been that sea star factor (SSF; a protein) can switch on suppression as it prevents immune responses to antigens fed subsequently. Ongoing immune reactions are not influenced by SSF.

Technically it is now possible to isolate inter-epithelial lymphocytes (IEL's) with a yield of 2-6.$10^6$ lymphocytes per mouse. In BalbC mice and in homologous SCID mice IEL's are found but in a higher yield in the SCID mice and the latter kill better infected epithelial cells. Target cells in this test have been Yac-1, D-8ir, HEPA-1a.

Germ-free mice monoassociated with *Morganella morganii* show no change in IEL-yield in comparison with germ-free control animals regarding FACS-analysis of IEL's. However, there is a significant difference between the two in the

humoral immune activity. Mono-association with SFB causes a significantly stronger population of the lamina propria with plasma cells and also expression of αβ-TCR and an increase in Thy1 bearing T cells. SFB can provoke activation of CD8+ T cells.

IgA coating of bacteria finally, may have to do with an escape of coated bacteria from further immune reactivity to the strain.

## Neuro-endocrine Interactions with the Immune System

Considerable evidence exists for neurite apposition with various effector cells of the nervous system. The best described so far consist of the nerve T cell interactions in the intestine and other lymphoid areas, especially those that are T-dependent, nervous connections with Langerhans cells in the skin and with mast cells, especially but not exclusively in the intestine. There are numerous descriptions now in the literature about neuropeptide effects on a variety of immune responses, both *in vivo* and *in vitro*, as well as descriptions of receptors expressed for these neuropeptides on a variety of effector cells. The communications appear to be two way. Most of the descriptions have centred around substance P and the represent positive or stimulatory effects. Other effects have an inhibitory nature and are found with CGRP and somatostatin. One of these effects, therefore, of stress by a nervous connection will be to have an inhibitory effect on Langerhans cells and the initiation or inflammatory response, promotion of mast cell activity or the reverse of T cell function. Nervous mast cell connections have been shown in many different systems and a reflex exists between antigen, mast cells, nerves and target organ such as the epithelial layer. This axon reflex has been demonstrated. Both in the intestine and respiratory tract and is accompanied by increased permeability as a consequence to opening of the tight junctions probably through cytokine effects. Such stress induced increased intestinal permeability, can enhance bacterial transfer.

## Role of Nutrition in Control of Bacterial Translocation

Translocation is an important part of the continuous interaction between host and microflora. In septic animals, a low protein diet appeared associated with a lower incidence of death than in animals on a high (20%) protein diet. This appeared to be related to an increase in unregulated cytokine production by the gut. Conversely, in injured and/or post surgical patients, high protein diets reduce the incidence of infections.

To measure translocation, the presently best known technique uses radio-active labelled microorganisms. $^{14}C$ and more recently $^{111}I$ have been used for this purpose. These labels remain in (living) bacteria. With the I-label it was found that 90% of the translocated bacteria are killed and cleared in the tissues. $^{14}C$ can also be used for measuring the translocation of parts of bacteria; i.e. endotoxin. Endotoxin has thus found to be taken up largely by macrophages and thus induce production of cytokines.

It is however, still difficult to measure (monitor) translocation in man. In such studies, blood cultures are taken and to find fragments of bacteria, probes for *E. coli*, *Bacteroides* and other species have been used. Detecting bacterial DNA is perhaps the most sensitive method available.

There is no evidence for selectivity in translocation. All (kinds of) intestinal micro-organisms appear to translocate, albeit perhaps at different rates and following (mucosal) colonisation in different minimal numbers. Translocation is affected by diet. Diets enriched with

Omega-3 fatty acids, glutamine or riboflavin will decrease translocation and arginine will increase the killing of organisms that do translocate.

## Role of Non-specific Opsonisation in Clearance of Microorganisms

Lectino-phagocytosis (non-specific preparation for phagocytosis) may often (normally) precede the phase of opsino-phagocytosis (binding of specific antibodies with microorganisms to more strongly connect them with Fc-receptors on phagocytes).

Lectins may not play a role in microbial uptake during infections. Lectins may not sufficiently bind bacteria to phagocytes as they opsonise only half as good as antibodies (30-40% vs. 7-0%). In the normal defence in man, the exact role of lectino-phagocytosis is still uncertain. Possibly, opsonins in the gastro-intestinal tract are required for binding of bacteria to enterocytes and thus may play a role in translocation?

For bacterial uptake by phagocytes in suspensions of bacteria and macrophages, opsonins (antibodies) are needed. Fixed (dead) bacteria do not need opsonisation for binding to the cell membrane of macrophages. Perhaps opsonins are not longer necessary because electrostatic forces then enhance binding to phagocytes. It is uncertain whether opsonins are also a prerequisite for rapid clearance by dendritic (Langerhans) cells. Possibly, binding to lectins is sufficient in this type of cells.

Stress proteins (heat shock proteins) may play a role in the repair of phagocytes (macrophages) during (any) immune response(s).

Antigens which are given orally, translocate escape from clearance in lamina propria but bind to dendritic cells in the lymph of the thoraxic duct and are thus presented to T cells. The exact role of this process is uncertain.

Endotoxin (Gram-negative bacterial cell wall) binds chemically to high density lipoprotein (HDL) and fibronectin to be taken up by CD1, CD14 and CD64 cells after translocation.

## Neutrophil Kinetics and the Response to Infection

Neutrophils are formed in the bone marrow and migrate to tissue sites of infection and inflammation. In normal persons with normal blood neutrophil counts, large numbers of neutrophils can be recovered from oral washings because these cells normally exude there to maintain healthy gums and mucosal surfaces. Most forms of chemotherapy depress neutrophil production and impair the oral neutrophil response after chemotherapy. Injection of the haematopoietic growth factor G-CSF will stimulate neutrophil formation by the bone marrow and accelerate the recovery of blood neutrophil counts. The cells which are formed in response to this stimulus have increased expression of CD-14 and CD-64, surface receptors important for their interactions with bacterial products.

## Intestinal Microflora and Neutrophil Production

Haematopoiesis and the formation of neutrophils is ordinarily confined to the bone marrow. In response to many stimuli, including infections and administration of haematopoietic growth factors, haematopoietic precursor cells/ stem cells are often found in the blood and these cells may migrate to other sites where they may temporarily contribute to the host response to infection.

## Applicability of Large Agricultural Animals in Models for Man

Currently, (large) animals, such as pigs, provide infection/metabolism models to study effects of feed composition (including antibiotics, prebiotics

and probiotics) on intestinal microbiological resistance. In such animals this can be done under more controlled conditions (such as in climate-respiration chambers) as compared to man. Gastro-intestinal diseases are among the highest ranking of disease entities in pigs including those diseases which have a zoonotic or food-hygiene impact.

The availability of genetically different and clarified strains or breeds of animals, allows the estimation of genetic influences on disease resistance and immune responses. These responses concern either a major factor or in interaction with environmental factors such as feed.

It appeared that the energy partitioning processes in the organism among maintenance, production (growth) and e.g. immune responsiveness, is highly influenced by infectious agents, be they e.g. bacterial or parasitic in nature. It also appeared, that different types of environmental stressors do play this role. Therefore, these animal models may serve as pre-models for man. There is, on the other hand, a need for future refinement or adjustment of these models to better meet the needs set by research in this field in human medicine.

## Antimicrobial Inactivation by Gut Contents

Most resistance to antimicrobials is induced/maintained in the gut. Antimicrobial activity in the gut may affect many members of the gut microflora and therewith suppress colonisation resistance. In this way, resistant microorganisms, living in the environment of the subject, can colonise proper niches in the digestive tract of antibiotic treated patients and thus put the patient in (potential) danger as well as others in the environment. Low varying antibiotic concentrations in the intestines may also select resistant microorganisms and thus give rise to development of new resistant strains.

Inactivation of antimicrobials in the gut (a site where their action s mostly not required), therefore, could reduce incidence and spread of resistance. Administration of oral cephalosporins is known to lead to an inversed correlation between the presence of inactivating enzymes in faeces compared with the intestinal concentration of the drug and the level of ecological disturbances.

Another approach to reduce the maintenance/induction of resistance to antibiotics, is short treatment (24 hours). During short treatment periods, no effective (suppressive) steady state concentration can develop in the in the intestinal lumen. This approach, however, has limited applicability, as it can only be used in some types of enteritis and for preventive treatment in surgery.

Inactivation of antibiotics can occur in two different ways in the gut:

1. by enzymatic break-down
2. by chemical binding to intestinal contents

Enzymatic destruction of antimicrobials is optimal at a certain pH. It is not present in the gut contents of every individual (man as well as animals). However, it is irreversible, once an antimicrobial is affected it can not longer act; even not following disconnection from the enzyme.

Chemical inactivating binding of antimicrobials occurs in all individuals (man and animals), however, in some stronger than in others. Furthermore there is no homogeneity in the distribution of inactivating factor(s); some days inactivation can be much stronger than in others in the same individual.

Antibiotic inactivation (AI) could be of great practical value in clinical practice. In order to make its use applicable, a reliable system should be developed. Patients under antibiotic treatment should obtain during treatment a sufficient oral supply of AI-substance to stop

any activity in the gut.

## Antimicrobial Peptides of Vertebrates

Antimicrobial peptides are found widely distributed in Nature and have been isolated from a variety of vertebrate sources. The magainins were isolated from frog skin and help to defend the mucosal surfaces of the animal from environmental microbes. Similarly, a class of antimicrobial peptides known as defensins are produced by the epithelia covering most exposed surfaces of the mammalian body including the respiratory and digestive tracts. In mammals, this system is dynamic and responsive to insult. This response is likely mediated by bacterial products such as LPS and cytokines such as TNF. Recent evidence suggests that dysfunction of epithelial antimicrobial peptides may play an important role in the lung pathology seen in cystic fibrosis. Antibiotic peptides kill pathogens by a unique mechanism of action involving pore formation in target membranes. These peptides typically are active against bacterial strains that are resistant to current antibiotics. Some synthetic analogues are resistant to degradation proteases. These features make this class of antibiotics attractive for pharmaceutical development. Such peptides are currently under development for the treatment of infected foot ulcers, in cystic fibrosis, and cancer.

## The Uses and Development of Computer Models

The computer model developed in the ISGNAS-pilot study provides evidence that modelling of interactions within the gut microbial ecosystem is feasible. One use of such a model is checking whether combined *in vitro* data obtained from, e.g., chemostat experiments could indeed mimic the *in vivo* behaviour of the complete system. Furthermore, as has been done with the Cybermouse *in silico* murine immune system, models can be used both to design *in vivo* experiments rationally and to assist in the interpretation of results. Before any experimentation begins, parameters which give the best discrimination between competing theories may be determined. This may lead to a reduction of the number of laboratory animals, healthy volunteers and patients needed.

What is unlikely to be accomplished with such a model is prediction of developments in the microflora of individual patients. The number of unknown and unmeasurable parameters is probably too high to ever achieve such accuracy in modelling.

The model as it stands in far from complete. A step-by-step plan to improve the model is proposed:

1. Add more different species of bacteria,
2. Add a wider variety of food substrates and toxins,
3. Allow bacteria to produce metabolites and toxins,
4. Combine to form food webs,
5. Add adherence sites on epithelium
6. Add chemotaxis,
7. Within this more complex model, study CR,
8. Add an immune system.

The current model already supports bacterial motility (without chemotaxis), by increasing the diffusion rates of certain species at the expense of growth rate (higher basal metabolism). For the first four steps, a more or less generic (meta)model of a bacterial metabolism must be made. Once this has been formulated it can de used in two very different ways: (i) tactically, to closely mimic existing bacteria by careful design, and to see whether this models the actual system, and (ii) strategically, by assigning random metabolisms (within certain reasonable limits) to species, entering various combinations of sub-

strates, and to see what type of food web emerges, and which types of survival strategies are feasible within this artificial environment. Evolution could also be mimicked by introducing random changes into the metabolic properties of bacteria. It is obvious that there are many possible *in silico* experiments within the current framework. An invitation is issued to researchers within the field of study of the ISGNAS to design and if possible conduct such experiments in collaboration with the ISGNAS effort.

Adding epithelial binding sites should be done using the model of *Freter* et al. (1983). The inclusion of an immune system should involve the use of one or more of the *in silico* model immune systems developed elsewhere (e.g. Cybermouse at UCSD, or the Theoretical Immunology Group at SFI). Links with competing models could provide a valuable test of the applicability of each model.

## LITERATURE


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