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OLD HERBORN UNIVERSITY SEMINAR ON IMMUNE SYSTEM AND MICROFLORA: MINUTES AND OVERVIEW OF THE DISCUSSIONS

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INTRODUCTION

Type of patients, type of infections in hospitals since the introduction of antimicrobial drugs

As outlined by Maxwell Finland in 1959 (*Finland* et al., 1959), both the spectrum of bacteria involved in serious infections in hospitalised patients and the type of patients have changed significantly since the introduction of antimicrobial drugs in 1935. In the pre-antimicrobial era, infections by pathogens dominated the scene. Since antimicrobial drugs became available, potentially pathogenic (opportunistic) bacteria and later yeasts caused most infections. In these years, also the more compromised type of patients gradually replaced the conventional type of infected patient with an infection by a pathogen.

Resistance to antimicrobial drugs

Since the introduction of antimicrobial drugs, it also became evident that, regardless many repeated attempts of the pharmaceutical industry, it has not been possible to master the resistance problem. It is for this and other reasons not to be expected that antibiotics will provide the ultimate answer to the problem of opportunistic infections in the increasing number of compromised patients in our hospitals.

Effect of antimicrobial drugs on intestinal microflora

Many antibiotics have appeared to affect the indigenous microflora of the digestive tract. Suppression of the microflora is an important factor in the transmission of resistance to other bacteria in the tract as well as in the spread of resistant bacteria to other patients.

The Old Herborn University Seminars

It was decided in 1985 to organise annually a meeting with more time for discussion than for presentations on the various aspects involved in the interactions between host (patient) and his microflora. This kind of meeting has become known as the Old Herborn University Seminar. It was hoped that, by collecting information around this theme by inviting experts in the various subsets of this issue, we might learn step by step in which manner healthy individuals interact with and withstand opportunistic microorganisms.

Recently, during the Third South Pacific Congress on Chemotherapy in Infectious Diseases, data have been reported about the incidence of multiply resistance. In a number of hospitals in some countries, antibiotic treatment is

not longer effective for this reason. This information has urged us to speed up our attempts to collect information supporting our search for other ways than antibiotic therapy for prevention and treatment of opportunistic infections.

The present meeting was specifically focussed on the physiologic routes of contacts and interactions between the immune system and microflora as well as on techniques by which these interactions could be measured with great sensitivity and accuracy. The outcome of the discussions of the meeting could possibly be used as a basis for the design of (standardised) protocols to study new ways of prevention and possibly even therapy of opportunistic infections in hospitals.

DISCUSSIONS

General

The program of the meeting was based on the fact that the antimicrobial defence system consists basically of two mechanisms which should both be measurable in greater detail:

a. *Inter-bacterial interactions* (including competition for nutrients provided by the host organism). Inter-bacterial interactions inside the digestive tract could be regarded as a first line of defence. This part of the defence system in which the host organism is also involved is called *colonisation resistance of the digestive tract.*

b. *The antimicrobial defence system of the host organism* consisting of an *a-specific part* (phagocytosis killing and digestion of microorganisms) and a *specific part* by cells of the immune system. The specific part is involved in both opsonisation of bacteria by coating them with antibodies and the subsequent cytotoxic killing of phagocytosing cells that have been paralysed by the ingested organisms.

The discussions concerned these fundamental points. In addition techniques to measure the activity of the interactions between host and microflora have been discussed.

Colonisation resistance

The *colonisation resistance* (CR) of the digestive tract was defined as the resistance encountered by a (non-viral) microorganism when it tries to colonise a particular niche in the digestive tract upon oral intake. The resistance would be the net resultant of a concerted action of the host organism (by feeding the indigenous flora with saliva/mucus and extruded cells in the mucosal layer) and his indigenous microflora (by competition for nutrients and by producing substances with some hostile [antibiotic] activity). Presumably, secretion of antibodies into the intestines plays a role. The combination of host and flora forms a stable ecosystem.

The CR may differ between individuals of an animal species. A lower CR may imply an enhanced risk of infection, especially in compromised individuals. Therefore, it is to be expected that it will become of practical value to measure the CR in individual patients. However, it is still difficult to measure the CR directly in patients. Firstly, this is so because it requires oral contamination with living potentially pathogenic bacteria. Secondly, for each bacterial species the CR may be different although values overlap. Apperloo-Renkema reported a linear correlation between the mean ^{10}log concentration of the *E. coli* strain used for oral contamination and the CR measured by comprehensive biotyping of Enterobacteria species in four faecal samples. Indirect

measurement of the CR by comprehensive biotyping of Enterobacteria species in minimally four faecal samples is the only way for the time being. However, this is a laborious and expensive way of measuring the CR. New techniques to determine the CR clinically should therefore be developed.

Suppression of the composition of the microflora by antibiotics during treatment of an infection as well as by modulation with food additives and diet may influence the CR to foreign bacteria respectively negatively and positively. In addition, it may influence the interactions between the flora and the immune system. In previous seminars, it appeared that flora suppression with antibiotics might play an essential role in the outcome of experimental treatment of tumours in oncology. In another previous seminar, the potential role of the microflora - and thus the effect of its modulation - on the development of autoimmune phenomena such as Graft-versus-Host Disease (GvHD) has become evident.

Methods to measure the CR

Factors, which can be used to determine the intactness of the indigenous microflora, are called *Microflora Associated Characteristics* (MAC's). Several MAC's have been reported, most of which concern chemical activity of bacteria, one MAC concerned the morphology of the faecal microflora. Many of the bacteria involved in MAC's are presumably involved in the CR. However, a study in which this relation has been specifically studied is not known. The use of β-aspartylglycine as a (negative) marker for intactness of the flora as a MAC was regarded most promising. However, its concentration may fluctuate rather strongly in a series of faecal samples of individual subjects without proven concomitant fluctuations in the composition of the intestinal flora.

The entropy of the micromorphological diversity of the faecal flora as determined by *Meijer* et al. (1991) in nigrosine stained faecal smear preparations with computer software designed for this purpose, theoretically also could be considered as a measure for CR. However, no study is known to date that indicates a correlation between the micromorphological entropy and the CR for one or more potentially pathogenic bacteria. The entropy however, is easy to determine. Furthermore it has not only shown a close relation between the outcome of anaerobic culturing of faecal samples during antibiotic treatment but also showed a slight but significant decrease after some time during oral treatment of volunteers with Symbioflor 1® (a mixture of ten different strains of living *Enterococcus faecalis*). This requires further study.

Correlation between antibody titres of Ig-isotypes and the degree of CR. The relation between the CR and an anti-potentially-pathogenic antibody titre has been studied indirectly. This means that a correlation was sought between the titre of circulating antibodies to an *E. coli* strain used for oral contamination in healthy human volunteers. There was however in that study only an indication that high IgM antibody titres to *Enterobacteriaceae* species in the faecal samples did correlate with a low CR. A significant correlation was not found for IgG or IgA antibodies.

THE DEVELOPMENT OF DEFENCE SYSTEMS IN THE COURSE OF EVOLUTION

Our planet was first colonised by autotrophic bacteria; later mutants developed which could make use the feederlayer formed by autotrophic bacteria.

In the course of billions of years, different ecosystems have thus been formed at different places. Their defence to newcomers (bacteria developed in another niche), which may have been moved in by the wind from time to time, may have been the same as it is nowadays: i.e. competition for nutrients and the presence of toxic substances (antibiotics) for which the local population was naturally resistant. Bacteria and other primitive cell-systems had - and still have - a strong capacity to adapt to external (environmental) changes in several generations. Development of resistance to antimicrobial drugs (including the man-made and man-modified antimicrobial drugs) was therefore to be expected. The design of an antimicrobial agent for which no resistance can develop may never be possible.

From the bacterial ecosystems, eukaryotic cells may have developed as suggested by Lynn Margulis in her book "Symbiosis in cell evolution" (ISBN 0-7167-7028-8). These cells may have been comparable to our present monocellular organisms in their capacity to defend themselves to micro-organisms, namely by phagocytosis, intra-cellular killing and -digestion. When later in evolution multi-cellular organisms developed a digestive tract, they may have attained a microflora in that tract similar to the ones present in primitive animals. More recent in evolution legs, wings or a tail to swim developed. New animals could actively move to different places. Arrival in new environments may only have been possible because the simultaneous development of an (primitive) immune system. The static system formed by the antibiosis by the digestive tract ecosystem may not have been adequate to protect the host organism to new bacteria encountered in other (microbial) environments. For the relatively rapid changes in the spectrum of microorganisms present in the environment, encountered by moving from one place to another, a specific flexible defence system was required. The antimicrobial defence had to become specific because it should not affect bacteria of their own gut ecosystem like antibiotics may do during treatment. The defence had to be dynamic and adaptable because of the development of resistance by bacteria. Microorganisms appear to have the capacity to vary antigens in their outer membrane so that they become less sensitive to the existing immune response.

It should be attempted to learn more from developments during the evolution, because these developments have been dictated by Nature and they may represent the most optimal format for long-term survival of the various species including man.

IMMUNE INTERACTIONS WITH MICROFLORA

General

Central in the discussion was the fact that in healthy individuals no signs and symptoms of inflammation are seen although bacteria appear to pass through the gut epithelium and therewith might provoke an inflammatory response.

Inflammation was defined as the response of white blood cells and their products to foreign antigens to such a degree that local vasodilatation occurs and more white blood cells (and their products) are attracted.

In severe cases, the inflammatory response may become systemic and involve many organs (multi-organ failure) being associated with an increase of the serum concentration of interleukins such as IL-6 and TNF.

Oral tolerance

The absence of inflammation upon penetration of bacteria (and food antigens) which have induced hyporeactivity after oral ingestion is assumed to be due to a mechanism called oral tolerance. In classical experiments, oral tolerance was induced by oral gavage of high numbers of sheep red blood cells in mice or rats. Upon subsequent parenteral challenge with sheep red blood cells, no or low titred antibody response occurred. This type of induced tolerance was transmissible with spleen cells. For this reason it is likely, that CD-8 T-suppressor cells are involved.

Interactions between intestinal bacteria and immune system

The bacteria of the resident flora which colonise the mucus of the intestinal epithelium are presumably in continuous contact with the Gut Associated Lymphoid Tissues (GALT) and/or the systemic immune system. Presumably predominantly cells of the GALT are responsible for:

1. The humoral immune hyporeactivity to bacterial and food antigens, and
2. The absence of delayed type hypersensitivity reactions to intestinal antigens (including bacteria).

In order to get in contact with the immune system, bacteria or bacterial fragments must cross the barrier formed by the epithelial lining. This transfer through the epithelial lining is called *translocation.* Translocation of bacteria may include bacterial fragments and even larger (dietary) molecules.

Translocation of pathogens such as *Salmonella typhi*, *Listeria monocytogenes*, and *Mycobacterium bovis*, may occur predominantly through the M-cells overlaying the Peyer's patches. Other bacteria however, as has become evident in EM-studies by Wells and coworkers may (also) translocate elsewhere. Translocation of intestinal bacteria may therefore also occur in the colon in which Peyer's patches are rare. *In vitro* studies with colonic epithelial cells have made likely that bacteria are actively embraced by the microvilli and once they are completely engulfed they get internalised in the cell. Intra-cellular transport to the basal membrane and the underlying lamina propria may *in vivo* complete translocation. Translocation is not confined to the digestive tract as it has also been observed in the bronchi into the Bronchus Associated Lymphoid Tissues (BALT) and by bacteria of the vaginal flora to the local draining lymph nodes.

Induction mucosal hyporeactivity

Induction of tolerance by bacteria, which are indigenous in the digestive tract, may play a role in the absence of inflammation in the lamina propria and the sub-mucosa. Disturbance of this balance between immunity and tolerance could cause local or more diffuse inflammation in the intestines upon translocation of resident (colonising) bacteria. There is indeed evidence, which makes likely that, among else, 'chronic inflammatory bowel disease' could be caused by improper function of the gut associated immune system.

Influence of circulating antibodies on the occurrence of translocation

In some publications it is reported that specific immunity would reduce translocation of corresponding bacteria. Observations reported by others, however, do question that. It was suggested that this phenomenon should be studied in SCID mice who have neither T nor B cells and in RAG-2 knock-out mice. Only in this way the precise contribution of both cell types to translocation could be studied.

Translocation in man

Translocation should also be studied more intensely in man. However, such studies are only possible in patients undergoing abdominal surgery and possibly in 'organ donors' following CVA.

In both types of subjects, however, several additional factors may influence the outcome. In surgical patients this could for example be the underlying disease and anaesthesia.

ORAL IMMUNE TOLERANCE

General

Oral tolerance (immune hyporeactivity) preventing cellular immune reactivity may result from the formation of suppressor-cells in the gut's parenchyma or lymphoid tissue. After migration of these cells to the periphery, systemic tolerance may be the result. A well-known example is the prevention of nickel allergy by oral intake of nickel chloride molecules.

Mechanisms involved

Two mechanisms were considered responsible for the absence *of delayed type hypersensitivity (DTH) reactions in the digestive tract wall*:

a. Upon intake of an antigen (could be a fragment of a bacterium) in Peyer's patches, T-helper cells instruct B cells to produce IgA. After transfer through the thoracic duct, these B cells migrate back to the gut and invade the lamina propria where they differentiate into actively IgA secreting plasma cells. Secretion of IgA into the gut lumen and its specific binding to bacteria (and other antigens), might cause enhancement (antigen-blocking) and thus prevent further immune (cytotoxic T-cell) activity to the antigen in question if translocation occurs.

b. The environment in the lymphoid tissue of the gut might be such, that only Th-2 reactivity can develop (e.g. high local production of IL-4 or IL-10). Such an environment not only prevents the development of Th-1 activation (and therefore local DTH reactivity) but might also be responsible for the formation of Th-2 cells. Antigen specific Th-2 cells will migrate to the periphery and may, after renewed antigen contact, prevent Th-1 reactions to occur by secreting IL-4 and IL-10 lymphokines.

Oral tolerance upon association with bacteria in the new-born and in adult germfree mice

In new-born mice, antibodies as well as maternal lymphocytes obtained passively by passage of the placenta and via the milk, may play an essential role in the adaptation to the development of an gastro-intestinal microflora. Conventionalisation of adult germ-free mice, however, also rarely causes disease or death due to infections. In the conventionalised adult germfree animal the idiotypic antibody network and NK cells may play an essential role in bridging the interval after conventionalisation until 'oral tolerance' has developed. Initially however, quite substantial bacterial translocation may occur. A period with enhanced bacterial translocation shortly after birth (conventionalisation) has not been described in baby mice. However, an initial phase with increased uptake of larger dietary molecules is known to exist. In both conventional new-born mice and in mice who are conventionalised at adult age, circulating antibodies to intestinal bacteria are formed. However, the titres are generally low.

This information about circulating antibodies to intestinal bacteria was reported in the fifth Old Herborn University Seminar.

VACCINES

The vaccines, defined as preparations of dead or life microorganisms, are meant to instruct the immune system. Instruction of the immune system may either result in a decrease of the response (suppression) to a subsequent challenge with the same antigen or to an enhanced response (activation). Two lines of administration have been discussed: Orally and parenterally.

Oral vaccines

Life bacteria

The results of the use of a life oral vaccine were reported on the first day of the meeting. It concerned Symbioflor 1®, a mixture of ten *Enterococcus faecalis* strains. Medical use of this vaccine would suppress inflammation in the oropharyngeal region. In nine of ten volunteers, a significant decrease of the titre of circulating IgG to *Enterococcus faecalis* was found. In addition, this vaccine appeared to influence the composition of the faecal flora of the volunteers according to a slight but significant decrease of the entropy of the micromorphology of the microflora. This change in the flora occurred concomitantly with a decrease in the IgG antibody titre to the autologous faecal flora during treatment.

The site at which *Enterococcus faecalis* may have been taken up was either in the tonsils or by the Peyer's patches in the small intestines. It was suggested to study whether the tonsils or the Peyer's patches are the predominant port of entry by application of the Symbioflor 1® mixture in enteric-coated capsules in future studies.

In this category of vaccines, the use of strains of potentially pathogenic bacteria isolated from the stools of an individual also urgently requires study. It seems plausible that these vaccines 'boost' negatively (suppressive) or positively, the pre-existing immune response to the individual strains in the vaccine. It seems plausible that these vaccines can be administered parenterally as well. However, since no information is available as yet, great caution should be taken with this mode of administration. Experience with single pure cultures of Gram-positive and Gram-negative bacteria is first required before the use of mixtures of pure cultures can be considered.

Dead bacteria

Also small peptides of intestinal bacterial (outer membrane) origin appeared to influence the immune system upon oral administration in mice. The influence of some of these molecules on the immune system was boosting, whereas the effect of other molecules was suppressive. The chemical analysis of these molecules is being done.

Parenteral vaccination

There is no information about the effect of parenteral vaccination on translocation of potentially pathogenic bacteria and subsequent stages of clearance of these bacteria. This subject requires study.

DEVELOPMENT OF TECHNIQUES TO MEASURE ANTIBODY TITRES TO COMPONENTS OF THE FAECAL FLORA

Fluoro-morphometry

With the help of a system designed and developed in Groningen, the GRID-system, it was possible to reproducibly determine the amount of circulating antibodies to objects (bacteria) in the

faecal flora. A study by Apperloo-Renkema has made likely that the amount of fluorescence measured per object correlates linearly with the classical (^{2}log) antibody titre to the same object(s). These studies have been published. Some discussion occurred on the desirability of the use of markers for bacteria. With the help of specific markers for bacteria, it may become possible to distinguish between bacteria and bacteria-like particles in faeces.

Analysis of antibody coating of faecal bacteria by flow-cytometry

Both *in vivo* coating and *in vitro* coating of bacteria has been found possible, provided the events (bacteria) are marked with propidium iodine (PI) and the correct filters are used. This technique correlated well with data obtained by fluoro-morphometry in corresponding faecal samples. Flow-cytometry analysis was reported to be faster than fluoro-morphometry.

CONCLUSIONS

Our future attempts to find alternative ways of prevention/treatment of infections could perhaps best base on the physiologic mechanisms, which have developed and survived during evolution. Only relatively little is known of the cells and substances involved in the normal control of potentially pathogenic microorganisms.

Attempts to study this new concept for prevention/treatment of infections by potentially pathogenic microorganisms should concern the design of an animal model to study the occurrence of bacterial translocation as well as studies of the mechanisms involved in the physiologic smooth (unnoticed) way in which translocated bacteria are cleared from the tissues/circulation in healthy subjects.

Meanwhile, methods have been designed and tested to measure and follow in the time, the interactions between various cells of the humoral part of the immune system and indigenous bacteria.

Because of the obvious role of intestinal bacteria in autoimmune phenomena, a study of ways to prevent/treat infections along physiologic lines, outlined by Nature during the evolution, seems important.

FORMATION OF AN INTERNATIONAL STUDY GROUP

Because there is an urgent need to search for other ways of prevention and therapy of infections, since the usefulness of antibiotics is rapidly declining, it was discussed whether an International Co-operative Study Group should be formed.

Two facts will form the basis for the study of ways for prevention/treatment of infections in the future:

a. The great majority of the infections in patients is caused by opportunistic bacteria. Changes in the physiologic defence mechanisms of the body provide these bacteria with a chance to cause clinical infections.

b. The development of resistance in opportunistic bacteria to specific immune responses has remained zero during evolution. This makes likely, that the immune system is sufficiently rapid and flexible to follow changes in the antigenic composition of opportunistic bacteria.

LITERATURE

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