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10. NEW ANTIMICROBIAL STRATEGIES

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INTERNATIONAL STUDY GROUP ON NEW ANTIMICROBIAL STRATEGIES (ISGNAS): MEETING THE CHALLENGE OF RESISTANCE TO ANTIBIOTICS

THE ISGNAS WRITING COMMITTEE*

SUMMARY

ISGNAS enables advancement of research through building a network of organisations and is also working to develop new antimicrobial strategies. Communication among participants is accomplished through published reports, E-mail, Internet, symposia, and special announcements.

INTRODUCTION

The International Study Group on New Antimicrobial Strategies (ISGNAS) was formed in response to the recognition that development of microbial resistance to antibiotics is becoming a serious, world-wide problem. The group met in 1993 for the first time to discuss the feasibility of developing rational alternatives to the use of antibiotics and prepared as a result a comprehensive overview of normal (physiologic) mechanisms involved in the control of potentially pathogenic (opportunistic) microorganisms.

One objective of ISGNAS is to understand the conditions which allow opportunistic microbes present among the symbionts to cause an infection. There is a need for more coherent information concerning the habitat, growth requirements and host and pathogen properties which allow opportunistic pathogens to cause life-threatening infections. In particular, information is urgently being sought to understand the complexity of the interactions between the vast number of microbial species, and the interactions between the microbes and their host.

Another goal is to inspire and enable basic and clinical research that will lead to the development of new therapies for regulating colonisation, translocation and infection by opportunistic microorganisms in patients during periods of decreased resistance. With a sufficient amount of knowledge of how healthy individuals keep opportunistic microorganisms under control, it may become feasible to iatrogenically maintain host-resistance and inter-microbial factors involved in containment of opportunistic microbes. Therapies aimed at bolstering natural resistance mechanisms will be of critical importance to individuals whose resistance has been compromised as a result of another clinical condition.

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CONCLUSIONS AND RESEARCH RECOMMENDATIONS BASED ON THE ISGNAS REVIEW

It was decided that the information in the review of ISGNAS would be used to identify gaps in the present knowledge. The subjects described in the gaps are described by ISGNAS as Research Priorities. These research priorities would serve as a lead for research in experimental animals as well as in man (patients) to be conducted by experts in well experienced centres in the world. The following paragraphs are based upon the ISGNAS review (*Areneo* et al., 1996).

I. The normal gastro-intestinal microflora (GIM)

Bacteria, resident in the intestinal microflora suppress multiplication of newly ingested bacteria and may (sterically) hinder their adhesion to the mucosal lining. Accordingly, ingested bacteria and yeasts either die or get gradually eliminated by excretion with the faeces. This first line of defence is called Colonisation Resistance (CR).

CR flora may release substances which interact/interfere with receptors and adhesion molecules for bacteria on host cells. They could also release/produce immunomodulating substances. This modulation obviously occurs predominantly in the 'gut associated part' but may also modulate the 'systemic part' of the immune system, the mononuclear phagocytosing system (MPS) and the bone marrow. CR flora may also interact with the neuro-endocrine system and condition the mucosal or epithelial barrier. Finally, the composition of the diet influences the actual composition of the intestinal microflora.

Research Priorities:

- Development of technique(s) to accurately determine the composition of the GIM and the quality of the CR in a short time and at low costs.
- Study of substances (nutritional and microbial) by intestinal microorganisms involved in interaction with receptors for bacterial adhesion on mucosal cells.
- Study of bacteria in the normal microflora which produce immuno-modulating substances, to chemically characterise the chemical composition of these substances and to identify target host cells and their responses.
- Study of indigenous and exogenous microbial substances which influence bone marrow haemopoietic activity.
- Study of the influence of the composition of the diet and probiotics on the microflora and on the host.
- Study of substances which influence the immuno-neuro-endocrine system.
- Study of substances which influence the GI-tract motility.

II. Intestinal barrier integrity

In vivo loss of intestinal barrier integrity has been reported in the myriad of clinical conditions noted to predispose a patient to bacterial translocation. Essentially, all the diverse clinical conditions are associated with bacterial translocation and nearly all these conditions are associated with altered intestinal flora, which mostly concerns bacterial overgrowth. Increased intestinal permeability (opening of epithelial occluding junctions) can clinically be assessed by several methods including urinary excretion of orally administered agents.

Research Priorities:

- The mechanism whereby loss of intestinal barrier function might permit intestinal bacteria to migrate out of the intestinal lumen to distal sites.

III. Microbial interactions with the intestinal mucosa

The mucosa produces a mucus layer which is a defence mechanism. The mucosa, under normal physiological conditions, would allow translocation of microorganisms to a limited extent.

The microflora stimulates (perhaps indirectly) the activity of the undifferentiated cells in the crypts of the intestinal mucosa.

Research Priorities:

- Identification of the [type(s) of] microorganisms and of [the type(s) of] host cells involved in the modulation of mucus production by mucosal cells (modulation of the quality of the feeder layer).
- Study of bacteria in the intestinal tract which modulate proliferation and maturation of undifferentiated crypt cells.
- Study of the process of translocation of bacteria as well as their bio-active fragments.

IV. Site of clearance of microorganisms as well as microbial fragments

The gut lamina propria, the mesenteric lymph nodes, the spleen, the liver and the bone marrow can be regarded as important clearance sites. In the lamina propria translocated microorganisms might be cleared in a non-inflammatory way. This system may be functioning even under normal physiological conditions. The liver, being directly supplied with blood from the intestines, is in an exceptional position and may be primed for the clearance of bacteria and bacterial products in the systemic circulation. Modulation of the GI-tract microflora may also influence the function of hepatocytes.

Research Priorities:

- To investigate whether the lamina propria is an important site at which a significant proportion of translocated bacteria and yeasts are cleared.
- To identify factors to stimulate the production of specific and non-specific opsonisation of translocated microorganisms and microbial fragments.
- To identify factors by which the intestinal microflora interacts with the function of hepatic cells.
- To identify factors which increase the systemic clearance capacity.

V. The gut associated lymphoid tissues (GALT) and the systemic immune system

The GALT includes lymphoid organs in the GI-tract (Peyer's patches), lymphocytes in the lamina propria. The systemic immune system involves the thymus, the bone marrow and the lymph nodes (not associated with the gut) and the spleen.

Research Priorities:

- Study of cellular and humoral immune responses which are induced by normal microflora as well as the induction sites normally used by bacteria. This includes opportunistic bacteria and yeasts as well as non-pathogenic microbes.
- Study of the implication of the type of immune response induced for the development of inflammatory (clinical) signs and symptoms. The relevance of the different isotypes of antibodies produced.
- Elicit the roles and function of IgA.
- Determine the importance of oral tolerance.
- Role of the immune system on the composition of the microflora (its modulating capacity and any possible replacements).
- Identification of members of the microflora in the development and activation of the mucosal immune system.
- Study of the effectiveness of vaccination; active or passive.

- Identification of factors released by the microflora which are chemotactic to phagocytic cells.
- Evaluate the efficacy of immuno-modulators in increasing both systemic and mucosal non-specific immunity to pathogens.

VI. The bone marrow

The bone marrow is the site of origin of, among else, specific and non-specific immune cells.

Research Priorities:

- To identify factors which may focus haemopoietic activity towards cells involved in the non-specific immune system with the purpose of eventually manipulating the system.
- To study the influence of flora modulation and other factors on the survival of stem cells (bone marrow as well as intestinal crypts cells) during chemotherapy/irradiation.

VII. Immuno-neuro-endocrine system

The Immuno-neuro-endocrine system is under influence of the gut microflora and vice versa, and controls on the other hand also the systemic resistance.

Research Priorities:

- To identify factors released by the microflora which locally or systemically modulate the neuro-endocrine system.
- To modulate the neuro-endocrine system to maximally control opportunistic pathogens.

VIII. Mathematical modelling of the host-microflora interaction

As new detection methods are developed and observational data on the gastro-intestinal microflora (GIM) and its interaction(s) with the host are improved, it will become increasingly necessary to develop a theoretical framework to explain the observations. As mathematical models have been used successfully in a number of different ecosystems, both microbial and of higher organisms, it is not unreasonable to assume that such models may increase our insight in this case. Especially in the case of complicated, non-linear systems (including the immune system), computer simulation can provide a powerful means to integrate knowledge obtained from studying various parts of the system in isolation, and to provide insights into the dynamics. Advanced time series analysis techniques on data obtained by other ISGNAS efforts must be used to validate the models.

Research Priorities:

- Development of time series analysis techniques to detect the presence of non-linear (chaotic) behaviour in the dynamics of the GIM and the defence system.
- Development of a computer model of the GIM based on bacterial physiology; comparison of theoretical and observed dynamics.
- Integration of the above model with models of the immune system, bowel motility etc. developed elsewhere (or currently under development).

RESOURCES OF ISGNAS

The international study group consists of biomedical scientists (both medical and basic), each an expert in a different subspecialty of host defence mechanisms (Table 1).

Table 1: Membership of ISGNAS

Member	Location	Expertise
J. Beuth	Köln, Germany	Bacterial immune stimulating products
J. Bienenstock	Hamilton, Canada	Neuro-immuno-endocrinology
J.J. Cebra	Philadelphia, USA	Intestinal immunology
P.J. Heidt	Rijswijk, The Netherlands	Gnotobiology
W.L. Manson	Groningen, The Netherlands	Infections in patients with decreased resistance
T. Midtvedt	Stockholm, Sweden	Microflora acquired characteristics (MACs) and Germfree associated characteristics (GACs)
J.P.T.M. Noordhuizen	Wageningen, The Netherlands	Zootechnology/Infectious disease control in agriculture
C.E. Nord	Stockholm, Sweden	Antibiotic resistance
P. Nieuwenhuis	Groningen, The Netherlands	Systemic immunity
V. Rusch	Herborn, Germany	Immune stimulation by live bacteria
D. van der Waaij	Groningen, The Netherlands	Medical microbiology
R.I. Walker	Rockville, USA	Vaccines
M.H.F. Wilkinson	Groningen, The Netherlands	Mathematical modelling

LITERATURE

B.A. Areno, J.J. Cebra, J. Beuth, R. Fuller, P.J. Heidt, T. Midtvedt, C.E. Nord, P. Nieuwenhuis, W.L. Manson, G. Pulverer, V. Rusch, R. Tanaka, D. van der Waaij, R.I. Walker, and C.L. Wells: Problems and priorities for controlling opportunistic pathogens with new antimicrobial strategies; an overview of current literature. Zbl. Bakt. 283, 431-465 (1996).

THE DEVELOPMENT OF THE DEFENCE SYSTEM DURING EVOLUTION

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SUMMARY

A progressively growing bulk of evidence has shown that the immunity is an integral part of a general homeostatic system maintaining the integrity of the internal environment of an organism. Eumetazoan animals respond to the pathogens invasion with a cascade of adaptive reactions involving neuroendocrine and immune co-operations. In all living animals today the immune mechanisms evolved into an effective device which enabled their successful survival, and above all, their adaptive radiation in the biosphere. The immune strategy common to every natural assemblage of animals (a phylum) represents an appropriate fundamental morphofunctional pattern according to evolutionary history, and is determined by the natural forces of the environment in which these animals have radiated. The important animal assemblages in their order of increasing complexity comparing their basic body plans in relation to their immune potential are depicted in this survey.

INTRODUCTION

There is no function without a structure, and there is no structure without a history. The history has always two faces, an ontogenetical and a phylogenetical one. The immunity is one of the basic attributes of all living creatures. The aim of this short overview is the reconstruction of its emergence from various points of view, and its evolutionary history. For this purpose we can theoretically admit the existence of major superassemblages of more interrelated, natural groups of animals. Within these groups, the immune capabilities of their representatives in the relation and dependence on their morphofunctional endowment will be determined.

GENERAL REMARKS

Evolution of homeostatic systems

Homeostasis is a major attribute of all living matter, which is realised by a row of orchestrated autoregulative processes, including immunity (*Austin*, 1978). All living creatures from the protists upward behave as open systems (*Bertalanffy*, 1953). We may accept them within the definition made by *Orgel* (1973) as "Complex - Information - Transforming - Reproducing - Objects - that - Evolved - by - Natural -

Selection". This definition can, from immunological point of view, be modified by adding - "and maintaining their integrity".

Even the most primitive ancient organisms had to integrate their simple processes into a harmoniously balanced form, and more than one function had to co-ordinate at the same time. Fulfilment of this condition is the basis for transition from inorganic matter to a living organism regulating its metabolism. The emergence of regulatory mechanisms was fundamental for establishment of the homeostatic control (*Bernard*, 1877; *Lahav*, 1985).

A role of hierarchisation in evolution of homeostatic systems

Obviously, the more complex an organism is, the more stratified is the organisation of its homeostatic devices ("the stratified stability"). The capacity to maintain stability increases with greater stratification of the biological system.

The genetic code, and the biochemical and physiological processes are virtually constant among both the lowest and highest animal phyla. More than 90% of total enzyme activities is common in both the prokaryotes and eukaryotes. Similarly, the cells as structural entities (tissues and organs as well) are constructed with the same uniformity. Thus the only evolutionary way of achieving the greater complexity is by increasing the structural and functional hierarchisation (*Bronowski*, 1970; *Bonner*, 1988).

Homeostasis has adaptive advantages

Each higher level of homeostasis ensures better adaptation capability of species (*Huxley*, 1953). The adaptive advantages of homeostasis have been obviously functioning in periods of evolutionary stasigenesis, when ensured a relatively long-lasting stage of stable survival of the species (*Rensch*, 1966). Qualitative changes of homeostatic mechanisms have passed during evolutionary dysbalance, within stages of anagenesis (progressive evolution) and cladogenesis (diversification).

Evolution of neuroendocrine regulation

The changes in the metazoan way of life from the sessile way to the active seeking of food were accompanied by the origination of new structures and organs that would ensure more developed locomotory functions. The increasing morphofunctional complexity required a considerable increase in energy supply. The ancestral metabolic system was not sufficient any more, but the new, more efficient mechanisms for obtaining energy from the food did not evolve as a consequence of this transition. It was precisely the evolving and consolidating of the system of hormonal hierarchical regulations that assured the more efficient stimulation of these traditional metabolic pathways to manufacture energy more effectively (*Czaba*, 1980; *Pertseva*, 1991).

Co-evolution of immune and neuroendocrine systems

Up to the present, the highest level of homeostasis has been reached by the mutual functional interconnection of highly hierarchised systems, the neuroendocrine and immune (*Ader*, 1981). Almost all hormones secreted in the organism have been shown to significantly influence the immunological reactions and humoral immune vectors like cytokines may affect their neuroendocrine targets. Moreover, many kinds of hormones are manufactured by immunocompetent tissues and cells. Immune and neuroendocrine systems are similarly organised and phylogenetically emerged in a similar way: from dispers-

edly localised cells and tissues towards highly structurally hierarchised organs.

Immune mechanisms have evolved as a defence of individuality

The existence of living organisms on the Earth is conditioned by their ability to maintain their own individuality, which means the ability to keep genetic self-stability. Every organism either extinct or extant is a product of a never ending co-evolution with other organisms, from microbial to multicellular, in the form of symbiosis, commensalism, or parasitism. The principle to maintain individuality of all eukaryotic multicellular animals, from the sponges upward, lies in the prevention of any other alien form of living system into the internal milieu.

From this point of view, immunity must be considered as a fundamental attribute of living organisms, similar to metabolism, irritability, or reproduction. All of the following eventualities could caused the emergence of the first immune phenomena among ancestral organisms.

Factors threatening the integrity

Parasitism

It was already mentioned that the uniqueness of an individual is ensured by the functional co-operation of neuroendocrine and immune systems. However, only immune system is able to neutralise and to destroy pathogens penetrating from external environment. Diseases accompany all living creatures from the very beginning of their lives and the faculty of falling ill (the pathibility) belongs to the general characteristics of life. Under the evolutionary pressures of pathogenic vectors, the immune mechanisms have been more precisely formed with the primary aim to increase the possibility of the individual to survive (*Doberstein*, 1951).

Fusion

The fusion of genetically different individuals or their parts means the threat of somatic parasitism and it can result in the lost of individuality. The transplantation reaction may be a phyletic consequence of the adaptations to the risk of that parasitism (*Buss* and *Green*, 1985).

Internal processes requiring homeostatic regulation

Growth, morphogenesis, ageing

Whereas the neuroendocrine system monitors the chemical characteristics of internal environment, the immunity perceives the antigenic expression on the cell surfaces throughout the body. The synchronisation of the processes of histogenesis (histolysis) during embryogeny requires a regulation by a system capable of both the recognition and elimination of disturbances (*Moscona*, 1974; *Stewart*, 1992). The same is valid for the surveillance and elimination of potential harmful changes during postembryonal stages of life (*Goya*, 1991).

Neoplasia

Mutant cells, which do not fulfil their functional role and may represent a potential danger for a steady-state within the internal milieu are recognised and eliminated by immune mechanisms. The molluscs, arthropods, and vertebrates are the only animal groups with clear-cut cases of neoplasia. In spite of that, all animals with at least cell-mediated immune system can be expected to have neoplasms (*Balls* and *Ruben*, 1976).

The complex role of homeostasis is shown in Figure 1.

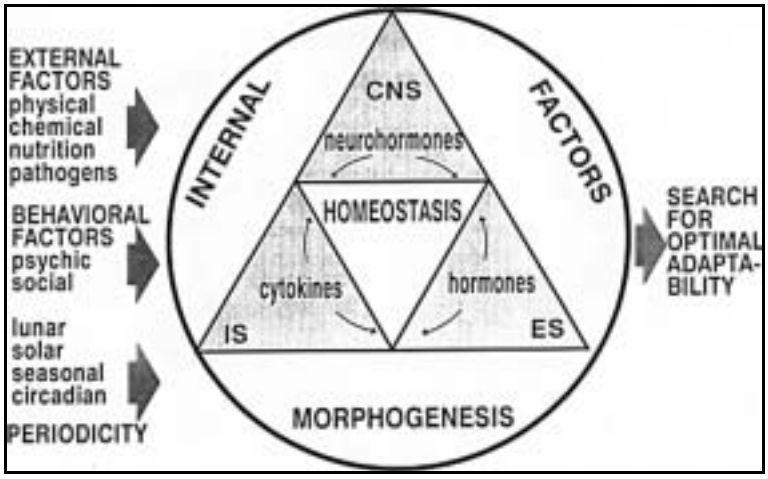

Figure 1: The complex role of homeostasis.

EVOLUTION OF IMMUNITY

Basic mechanisms of immunity

Two fundamental patterns of internal defence strategies evolved in the remote past: the constitutive (non-anticipatory) and adaptive (anticipatory) immunity (*Klein*, 1989). The constitutive immunity involves innate mechanisms utilising predominantly the phagocytic cells, killer cells, and non-specific humoral substances. To be endowed by the adaptive type of immunity means to possess the specific receptors recognising and binding a vast number of antigenic epitopes, and as a consequence of these events to manufacture a row of cellular and humoral reactions realising the specific response.

All main taxa of eumetazoan animals have developed proper immune strategies constricted by their morphofunctional possibilities only. However, three basic immune phenomena are common for all animal assemblages: the capability of recognition (of self or nonself), processing of immunogenic material, and response to it.

Recognition

Recognition should be understood as an affectory phase of the immune reaction. Generally, it means a type of communication of an individuum with the surrounding environment. All organisms, even the unicellular ones, are capable or recognition *via* their cellular surface receptors. The protostomians do not possess variable-region molecules, e.g. immunoglobulins or TCR. Emergence of these molecules among ancestral vertebrates was a crucial novelty for the evolution of adaptive immunity (*Cooper*, 1992).

Processing

The processing can be understood as the transport of the signal received from the receptor to other molecules, and subsequent analysis of the information from the signal (*Werdelin* and *Mouritsen*, 1992). The endocytosis is generally regarded as the first stage of immune processing. All eukaryans are capable of phagocytosis. In more ad-

vanced animals with more hierarchised immune system, various types of specialised populations of accessory and effectory cells evolved to meet the needs of efficient effectory parts of the immunity (*Síma* and *Vêtviçka*, 1992).

Response

The effectory phase of the immune reaction is the response. As a large diversity of animals exists, it is not surprising that various forms of immunological response can be found. These forms differ from each other in several respects: specificity, rapidity, efficiency, and existence of immunological memory.

Analogy and homology

The elucidation of questions concerning the evolution of immune systems is connected with the problems of origin and relationships between the metazoan phyla. Different ancestry resulted in different consequences even under the same selective pressures. The research of the analogy and homology of immunity should generally be subordinated to the following considerations (*Síma* and *Vêtviçka*, 1990):

1. The emergence of qualitatively new immune properties occurs at all phylogenetical levels, and many properties are passed on vertically from ancestral taxon to another as apomorphies.
2. The principal variations in immune characters cannot be presupposed within a single natural group of animals (a monophyletic taxon).
3. The more evolutionary remote natural groups of animals (sister groups), and the more remote animals are from their ancestors, the greater the differences will be found in their more complex immunity.
4. The more evolved (or younger in evolutionary terms) such natural assemblage is, the more complex may the immune pattern be. This is not valid for some specialised groups, e.g. the parasites. These animals did not evolved a more progressive types of immunity, but rather conserved the ancestral type, or even partially lost some of its functions.

The basic body plan

Multicellular organisms with whatever body arrangement, shape, and size had to ensure the availability of chemical substances for life of every cell, and the removal of the wastes. The larger organisms evolved networks of channels directly connecting their internal tissues with the exterior (water channels in sponges), or replacing of external environment by fluids or "blood" circulating within the systems of body spaces, channels or vascular system.

All animals belonging to the main phyla are built on the same fundamental body plan, which represents the most convenient evolutionary compromise between the morphofunctional possibilities determined by the genome and selective pressures coming from the environment. Larger steps in the phylogeny of homeostatic systems always began with the appearance of the new basic body plans in the main animal phyla, especially with the emergence of the first coelomate and metamerised metazoans, further with the onset of chordate-vertebrate basic body plan and endothermy (*Clark*, 1964; *Romer*, 1971). Similarly, all variations of immune apparatuses within a phylum are the variations of one ancestral scheme, more or less specialised according to developmental changes associated with the major morphological novelties accumulated during the evolution (*Conway Morris*, 1993; *Valentine* et. al., 1996).

THE OVERVIEW OF IMMUNE PHENOMENA AMONG MAIN ANIMAL TAXA

DIBLASTIC PHYLA

I. Irregular or radial, cell-aggregate basic body plan

The poriferans

The sponges are most primitive multicellular creatures. Neither true tissues nor organs are present, and the cells display a considerable degree of independence. To summarise the immune pattern of the sponges they have a highly discriminative and, in the same extent, co-operative cellular defence system. Phagocytosis is the major defence reaction, and the free-wandering amoeboid cells, the archaeocytes, are the main effector cell population. They are active also in quasi-immune recognition, cytotaxic reaction, and graft rejection. The various lectins are involved in aggregation phenomena allowing cell to cell contacts (*Vasta*, 1991). The histoincompatibility and cytotoxicity may seem to be correlated with the presence of inducible specific alloimmune memory (*Hildemann* et al., 1980; *Van de Vyver* and *Barbieux*, 1983).

II. Radially symmetrical sac-like basic body plan at the tissue grade of construction

The coelenterates and the ctenophorans

The functional specialisation of cells can be seen for the first time in phylogeny. The gastrovascular cavity is in larger species transformed into a series of narrow canals functioning similarly to a circulation system. The simple general body plan of coelenterates was not sufficiently plastic, thus the more complex immune structures or organs could not develop. Therefore, the immunological defence of these animals remained limited only on phagocytosis and histoincompatible reaction accompanied by certain alloimmune memory (*Bigger* and *Hildemann*, 1982; *Buss* et al., 1985). A humoral component of the coelenterate immunity could be represented by an external mucus secreted by most of these animals. It may play a role in the protection against pathogens (*Burkholder*, 1973), recognition processes, as well as in cytotoxic reactions (*Muscatine*, 1989). It is possible that the coelenterates avoid other more complex evolutionary solution of their immune potential by developing a very effective capability of regeneration which is regarded to be the most powerful in the entire animal kingdom. The speed of healing following a damage may significantly reduce the possibility of pathogen invasion (*Sparks*, 1972).

TRIBLASTIC ACOELOMATE PROTOSTOMIAN PHYLA

III. Bilateral sac-like basic body plan at the triploblastic grade of construction

The platyhelminths

The platyhelminths are endowed with a third layer, the mesoderm, which represent an important advance in phylogeny: it forms an evolutionary background for later emergence of various organs including those devoted to immunity. Mesodermal parenchyma contains free wandering cells, the neoblasts, which have features resembling some vertebrate haemocytoblasts. Planarians are still devoid of a definitive blood-vascular system and no specialised structures for the immune functions are known. Immune capability of these mostly minute animals are scarce, and only phagocytosis and graft rejection have

been described. Most species of flatworms have also considerable powers of regeneration (*Valembois*, 1982).

IV. A tube-within-a-tube basic body plans

The nemerteans

In comparison to flatworms, there are two evolutionary progressive features in nemerteans: Their digestive tract has a second opening (the anus) situated at the posterior part of their body; Secondly, even more important, they are the first animals in phylogeny possessing a true closed circulatory system. Their vessels are lined by an epithelium, which is exceptional among the invertebrates. Cells resembling vertebrate macrophages and small lymphocyte-like cells can be found among several categories of blood cells. The nemerteans are able to reject xenografts with some sort of short immunological memory (*Langlet* and *Bierne*, 1977).

PSEUDOCOELOMATE PHYLA

The aschelminths

The common feature of these animals is the possession of a body cavity derived from a persistent blastocoel - the pseudocoel. These animals are devoid of any circulatory system. The role of free-wandering amoeboid cells inside the mesenchymatous tissue is unknown. Practically, no information is available about their immune patterns (*Van de Vyver*, 1981).

EUCOELOMATE SCHIZOCOELIC PHYLA

Origin and importance of the coelom

The acquisition of the secondary body cavity (the coelom), in eumotazoic body pattern conferred further progress upon the hierarchised control of homeostatic processes. The originally pluripotent mesodermic cells composing parietal or splanchnic layers of coelomic linings may differentiate under the inductive influence of ectoderm or endoderm into various functionally specialised tissues or organs. The development of organs within the sufficient space and their stratification along the alimentary tract could secure better effectivity of digestion and better utilisation of nutritions, and by that way it allowed higher income of the energy for more active life. These new conditions, together with growing body size, made the previous mechanisms of transportation of nutrient or waste less effective (by diffusion or cellular distribution) or fully inefficient, and could concomitantly induce the development of independent transport system. A well-established circulatory system is therefore obligatory for almost all eucoelomate phyla.

The representatives of annelids, arthropods, and molluscs forming an artificial superassemblage, the "annelid superphylum", are regarded with a build-up of their secondary body cavity according to the schizocoelic mechanism. The echinoderms, hemichordates, and chordates ("echinoderm superphylum") form their coelom in enterocoelic way (*Kerkut*, 1960; *Clark*, 1964).

In eucoelomate animals the first defence structures evolved in connection with rostral part of the digestive tube, where the exposition to the environmental antigens induced development of such organs like typhlosole, a common organ present not only in invertebrates but also in some less advanced vertebrates.

The sipunculans

The sipunculans possess a well developed coelom but no real vascular system has been formed. No haemopoietic structure has been discovered in the sipunculans, so that the differentiation of free coelomocytes probably occurred from the peritoneal epithelia (*Dybas*, 1981). These cells are vectors of the immune phenomena like recognition, phagocytosis, and elimination of particles from the coelomic space. Moreover, they possess enzymes used in vertebrate cells for killing the bacteria. From the humoral defence factors, lysins and agglutinins have been determined in the coelomic fluid. Some signs of spontaneous cytotoxic activity of sipunculid leucocytes against various allogeneic and xenogeneic cells has been found, in contrast with previous acoelomate or pseudocoelomate animals.

EUCOELOMATE SCHIZOCOELIC METAMERISED PHYLA

Origin and importance of metamerism

The second key event in phylogeny, comparable in its importance to the evolutionary emergence of the coelom, is segmentation or metamerism. At this crucial event, the branching into two main directions of evolution within the animal kingdom established the differentiation of the annelid and the echinoderm superassemblages. All eumetozoic animals above this evolutionary level are segmented and have a coelom. It is important to realize the evolutionary importance of metamerism for the immune phenomena: the sophisticated defence systems in both members of annelid or echinoderm superphyla could evolve only in coelomatic segmented animals in which the regional specialisation of their body patterns has been the *conditio sine qua non* for functional specialisation *(Clark*, 1964).

The echiurans

The spoonworms represent the last evolutionary solution of the body construction antecendent the splitting of eucoelomic body plans into the modifications seen among representatives of annelid and echinoderm superassemblages. For the first time in phylogeny, segmentation appears to be a transitional phase during ontogenesis. Moreover, most of adult forms possess a closed simple blood-vascular system. No data are available about the immune pattern of these animals nor about the defence role of their free cells with exception of a clumping-like reaction in presence of bacteria (but no accompanying phagocytic reaction was observed). Only some bactericidal activity in the coelomic fluid has been described (*Dybas*, 1981).

THE "ANNELIDE SUPERPHYLUM"

The annelids

Generally, all structural novelties emergency of which we have followed and which are unambiguously considered to be fundamental, (i.e., the coelom, the closed blood vascular system and the metamerism), together with all major organs and organ systems are present. A relatively high number of free-wandering cells can be found both in the coelomic fluid and inside the vascular system. They arise from specialised parts of coelomic or blood vessel epithelia, or from the distinctive structures described as "lymph glands" (*Dales* and *Dixon*, 1981). The main cell types involved in the defence processes (recognition, phagocytosis, encapsula-

tion, and histoincompatibility reaction) are various categories of amoebocytes (*Cooper* and *Stein*, 1981). With regard to the humoral immunity, substances with haemolytic, haemagglutinating, antibacterial, and antiviral properties have been detected in coelomic fluid. Moreover, the molecules of nonimmunoglobulin nature with enhancing effect on the phagocytosis, amounts of which can be increased by antigenic stimulation, and the primitive cytokine activity (IL-1 and IL-2- like) have also been found. It may be concluded that at least some representatives of annelids have evolved a highly sophisticated system of mutually collaborating cellular and humoral defence components, in many respects comparable to those of vertebrates *per analogiam.* Earthworms can be considered to be the first invertebrate animals having a specific anamnestic immune response (for review see *Vêtviçka,* et al., 1994)

The arthropods

The arthropod's body plan has been proved to be enormously plastic and its modifications paved the way for the immense number of various species. The real coelomic cavity exists only in very early ontogeny. In later stages it gradually fuses and forms a mixocoel. The blood circulation system can be considered practically as open. The immune system of arthropods is capable of specific recognition of a wide range of antigenic material, and mounting of a vigorous response against it. Haemopoietic organs were found in larger species, and many morphologically distinct types of free or sessile cells manufacturing a wide range of the immune functions like phagocytosis, encapsulation, or rejection of transplants were described. In addition, an advanced system of humoral defence factors collaborating with the cells can be found. Bacterial agglutinins, the prophenoloxydase, and lectins could serve as examples of defence factors naturally present in the haemolymph. The lysozyme, cecropins, attacins, dipteridins, defensins, and other molecules are among the inducible factors (*Sherman*, 1981; *Bauchau*, 1981; *Gupta*, 1991). It may be concluded that in this enormous and varied animal superassemblage, the very effective immune mechanisms based upon the proper design of co-operating components have been created.

The molluscs

The molluscs represent a sister group to the common stock of true coelomate groups, resembling them in few synapomorphic characters. They have rather mesenchymate organisation of their body design with very limited coelom due to the enormous development of the primary body cavity which has been transformed into a large vascular system, the haemocoel. All molluscs have this open system, with the only exception of cephalopods. These animals reached a maximal level of complexity of their blood vessel system which cannot be found in any invertebrate phyla. A steady state of renewal of haemocytes inside the vascular system, the epithelia and connective tissue, takes place. In case of emergency, the recruitment of haemocytes from connective tissue occurs. This phenomenon is analogical to that in the murine peritoneal cavity. Some haemocytes display macrophage-like properties. These cells produce a number of defence humoral substances with strong lytic, cytotoxic, agglutinating, and opsonising activities. Moreover, the presence of cytokine-like molecules (IL-1, IL-2, IL-6, TNF-α, and TNF-β) in the molluscan haemocytes has been observed (*Franceschi* et al., 1994). The existence of two effective phagocytic systems, the free cells and fixed cells, approximate the molluscs in analogy to that of vertebrates.

All molluscs recognise and respond to foreign materials, and in some species a surprisingly high degree of specificity occurs. On the other hand, the molluscs seem to lack the recognition of allograft tissue as non-self, but xenografts were always rejected. The majority of humoral substances and cell-surface factors manifest a lectin character (*Suzuki* and *Mori*, 1990) which may attach to or agglutinate microbes and parasites, facilitate the processes of phagocytosis and encapsulation, or possess opsonising capabilities. In conclusion, with the exception of the above-mentioned analogies, nothing permits speculation about the homology of the mollusc immune pattern with that of vertebrate or any other invertebrate phyla (*Fletcher*, 1982).

ENTEROCOELIC DEUTEROSTOMIAN PHYLA

The "echinoderm superphylum"

The echinoderms

The echinoderms represent a sister group with the chordate-vertebrate lineage. A number of crucial developmental characteristics are common to both assemblages. The coelom of echinoderms is well-developed and structurally specialised in the functions of vascular and haemocoelic systems. The echinoderms have developed the distinct organs with coelomocyte-poietic activity from which the axial organ appears to be ancient "lymphoid" organ. This organ is often considered to be a homologue of the vertebrate spleen. The axial organ cells were shown to be heterogeneous and its adherent cell subpopulation is regarded to express B cell-like characters. These cells were shown to produce a lytic protein factor having antibody-like properties. Another protein, the IL-1-like factor, was isolated from coelomic fluid (*Beck* et al., 1990). The echinoderms are able to reject allografts in a manner similar to that of vertebrates (*Karp* and *Coffaro*, 1982). Second-set allografts are destroyed in an accelerated manner. These phenomena indicate a clear-cut evidence for a specific memory which may represent an ancestral discriminative type of vertebrate transplantation immunity. The killing reaction against both allogeneic and xenogeneic echinoderm cells, as well as against normal and tumour vertebrate cells, can be regarded as an analogue of the spontaneous cytotoxic reaction of vertebrate NK cells. Conclusively, the basic cellular defence reactions of echinoderms are still phagocytosis and encapsulation (*Smith* and *Davidson*, 1981). On the other hand, they possess remarkable immunological features that cannot be found in any other invertebrate phylum.

The cephalochordates

The cephalochordates are considered to be the true transitory group on the evolutionary pathway to vertebrates. The free coelomocytes seem to play no role in the defence except phagocytosis. Natural lectins of the invertebrate type have been demonstrated, but neither their recognition role nor co-operative role with phagocytes has been elucidated (*Millar* and *Ratcliffe*, 1990). Despite the apparent lack of any immunocompetence, these animals posses the ability to amplify their population of free cells by a mechanism common lately to all vertebrates, i.e. the proliferative multiplication of lymphoid cells. Moreover, cells morphologically similar to vertebrate lymphoid cells have been documented in the pharyngeal region (*Rowley* et al. 1984).

The urochordates

The haemocoelic vascular system of urochordates contains more types of

free cells capable of co-operation during a very specialised phagocytosis and encapsulation (*Wright* and *Ermak*, 1982). The histopathology of encapsulation resembles the formation of vertebrate granulomas. The urochordate lymphocyte is often regarded to be the homologue predecessor of all vertebrate lymphoid cells. The haematogenic tissue is organised in discrete structures (called "lymph nodules") localised in the vicinity of branchial region and digestive tract. A homology between those structures and haemopoietic tissues of vertebrates is supposed. The blood cells are involved in allogeneic rejection associated with a colony specificity and programmed senescence. It was suggested that the transplantation reaction is governed by a MHC-like gene locus (*Weissman* et al. 1990). The lectins and other natural factors with an antibacterial activity in body fluid, together with the presence of some molecules of Ig superfamily (Thy-1 or a disulfide-linked heterodimer surface protein resembling mammalian lymphocyte receptors) has been described. Interleukin-like molecules also have been identified (*Raftos*, 1994). In summary, the urochordates possess main features of adaptive immunity: a structural basis resembling both the haemopoietic and the macrophage-phagocytic systems of vertebrates, and a capability for continual renewal of immunocompetent cell populations co-operating with humoral cytokine-like factors.

THE VERTEBRATES

The agnathans

Jawless fish are considered to be first true vertebrates. No final separation of the primary and secondary lymphoid organs exist, even if the presence of true plasma cells was revealed. Agnathans are the first animals able to react to an antigenic challenge by the production of "antibodies" with some degree of homology to mammalian Ig molecule. According to the new molecular analytic studies, the agnathan "antibodies" appeared to be rather the C3 complement component than true immunoglobulin (*Nonaka* and *Takahashi*, 1992). Nevertheless, these creatures express for the first time in immunophylogeny many progressive immunological features which cannot be found in any previous animal assemblage, indicating the similarity of their defence capacity to that of a common adaptive type of advanced vertebrates (*Fujii* et al., 1992; *Zapata* et al., 1981; *Zapata* et al., 1984).

The chondrichthyans

The cartilaginous fish, regardless of their rather archaic and simple vertebrate body pattern, are first vertebrates characterised by well-developed cellular and humoral immune mechanisms. They possess distinct spleen (white and red pulp) and thymus, and important lymphohaemopoietic organs (Leydig's organ, spiral valve, epigonal organ). Besides the antibodies of the IgM isotype which molecular complexity is comparable to that of mammals, the skates evolved proper immunoglobulin class, the IgR isotype, which is not found anywhere else (*Kobayashi* et al., 1984; *Rast* et al., 1994). The question of the T and B lineage divergence remains unresolved similarly to the agnathans. The chondrichthyans can be considered as evolutionary critical animals. From chondrichthyans up, on the evolutionary scale, all basic molecular and cellular vectors of immunity, and all main immunocompetent structures can be

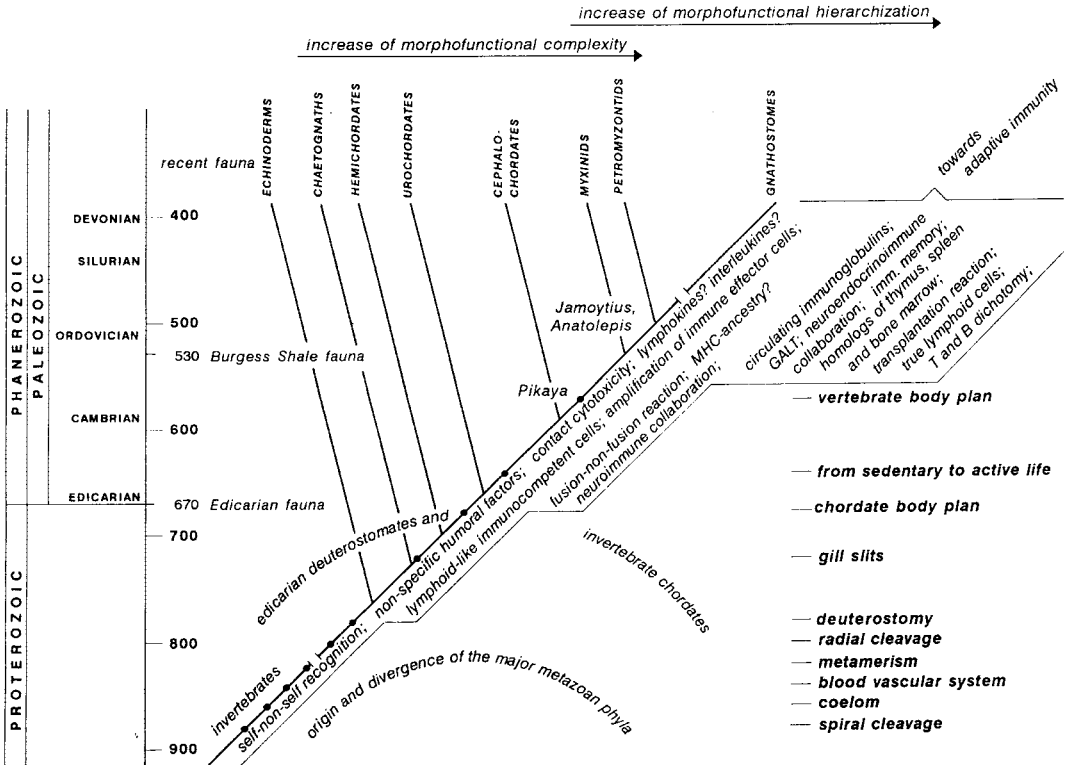

Figure 2: The comparison of the mutual evolutionary relationship of morphofunctional and immune phenomena.

found. Among chondrichthyan ancestors, in the deep past, started the immune strategy that we are accustomed to call the adaptive, anamnestic, inducible, and anticipatory immunity.

The comparison of the mutual evolutionary relationship of morphofunctional and immune phenomena is shown in Figure 2.

CONCLUSION

When thinking about evolution of immunity, it must be kept in mind that all types of defence reactions of the present-day animals are optimal for them as these reactions made possible their phylogenetic survival and adaptive radiation.

The invertebrates do not show as a high degree of defence specificity as vertebrates, the discrimination may generally be so poor that allografts and sometimes even xenografts are not rejected. This may be an advantage as long as a reaction against self is avoided, or may be disadvantageous, e.g. in the case of autoimmunity or allergy. Those species whose bodies evolved to be minutes had reduced their structural organisation and also necessarily simplified their immune strategy. They have been looking for other evolutionary solutions to this problem and found them in the shortening of their life spans or in rapid changeover of

generations. In more advanced invertebrate taxa both the cellular and the humoral components are co-operative and highly specialised. The only missing component is the immunoglobulin molecule.

Besides elucidating how evolution built up the immune mechanisms piece by piece, the effort spent on the research of non-traditional models may be of extreme importance with even a practical use at least in two branches of biomedical science: "Once we comprehend the strategies used by primitive animals, we hope to apply our understanding to mammalian host defences" (*Habicht*, 1993); secondly, it may be quoted *Sir F. M. Burnet* (1960): "As long as there are useful invertebrates like earthworms, oysters, or honey bees to be protected, and others like tapeworms, slugs, and mosquitoes to be destroyed, there will be a utilitarian justification for studying invertebrate pathology and whatever is equivalent in them to what we study in vertebrates as immunology".

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NEURO-ENDOCRINO-IMMUNOLOGY

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INTRODUCTION

Neuro-endocrino-immunology is a point of intersection in immunology. It is also referred to in the literature as psychoneuroimmunology. The emerging concept is that cells of the immune and inflammatory systems communicate directly with the peripheral and/or central nervous system. This connection or communication pathway is also mediated via the bloodstream and, therefore, involves hormonal communication. The term hormones does not only signify the classical endocrine system, but also molecules released by the nervous or immune systems which have functional effects at some distance, such as, for example, catecholamines and a variety of neurotransmitters. Thus, the brain and the nervous system are part of the neuroimmuno-regulatory network in which the various components of the network not only communicate with each other and affect each other, but also regulate additional sites through this mechanism.

ROUTE OF INTERACTIONS BETWEEN NEURO-ENDOCRINE SYSTEM AND DEFENCE SYSTEM

Thus, antigen can interact with a T or B cell, macrophage, dendritic or mast cell through antigen specific receptors (antibodies) which, when bridged, will cause release of a host of immune and inflammatory mediators including:

1. cytokines,
2. growth factors,
3. eicosanoids,
4. histamine,
5. serotonin,
6. neurotransmitters, etc.

These can act locally on the nervous system which then transmits information through the normal efferent pathway, and results either in local axon reflexes or transmission of information via ganglia to the spinal cord, and thence to the brain. In turn, the brain, can initiate events peripherally through such peripheral afferent signalling mechanisms or through intrinsic mechanisms, initiated for other reasons of communication within the central nervous system. These pathways either use traditional systems of nervous conduction or the hypothalamic-pituitary-adrenal (HPA)-axis. This axis involves corticotrophin releasing factor (CRF) and this axis is operative in a series of situations in which "stress" appears to play a role. These include responses to environmental and social factors, psychiatric and psychological adaptation, and immunologic and inflammatory responses to infectious agents such as viruses, bacteria and parasites.

INFLUENCE OF STRESS ON THE QUALITY OF THE DEFENCE RESPONSE

Stress is stated by *Black* (1995) to be "a state of disharmony or threatened homeostasis provoked by a psychological, environmental or physiological stressor". Stress is also invoked as part of the normal adaptive response to stimuli and should not be thought of as an abnormal reaction, since it involves the adaptation to "fight or flight". Much of this response is mediated through the HPA and is integrated via the hypothalamus by adjustment to certain functions such as the sympathic nervous system and endocrine secretion. *Blalock* (1989) defined this as an active bi-directional communication pathway between the nervous and immune systems. While the normal stress response is also characterised by secretion of corticosteroids a myriad of chemical messages in addition are involved in these responses.

Classical Pavlovian psychological conditioning has also been shown to regulate immune responses and is not only capable of up-regulation, but also down-regulation or inhibition. These are extensively reviewed in a book on the subject (*Ader* et al., 1991) since the nervous system has both inhibitory (negative) and activating (positive) pathways. Many neurotransmitters such as somatostatin and calcitonin gene-related peptide are both inhibitory and activating.

CONCLUSION

These systems are extremely complex but are known to regulate biological activities as diverse as neuronal repair and regeneration, and homeostasis of the internal environment. At times, these systems may act entirely separately from each other, and at other times intimate and very active regulatory interactions come into play, ranging from fine tuning of reactions to major participation in physiological and dysfunctional events. The role of these factors in initiation or control of disease is only just beginning to be clarified.

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THE ROLE OF NUTRITION AND DRUGS IN PREVENTING BACTERIAL TRANSLOCATION AND ASSOCIATED SYSTEMIC INFECTIONS

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SUMMARY

The addition of pharmacologic amounts of arginine or glutamine to balanced diets, as well as substitution of Omega-3 for Omega-6 fatty acids, will independently result in improved resistance to infection and/or enhanced gut barrier function to translocation of intestinal microbes. Combinations of these immunonutrients in diets have further beneficial effects. There are now 7 prospective randomised clinical trials of the use of immunonutrient enriched diets in surgical patients. In the aggregate, these have shown that the use of such diets can reduce wound complications and infection by 50-75% and hospital stay by 20%, also providing considerable economic savings.

However, in individuals who have established sepsis with or without multiple system organ failure (MSOF), aggressive feeding may be harmful. In animal studies, high protein diets (20% of energy) and the same dietary formulas that improve resistance to infection in normal animals will often have adverse effects because they may serve as a substrate for bacterial growth in the intestine, support excessive cytokine synthesis by intestinal cells, and increase the amount of bacterial translocation from the intestine.

The role of specific immunonutrients in disease is complex. Their influence in regulation of immune responses via interactions with chemotherapeutic agents are largely unknown but worthy of intensive research.

INTRODUCTION

Microbial translocation can be defined as the passage of both viable and nonviable microbes and microbial products across the intestinal barrier (*Alexander* et al., 1990). Translocation occurs through M-cells (which is a normal pathway for processing antigenic material from the intestinal lumen), directly through epithelial cells (which increases after systemic injury) or through ulcerations in the intestine (which may become an important pathway after cellular injury, such as may occur after the administration of cytotoxic drugs or irradiation). Theoretically, translocation could occur through the tight junctions between cells, but this has never been documented *in vivo*. Most microbial translocation is felt to occur through intact enterocytes. After

injury, such as a thermal burn, this may occur very quickly, i.e., within 5 minutes of injury, peak within a few hours, and persist at an increased level for many days. Once the microbes penetrate through the mucosa, they pass through the enterocyte into the lamina propria where they are phagocytised by macrophages or granulocytes or pass without ingestion through the lymphatics or vascular systems to distant tissues and organs where they may cause disease or affect biological processes.

There are numerous studies that support the concept that microbial translocation occurs in man, but perhaps the clearest demonstration is a single study by *Kraus* et al., (1969). In this study, a healthy human volunteer ingested approximately 10^{12} *C. albicans* orally. He developed positive blood cultures for *Candida albicans* three hours after ingestion accompanied by a funguria and systemic symptoms of sepsis which lasted for 12 hours. In *ex vivo* experiments using human intestine, we have shown that candidal bodies pass directly through enterocytes, similar to the mechanism identified in animals. *Sedman* et al (1994) took samples of intestinal serosa and mesenteric lymph nodes for a culture from 267 consecutive surgical patients subjected to laparotomy. Growth of organisms in these samples was documented in 25 of 242 evaluable patients (10.3%). Postoperative sepsis occurred in 28% of those demonstrating translocation compared to 11.5% of those without translocation ($p<0.05$). However, there was no influence of the occurrence of translocation on mortality. More recently, our laboratory has used PCR techniques for detecting the presence of microbial DNA in the blood of surgical patients, and this appears to be a more sensitive method for detecting translocation in humans than previous techniques (*Kane* et al., 1997). The precise relationship between the incidence of translocation and the development of secondary complications such as multiple organ failure in humans is currently under study.

Translocation is not a new concept. In 1891, *Fraenkel* (1891) suggested that organisms could pass directly through the entire wall of the intestine to cause peritonitis, and various authors following that time supported this concept. However, it was not until 1928 that *Arnold* (1928) was able to demonstrate that viable bacteria could be recovered from the thoracic duct after translocation occurred through the intact intestinal epithelium. His work was extended by *Fisher* (1931) who demonstrated for the first time that yeast could be demonstrated to translocate from the intestine to distant organs. *Flory* (1933) was the first to demonstrate that translocation of bacteria occurred directly through epithelial cells. The importance of translocation was largely ignored, however, for the next two decades until *Fine* and his colleagues (1952) postulated that translocation of bacteria and endotoxin after haemorrhagic shock was a major cause of morbidity and mortality. More recent interest in the process of translocation has shown that there are several diseases clearly associated with translocation in man (*Alexander* and *Gennari* 1996). These include pneumatosis intestinalis, nonocclusive intestinal gangrene, necrotising enterocolitis, gamma radiation, cytotoxic drugs, the cytokine release syndrome, Crohn's disease, ulcerative colitis, haemorrhagic shock, severe trauma, thermal injury, severe neutropenia, and colon cancer. Experimental studies have shown that translocation is clearly important in the hypermetabolic response to injury in sepsis (*Gianotti* et al., 1994), the septic state in the absence of a defined focus, and multisystem organ damage, but these relationships are less clear in man.

Table 1: Partial list of conditions that increase microbial translocation as measured by cultures of tissues or organs

A.	Diminished blood flow or O_2 delivery
	Hypoxia
	Fever
	Vasoactive agents; e.g., platelet activating factor, zymosan, endotoxin
	Thermal injury
	Hypovolemic shock
B.	Improved host defense
	Neutropenia
	Phagocytic dysfunction; e.g., blood transfusion
	Corticosteroids
C.	Increase in luminal microbes
	Antibiotic therapy
	Elemental diets
	Intravenous hyperalimentation
D.	Epithelial Damage
	Irradiation
	Cytotoxic drugs
	Irritants
	Infection - e.g. CMV
	Mucosal disease; - e.g. Crohn's disease
	Bowel manipulation
	Reperfusion injury

IMPAIRMENT OF GUT BARRIER FUNCTION

Most of the studies that have defined conditions that increase the incidence of microbial translocation have been done in animals. For the most part, these studies have measured viable bacteria in tissues without separating the contributions of the intrinsic barrier function of the mucosa from alterations in host defence. There are three factors which will increase the numbers of viable bacteria in the tissues:

1. the intrinsic barrier function of the mucosa,
2. the numbers and types of microbes in the intestinal lumen, and
3. the ability of the host defence mechanisms to kill bacteria that have already translocated.

The conditions that increase microbial translocation are measured by bacterial culture of tissues as shown in Table 1. In general, these can be divided into four broad categories, those conditions which decrease blood flow or oxygen delivery to the mucosa, impaired host defence, an increase in the number of microbes within the bowel lumen and direct damage to the epithelium.

ENHANCEMENT OF GUT BARRIER FUNCTION

The ability of various agents to impair the passage of microbes through the epithelial barrier is shown in Table 2. Various analogues of prostaglandins,

Table 2: Enhancement of gut barrier function by drugs

Prostaglandin E analogues
Growth factors
Epidermal growth factor
GM-CSF (but not G-CSF)
bFGF
Bombesin
IGF-1
Mucous enhancing agents - sucralfate
Vasoactive agents
Heparan sulfate
Enalapril

particularly the E-1 series, have been shown to improve barrier function. A part of this may be related to increased mucous production, as well as an increase in the ability to improve blood flow to the mucosa. Most of the factors which accelerate growth of the epithelium will decrease translocation rates. Notable among these is GM-CSF which both decreases the rate of translocation and improves the killing of bacteria that do translocate. Mucous enhancing agents, such as sucralfate, will also decrease translocation, but there may be other effects as well. In addition, vasoactive agents which increase the circulation, such as heparan sulfate, and enalapril will improve the barrier function. The effect of GM-CSF on gut barrier function and survival provides an example of how drugs may have a dual effect (*Gennari* et al., 1994). Normal mice were given a transfusion of 0.1 ml of allogeneic blood to produce a non-specific immunosuppression. Two days later, they were randomly divided to receive 10 μg/kg GM-CSF or saline as a placebo control daily for three days. All animals were then given a gavage of 10^{10} *E. coli* and a 20% burn was inflicted. They were then followed for survival for 10 days. All mortality occurred within 48 hours. The mice treated with GM-CSF had a 90% survival compared to a 35% survival in the placebo treated controls ($p<0.05$). In another study, animals were treated similarly except that they were gavaged with ^{111}Indium labelled *E. coli* and sacrificed 4 hours after burn injury. At the time of sacrifice, mesenteric lymph nodes, liver and spleen were harvested for the determination of radionuclide counts and the persistence of live bacteria. Translocation as measured by the amount of radioactivity reaching the tissues was greater in the control animals than animals treated with GM-CSF in all tissues examined (mesenteric lymph nodes, liver, and spleen), indicating that GM-CSF improved the barrier function of the gut, i.e., inhibited the ability of the organisms to translocate. Cultures of each of these tissues also showed that there were fewer colony forming units in the tissues in animals treated with GM-CSF. Furthermore, calculation of the percentage of translocated bacteria that remained alive showed that killing was enhanced in the tissues by GM-CSF. Thus, this drug has a clear cut dual effect in improving survival from gut origin sepsis: improvement of the gut barrier function and improvement of the ability to kill organisms that do translocate.

Table 3: Effects of nutrients on translocation

	Effect on Barrier function	Effect on Bacterial killing	Effect on Survival
Amino Acids			
Arginine	—	↑	↑
Glutamine	↑	↑	↑
Glycine	—	—	—
Lipids			
Omega-3 PUFA	↑	↑	↑
Omega-6 PUFA	—	—	—
MCT	—	—	—
RNA	—	—	—

EFFECTS OF NUTRIENTS ON TRANSLOCATION

During the last decade and a half, there have been several nutrients which have been identified that have pharmacologic effects, particularly on the immune system. These include the amino acids arginine and glutamine, ribonucleic acid, and the polyunsaturated fatty acids (*Alexander*, 1995). Studies in laboratory animals have shown that these nutrients often have an effect on translocation (Table 3). When either gut barrier function is improved or there is an improvement of bacterial killing, there is usually an enhancement of survival of the animal when subjected to a septic challenge. Arginine improves the ability to kill translocated bacteria and this is related to an effect of nitric oxide (*Gianotti* et al., 1993). However, arginine does not affect the barrier function. In contrast, glutamine improves both the barrier function of the intestine and the ability to clear bacteria (*Gianotti* et al., 1997a). Lipids of the Omega-3 fatty acid family slightly improved barrier function but have a greater effect on bacterial killing (*Gianotti* et al., 1997b). The Omega-6 fatty acids have usually been used for controls, but high doses of the Omega-6 fatty acids will impair bacterial killing and decrease survival of animals. Combinations of the immunonutrients, when given in excess to animals, have an additive or sometimes synergistic effect (*Gennari* et al., 1995). In particular, fish oil and arginine, fish oil and glutamine, or arginine and glutamine are superior to individual combinations.

When prednisone is given to animals in high doses (10 mg/kg/day) for three days, there is an augmentation of mortality from gut derived sepsis, largely because it impairs the ability to kill bacteria that translocate from the intestine (*Gianotti* et al., 1996). This susceptibility can be reversed by diets containing either arginine or glutamine, indicating that the immunonutrients may have clinical applicability in patients who are immunosuppressed for a variety of reasons (*Gennari* et al., 1997).

Table 4: Controlled clinical trials that show a benefit of immunonutrition

Authors	Subjects	Results
Gottschlich et al., JPEN 1990	Burn patients	75% fewer wound infections 67-78% fewer infections overall 31% shorter hospital stay
Daly et al., Surgery 1992	Surgery for UGI & pancreatic malignancies	70% reduced wound complications 22% reduced hospital stay
Moore et al., J. Trauma 1994	Trauma victims	Fewer intra-abdominal abscesses and MOF (0% vs. 11%)
Bower et al., Crit. Care Med. 1995	Surgical ICU patients	Hospital stay reduced by 27%; 40% in septic patients; reduced acquired infections
Kemen et al., J. Crit. Care Nutr. 1995	Major abdominal surgery	After PO5: 53% reduced wound complications 3.6 day reduced hospital stay
Daly et al., Ann. Surg. 1995	Surgery for UGI and pancreatic malignancies	77% reduced wound complications 23% reduced hospital stay
Kudsk et al., Ann. Surg. 1996	Major abdominal trauma	85% fewer major infections 44% shorter hospital stay 27% reduced hospital charges

CLINICAL STUDIES OF IMMUNONUTRIENTS

The physiologic principles that evolved from early animal studies resulted in the development of therapeutic dietary formulations which contained immunonutrients. The first of these was tested in burn patients (*Gottschlich* et al., 1990) and subsequently, two commercial immunonutrient formulas that were outgrowths of our initial formula, Impact® (Sandoz Nutrition, Minneapolis, MN) and Immune-Aid® (McGaw Inc., Irvine, CA), have been tested in patients. There are now 7 prospective randomised controlled clinical trials (6 of them double blinded) which have shown a striking benefit of immunonutrition in surgical patients at a high risk of infection (Table 4). In aggregate, these trials have shown that aggressive enteral feeding with an immunonutrient formula will reduce hospital stay by approximately 20%, reduce wound complications and infection rates by 50-70% and significantly reduce the cost of care.

ADVERSE EFFECTS OF NUTRITION IN ESTABLISHED SEPSIS

While the benefit of aggressive enteral feeding is well established in both experimental animals and patients at risk for the development of infection, their benefit in patients with MOFS is questionable at best and may be harmful. In 1989, we developed a model of prolonged peritonitis to study the effect of

nutrition on outcome (*Alexander* et al., 1989). This model utilised implantation of a bacteria filled mini-osmotic pump into the peritoneal cavity. This pump delivered a mixture of bacteria into the peritoneal cavity over the course of a week, establishing a severe, but prolonged peritonitis, in which the animals could survive for 14-21 days, thus allowing time for nutritional intervention. By feeding via previously placed gastrostomies, it was possible to show that both restriction of caloric intake (*Alexander* et al., 1989) and provision of protein deficient diets have beneficial effects on survival (*Peck* et al., 1989). It has since been possible to demonstrate that the high protein diets were associated with an increase in the numbers of bacteria within the intestinal lumen and impairment of the barrier function of the intestine to microbial translocation (*Nelson* et al., 1996). The increased translocation in animals fed a high protein diet was associated with increased expression of message for the inflammatory cytokines in the intestine (IL-6) as well as down regulating cytokines (IL-10, TGF-β). Treatment of the animals with peritonitis with GM-CSF, sucralfate or epidermal growth factor improved survival in animals that had ongoing peritonitis that received a high protein diet, whereas the addition of glutamine or arginine to the diet did not improve survival. Together, these studies provide clear evidence that certain nutrients have pharmacologic effects on the immune system which are generally beneficial but may be harmful, depending upon the underlying disease.

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SPECIFIC AND NON-SPECIFIC OPSONISATION; ITS ROLE IN THE (NON-INFLAMMATORY) CLEARANCE OF TRANSLOCATED MICROORGANISMS*

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SUMMARY

Once bacteria invade the tissues phagocytosis is a crucial step in containing the microbes. Phagocytosis can only occur when bacteria are properly opsonised; loaded with antibody molecule and complement. Only when IgG and C3b and/or iC3b are present on the bacterial cell wall bacteria are recognised by phagocytic cells. Because phagocytic cells have receptors for the Fc fragment of the IgG molecule and for C3b it is possible that the phagocyte has also other receptors that could be involved in uptake of bacteria by the phagocytic cells, e.g.: mannose sensitive receptors, CD14 molecules, fibronectin binding receptors, etc.

However, the process of phagocytosis mediated by these receptors is much less efficient. Because the internal signalling pathway within the phagocyte is not known when bacteria are attacked via other receptors, efficiency of killing of bacteria via those alternative opsonins is unknown.

Many bacteria have developed strategies to prevent the binding of IgG and C3b. Capsules hinder binding and complement activation; many human pathogens have surface capsules e.g. *E. coli*, *H. influenza*, *S. aureus*. Many of the different inhibitors of opsonisation are discussed.

Finally, the role of opsonisation in the process of translocation is touched upon. However, data on the role of specific opsonins in eliminating translocating microbes are limited.

INTRODUCTION

The outcome of the interaction between certain microorganisms and PMN determines health or disease. Once the barriers of the skin and mucous membranes have been breached the host's health depends on PMN and other host resistance factors to combat invading microorganisms that can cause infection

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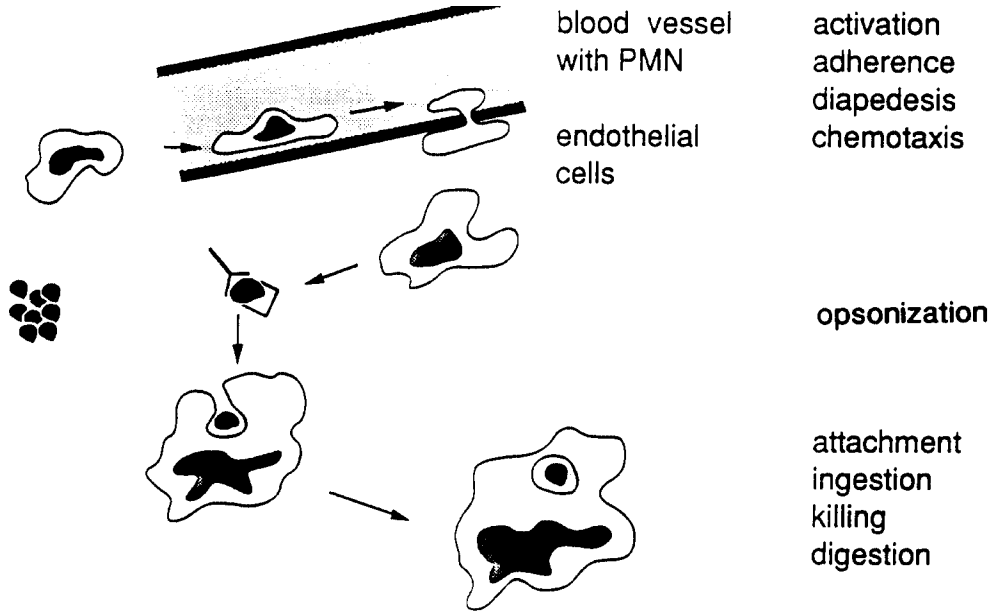

Figure 1: Processes involved in phagocytosis of bacteria by polymorphonuclear leukocytes.

(*Densen* and *Mandell*, 1990). PMN originate in the bone marrow and are continuously discharged in vast numbers into the bloodstream. They only live for a few days and each day about 10^{11} cells disappear from the body, even in the absence of infection.

For many infections the gastrointestinal tract can be a portal of entry for human pathogens. It is assumed that microorganisms can translocate from the gut into the regional lymph nodes (*Wells* et al., 1988). The mechanism of this process is still largely unknown. But there is some evidence that bacteria are taken up by phagocytes and then carried within the phagocytes to the mesenteric lymph nodes across the mucosal lung. Although it has also been shown that some bacteria invade certain mucosal cells and travel through these cells or between these cells (*Ménard* et al., 1994). It is now established *in vitro* that *Shigella* enters the epithelial barrier through M cells (*Phalipon* et al., 1995) that cove the dome of lymphoid follicles; subsequent invasion is primarily due to immigration of PMN which destroy the cohesion of the epithelial barrier.

As soon as microbes invade the tissues, circulating PMN are activated, leave the bloodstream, adhere to activated endothelial cells, and move through the endothelial barrier to the site of the infection. This process of migration under guidance is called chemotaxis and is defined as directed cell movement in one direction in response to an agent which signals and induces the cell to move. While PMN migration occurs, the microbes are opsonised; that is, the microbial surface is coated with antibody and complement factors for recognition by PMN. PMN have receptors specifically designed to bind to the Fc fragment of the IgG molecule present on the surface of the opsonised bacteria and other receptors designed to bind to the activated complement factors. The complement factors and the antibody molecules are ligands that promote

attachment of the microbe to the cell enhancing an otherwise inefficient microbe-phagocyte interactions. After this receptor-mediated attachment, PMN engulf the microbes and ingestion takes place. Once a microbe is phagocytised by the PMN, it is usually rapidly killed and digested.

During the last decade, our understanding of the molecular basis of the different steps involved in the process of phagocytosis and killing (shown in Figure 1) has greatly increased. It is now known that the outcome of the interaction between PMN and microbes is determined not only by PMN but also by the microbes.

OPSONISATION

Opsonisation in the present of antibody and complement

Generally microorganisms are only phagocytised after they have been properly opsonised; that is, loaded with activated C3 and IgG. Opsonisation through activation of complement is primarily a function of C3b and iC3b. The PMN receptor that recognises C3b is CR1, while the receptor that recognises iC3b is CR-3 (CD11b/CD18) (*Metzger*, 1990). For most opsonised particles especially (including) encapsulated organisms, the iC3b-CR3 interaction enhances attachment of the particle to PMN but not its ingestion. Ingestion only occurs in the presence of antibodies (*Metzger*, 1990). Antibodies bind to specific antigens on the cell wall of bacteria; these antibody molecules also serve as ligands for the attachment of bacteria to PMN. Two receptors for IgG are present on the PMN cell membrane: FcRII and FcRIII. FcRII can bind IgG1 and IgG3 equally well and better than IgG2 and IgG4. FcRIII binds only to monomeric IgG (*Sawyer* et al., 1989).

Many bacteria have developed a defence against opsonophagocytosis and are thus able to escape phagocytosis by PMN. The most important anti-phagocytic defence of bacteria is an enveloping capsule. These capsules protect the microbes against PMN by interfering with opsonisation (*Finlay* and *Falkow*, 1989). For example, pathogens that cause pneumonia and meningitis, such as *H. influenzae, Neisseria meningitides, E. coli, Streptococcus pneumoniae, Klebsiella pneumoniae*, and group B streptococci, have polysaccharide capsules on their surface. Non-encapsulated derivatives of these organisms are less virulent. Although the chemical composition of these capsules can vary significantly between strains and species, most capsules are composed of polymers of repeated sugar residues. However, only a few types of capsules are commonly associated with disease. *H. influenzae* isolates can produce one of six different types of polysaccharide capsules, yet those organisms expressing type b capsules are predominantly isolated from serious infections. Capsules from bacterial pathogens prevent complement deposition on the bacterial surface, while capsules from non-virulent strains are less efficient at preventing this deposition. The capsules are only weakly immunogenic and mask the more immunogenic underlying bacterial surface structures that would directly activate complement. Thus, the capsule prevents opsonisation of the organism, conferring resistance to phagocytosis (*Finlay* and *Falkow*, 1989).

Some other cell wall components that help microbes evade the phagocytic defence of the host are peptidoglycans, protein A (*Staphylococcus aureus*), and protein M (group A streptococci). The

role of each of these cell wall components are discussed below in conjunction with the microbes on which they are found.

Opsonisation in the absence of antibody and complement

Some bacteria are able to adhere to PMN in the absence of antibodies and/or complement. *E. coli* adhesins, for example, are important components that may mediate adherence of *E. coli* directly to PMN without antibodies and complement. These adhesins can be divided into two groups: one where D-mannosides inhibit the adherence and one where they do not. The mannose-resistant (MR) phenotype is mediated by cell-bound adhesins or by specific protein fimbriae. Among the *E. coli* strains with MR haemagglutination, the P adhesins recognise the sequence α-D-Gal-(1-4)-β--D-Gal on the target cell receptors. P adhesin-expressing *E. coli* are frequently involved in human urinary tract infections.

E. coli possessing mannose-sensitive (MS) adhesins adhere avidly to urinary slime. In addition, a number of other adhesins have been detected (M, X, and S adhesins, type 1c and G fimbriae). MS fimbriae (type 1 adhesins) increase the susceptibility of *E. coli* to PMN phagocytosis in the absence of specific opsonins (*Sobel* and *Kaye*, 1990). MS adhesins recognise the mannose residues of three different membrane glycoproteins in the PMN: gp 150, gp 70-80, and gp 100. Gp 150 (CD11/CD18) is the major receptor for type 1 fimbriae (*Rodriguez-Ortega* et al., 1987). Adherence of *E. coli* to PMN via the interaction of type 1 fimbriae and mannose-containing receptors leads to phagocytosis and killing by PMN (*Sobel* and *Kaye*, 1990). In contrast, PMN lack receptors for P fimbriae, which block phagocytosis (*Svanborg* et al., 1984). S adhesins are widespread among *E. coli* isolates that cause sepsis or meningitis. These adhesins recognise a structure containing neuraminyl acid derivatives. In many strains these derivatives appear in the form of neuraminyl-α-(2-3)-galactoside.

A possible serum factor other than complement or antibody that functions as an opsonin is the lipopolysaccharide binding proteins (LBP). LBP is an acute-phase reactant that binds bacterial LPS. LBP can bind to the surface of Gram-negative bacilli and strongly enhances attachment of these particles to the CD14 molecule in the cell membrane of phagocytic cells. LBP bridges LPS-coated particles to PMN and macrophages by first binding to LPS and then to the CD14 receptor. This binding leads to enhanced phagocytosis (*Wright* et al., 1989).

Gonococci possess antigenically variable outer membrane proteins, termed PII proteins, that appear to mediate adherence to human PMN (*Farrell* and *Rest*, 1990). These PIIs in the membrane of bacteria (outer membrane proteins) bind to carbohydrate moieties of glycoconjugates in a lectin-like manner. Anti-PII monoclonal antibodies abrogate adherence of non-piliated gonococci to human neutrophils. The neutrophil receptors for PII^+ gonococci appear to be stored in a subcellular granule population (*Farrell* and *Rest*, 1990).

In plasma there is a high molecular weight glycoprotein, called fibronectin, that aids the reticuloendothelial system in clearing the cell of microorganisms and helps maintain vascular stability. Several bacteria, for example, *S. aureus* and groups A, B, C, and G streptococci, have receptors for fibronectin. Because PMN can also bind fibronectin, it is possible that this glycoprotein acts as a bridge between PMN and the bacteria and therefore facilitates phagocytosis in the absence of specific opsonins (*Proctor* et al., 1984). It is

also possible that fibronectin enhances the opsonic and protective activity of antibodies and complement (*Hill* et al., 1984).

MECHANISMS FOR AVOIDING OPSONISATION

Microbial adaptation to avoid opsonisation

Many microorganisms can escape from opsonisation by varying their surface antigenic structure. Some bacteria are master chameleons. For example, *N. gonorrhoeae* possess at least two mechanisms for altering surface antigens:

1. They can change PII proteins. Most *N. gonorrhoeae* express several different PII proteins at any given time, and a given strain can potentially express up to seven different PII proteins. The genetic control of each PII gene appears unrelated to other PII genes, which results in many different combinations. The regulation of PII gene expression depends on the repeating five nucleotide CTCtt, which is located within the PII leader sequence. Variations in the number of repeats of this pentamer will vary the reading frame of the downstream PII gene.
2. They can change pilins. There are usually many silent pili gene sequences. The gonococcus can undergo gene conversion by placing one of these incomplete sequences into the expression site, thus, synthesising a new antigenically distinct pilin molecule. Antigenic variation occurs in many other bacteria as well: e.g., Group B streptococci, *H. influenzae, P. aeruginosa, Salmonella, Borrelia*, etc.

Interference of antibiotics with opsonisation

Exposure of bacteria to antibiotics below the minimal inhibition concentration increases their susceptibility to the antimicrobial action of normal human PMNL. Low concentrations of antibiotics influence cell wall composition. Clindamycin, for example, has an inhibitory effect on the M protein of streptococci and protein A of *S. aureus* and thereby facilitates opsonisation and subsequent phagocytosis. Antibiotics may also interfere with K antigen synthesis and LPS assembly in *E. coli*. (During antimicrobial treatment K antigen synthesis may be inhibited). Therefore, bacteria are more readily opsonised and subsequently phagocytised. Thus, during infections antibiotics may act in different ways: they may either kill the microbe directly or change the cell wall composition in such a way that an increased number of receptors for IgG and C3b is produced, thereby enhancing opsonophagocytosis (*Gemmell* and *O'Dowd*, 1983; *Milatovic*, 1983; *Veringa* and *Verhoef*, 1987).

NORMAL OPSONISATION AND EVASION OF OPSONISATION BY SPECIFIC MICROBES

Staphylococci

The major cell wall components of *S. aureus* are peptidoglycan, teichoic acids, and protein A. Peptidoglycan is a polysaccharidic polymer composed of B-linked (*Densen* and *Mandell*, 1990; *Sawyer* et al., 1989; *Berridge* and *Irvine*, 1984) chains containing alternat-

Table 1: Opsonins for *Stapylococcus aureus*

Unencapsulated strains:
- Antibodies against peptidoglycan
- C_3b and iC_3b generated by antigen-antibody reaction via the classical complement pathway
- C_3b and iC_3b generated by the classical and alternative pathway interaction with peptidoglycan
Encapsulated strains:
- Antibodies against polysaccharide capsule
- C_3b and iC_3b generated by the interaction of anticapsular antibodies with capsule

ing subunits of N-acetylmuramic acid and N-acetylglucosamine. Penta-peptide chains are linked to the muramic acid residue and are cross-linked by a pentaglycine bridge attached to L-Lysine on one chain and D-alanine on the other (*Davis* et al., 1990). Teichoic acids are simple glycerol or ribitol phosphates in repeating units, while protein A is a 42 kD protein with the capacity to bind human IgG subclasses (except IgG3) via their Fc terminals. Antibodies against peptidoglycan are opsonic (*Verbrugh* et al., 1980). When antibodies against peptidoglycan were isolated from serum and incubated with staphylococci, these bacteria were readily phagocytised. Peptidoglycan was also able to directly activate the complement system leading to deposition of C3b on the surface of the bacteria. However, more than 50% of *S. aureus* isolates obtained from blood cultures of patients are encapsulated. These capsular polysaccharides may interfere with the effective opsonisation by anti-peptidoglycan antibodies and hinder the interaction of complement with peptidoglycan (*Wilkinson* et al., 1979; *Verbrugh* et al., 1982; *Karakawa* et al., 1988). Anticapsular antibodies are needed for the efficient phagocytosis of these encapsulated bacteria.

Four mechanisms of opsonisation of unencapsulated *S. aureus* strains are described: the interaction of PMN with *S. aureus* through antibodies against peptidoglycan; the interaction through antibodies and C3b; the interaction through C3b generated by direct interaction of peptidoglycan with complement (classical pathway); and the interaction through direct activation via the alternative pathway (*Verbrugh* et al., 1980; *Wilkinson* et al., 1979; *Verbrugh* et al., 1982; *Karakawa* et al., 1988; *Nelles* et al., 1985). Antibodies against the 0-acetyl group of capsular polysaccharide are most efficient in opsonisation. While anti-peptidoglycan antibodies promote phagocytosis *in vitro*, their opsonic capacity *in vivo* is unclear, as most strains grown under *in vivo* condition contain a capsule that shields the peptidoglycan from specific antibodies (Table 1).

It would be of interest to test the protective capacity of both antibodies against peptidoglycan and capsules in an animal model.

During *S. aureus* infection of the host antibodies against teichoic acid are also produced. Their role in opsonisation is questionable and is probably indirect via activation of the complement cascade. In contrast, protein A probably does play a triple anti-phagocytic role in the bacteria-cell recognition process by virtue of its binding to the Fc portion of IgG: 1) extracellular soluble protein A

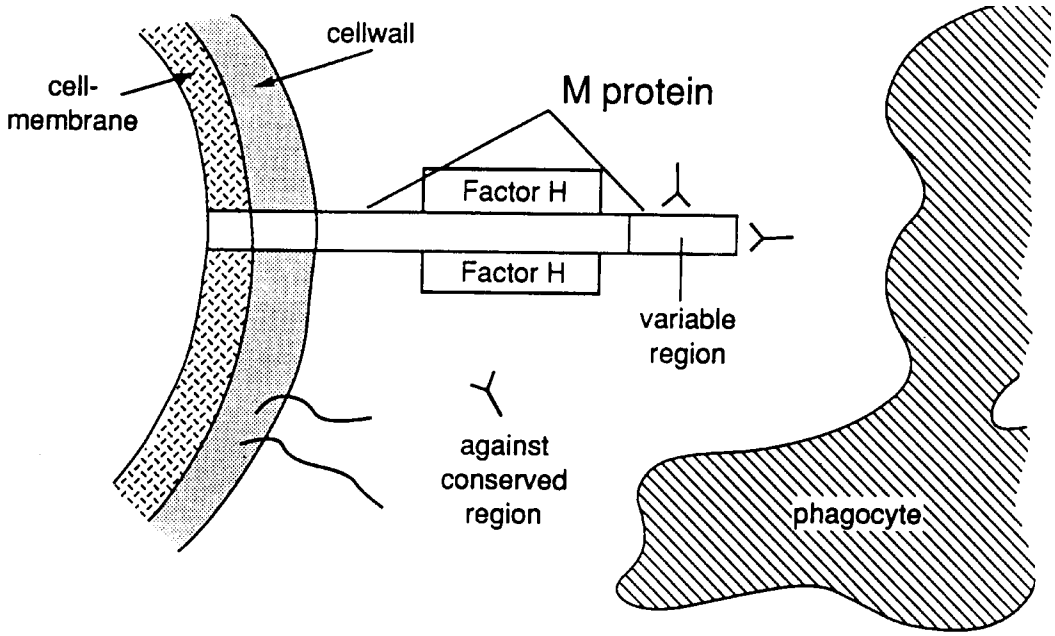

Figure 2: Interaction of M-proteins of streptococci with opsonic antibodies and factor H of the complement system.

can react with the Fc terminal of IgG molecules of human serum, thereby producing immune aggregates that consume complement. 2) extracellular protein A can bind to the Fc portion of specific anti-staphylococcal antibodies coating the microorganism with their Fab fragment, thereby preventing further interaction of the complex with the Fc receptor of phagocyte, and 3) cell-bound protein A binds to the Fc fragment of any IgG molecule in its neighbourhood, thereby eliminating non-specific and specific antibodies.

In recent years *S. epidermidis*, and other coagulase negative staphylococci has become major pathogens in hospitalised patients. Because of its ability to adhere to plastics, these organisms are formidable pathogens in the presence of foreign bodies. Principal adhesins that are responsible for the binding of *S. epidermidis* to catheters are a capsular polysaccharide and a protease-sensitive surface constituent from the slime-producing strains of *S. epidermidis*. In addition to promoting adherence to foreign bodies, these adhesins may also protect coagulase-negative staphylococci against phagocytosis. Antibodies to these adhesins may neutralise this shield and provide opsonisation of the bacteria. Monoclonal antibodies against *S. epidermidis* adhesins facilitate phagocytosis of homologous and heterologous *S. epidermidis* strains (*Kojima* et al., 1990; *Timmerman* et al., 1991). The major PMN receptor for *S. epidermidis* opsonins is the FcRIII receptor (*Schutze* et al., 1991). However, for strains that have a hydrophobic surface structure, antibodies by themselves are not sufficient for opsonisation. These strains also need C3b or iC3b (*Pascual* et al., 1988).

Streptococci

A major component of the *S. pyogenes* cell wall is M protein. M proteins interfere with opsonisation and therefore

can be regarded as important virulence factors. They form hair-like projections on the surface of group A streptococci. About 80% of M protein consists of blocks of repeating amino acid sequences. The amino acids near the C terminus bind M protein to the bacterial cytoplasmic membrane and, together with a region rich in prolines and glycines, they anchor M protein in the cell wall. This part of M protein is highly conserved and is also present in other cell wall proteins of Gram-positive bacteria (e.g. staphylococcal protein A; streptococcal protein G). The N-terminal end is the variable part of M protein and has an excess of negatively charged amino acids. PMN also exhibit a net negative charge on their surface. The negative charge of M proteins may thus have evolved to hinder contact between streptococci and phagocytic cells.

To add to this anti-phagocytic effect, M protein-positive streptococci do not bind C3b efficiently and thus evade opsonisation (Figure 2). This appears to be due to complement factor H binding of to the M protein, which prevents the deposition of C3b on the surface of *S. pyogenes*. In a sense, the M protein-bearing streptococcus cleverly disguises itself as a normal human cell to evade the complement system. During infection antibodies to the conserved and variable parts of the M proteins are made. Antibodies against the conserved part cannot bind when factor H is present and therefore do not have an opsonic capacity. Antibodies against the variable region are opsonic and also neutralise the region's negative charge and thereby further assist phagocytosis. However, they are only type-specific. Different streptococci with serologically different M proteins need different antibodies for efficient phagocytosis. One can thus be infected repeatedly with different group A streptococci.

Group B streptococci have cell wall components designed to evade the premature host defences of the neonate. These streptococci have polysaccharides and proteins in their cell walls which allow the strains to be differentiated into serotypes (Ia, Ib, Ic, II and III). The capsular polysaccharide is type-specific, while the C polysaccharide is group-specific and common to all strains of group B streptococci. Surface proteins are additional antigenic markers (*Baker* et al., 1982).

Most late-onset infections (onset of infections 6 days to 3 months after birth) are caused by group B streptococci belonging to serotype III, while early onset of infection can be caused by serotypes I, II, or III. Infections with meningitis are almost always caused by serotype III strains. The classical complement pathway and heat-stable opsonins are required for maximal opsonic activity by human sera for type I, II, and III strains (*Baker* et al., 1986). For clinical isolates of type Ia group B streptococci, opsonisation and phagocytosis may proceed via the classical complement pathway in an antibody-independent fashion, and C1 activation may be initiated by interactions with surface-bound capsular polysaccharides in these strains (*Baker* et al., 1982; *Levy* and *Kasper*, 1986; *Edwards* et al., 1982). Deficient opsonic activity of neonatal sera for clinical isolates of this serotype correlates with low levels of the classic pathway components C1q and C4. Since complement proteins in the neonate are not maternally derived and since levels of components in both the classical and alternative pathways are only 30-50 % of those in maternal or adult control sera at term, physiologically low levels of complement components or their receptors on phagocytes may provide a partial explanation for age-related susceptibility to group B streptococcal disease.

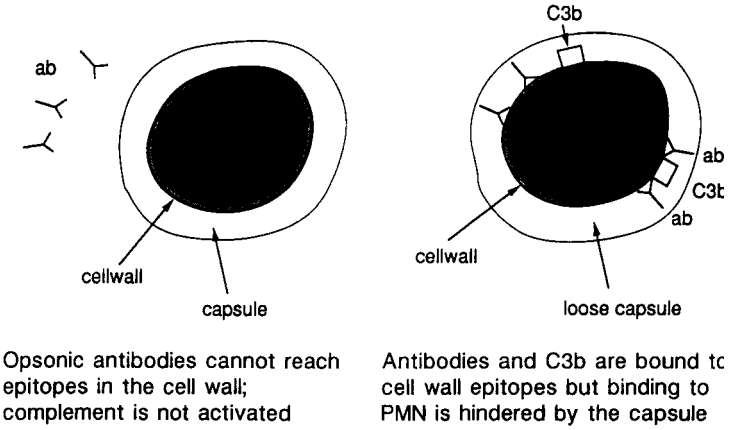

Figure 3: Ways capsules can interfere with phagocytosis.

Direct activation of C_1 may paradoxically be an extra virulence factor of the capsular polysaccharides. During infection large amounts of capsular material may be liberated from the bacteria. These fluid-phase macromolecules may then deplete complement by activating of C_1. Complement components necessary for opsonisation may then be absent.

Monoclonal antibodies (IgM and IgG) against type-specific antigens have been shown to be opsonic and to protect mice against lethal challenge with group B streptococci (*Egan* et al., 1983; *Hill* et al., 1984). Interestingly, IgA monoclonal antibodies were also shown to be opsonic (*Bohnsack* et al., 1989).

Opsonisation is an important factor in host defence against *S. pneumoniae*. Again, a polysaccharide capsule is the important virulence factor that hampers opsonophagocytosis. Of the 83 different serotypes of *S. pneumoniae* 23 cause nearly 90% of the pneumococcal infections. Like most bacteria, pneumococci are opsonised in the presence of complement through C3b and iC3b. Both activated complement factors are recognised by their specific receptors in the membrane of the PMN (CR1 and CR3). CR1 can specifically recognise C3b, while CR3 recognises iC3b. It is possible to differentiate between the C3b-CR1- and iC3b-CR3-specific interactions with monoclonal antibodies (*Gordon* et al., 1986). For example, the monoclonal antibody OKM10 that is able to block CR3 mediated phagocytosis of type 6A and 14 strains by 50-80%. These strains bear almost exclusively iC3b. Blockade of the CR1 receptor had no effect. For serotype III strains that bear C3b, iC3b, and C3d on the capsule, CR3- mediated phagocytosis accounted for only 20% of the uptake. Again, there was no evidence for CR1-mediated phagocytosis. The iC3b ligand elicits more release of superoxide, myeloperoxidase (MPO), and lactoferrin than C3b. The iC3b-CR3 interaction is thus the primary trigger for phagocytosis of iC3b-bearing pneumococci and for stimulation of intracellular bactericidal processes (*Hofstetter*, 1986). For comparison, it was shown that C3b, iC3b, and C3 make up 17%, 64% and 19% respectively, on *S. aureus* and 53%, 44%, and 2%, respectively, on *E. coli* (*Gordon* et al., 1988). Even among capsulated pneumococci a diversity exists in opsonisation. C3b and iC3b can be bound to the cell wall via a covalently linked thiolester-reactive

binding or via an amide linkage. Interestingly, the C3b molecules are bound almost exclusively to the capsule via the thiolester-reactive site, while the amide linkage is used for unencapsulated surfaces. The amide-linked molecules are far more potent activators of phagocytosis than the thiolester binding ones. This explains the ready phagocytosis of unencapsulated pneumococci and provides the capsule with another virulence mechanism.

Capsular polysaccharides may thus be regarded as virulence factors because they interfere with phagocytosis. This interference may be due to a variety of mechanisms preventing complement consumption (Figure 3). For example, binding of inefficient opsonins such as C3b, or thiolester-active binding of the C3b or iC3b molecule which makes the opsonisation less active by shielding or binding specific antibodies to cell wall antigens.

Haemophilus influenzae: Studies with *H. influenzae* have shown the crucial role for antibodies and complement in host defence. *H. influenzae* is the cause of respiratory tract infections in children and adults. In young children this microorganism can also cause bacteraemia and meningitis. Although unencapsulated *H. influenzae* strains can cause serious infections, most invasive infections are caused by the encapsulated strains. The capsule of *H. influenzae* type b is a polyribosyl-ribitol phosphate (PRP). Not only are antibodies against PRP necessary for opsonisation and protection of the host against recurrent infections (*Cates* et al., 1985), but classical complement components are also important for adequate opsonisation. This was shown in experiments with C1q-deficient serum, which demonstrated that opsonisation of *H. influenzae* type b may proceed through activation of the alternative pathway of complement, but that opsonisation via the classical pathway is much more efficient (*Roord* et al., 1983). The addition of C1q to C1q-deficient serum greatly enhanced opsonic activity. Also, there is an increased risk for *H. influenzae* infections in patients with a deficiency of other early components of the classical pathway of complement. In contrast, serum from patients with factor D (alternative pathway component) deficiency shows no impairment in opsonic activity for *H. influenzae*. This again underlines the predominant role of the classical pathway of complement in opsonisation of *H. influenzae* type b.

The third complement component C3 assumes a central role in the complement system. It participates in both the classical and the alternative pathways as well as in the amplification loop and is one of the major opsonins. Patients with C3 deficiency suffer from recurrent and often severe respiratory tract and systemic infections, that are frequently due to *H. influenzae* type b (*Roord* et al., 1983).

Enterobacteriaceae

Enterobacteriaceae are able to evade host defence. This capacity is mainly determined by properties of the bacterial cell wall. As shown in Figure 4, the Gram-negative bacterial cell wall consists of an inner cytoplasmic membrane, an intermediate murein or peptidoglycan layer, and an outer phospholipid-LPS bilayer in which proteins are inserted. LPS is anchored by the lipid region (lipid A) in the outer leaflet of the outer membrane with a covalently bound core-oligosaccharide structure directed outward. In addition, the outer part of the LPS of most strains, that are present in nature (wild-type strains), contains a polysaccharide chain (O antigen) bound to the distal terminal of the core-oligosaccharide. Some strains (e.g., many types of *E. coli* and *Klebsiella*) contain a surrounding capsular polysaccharide

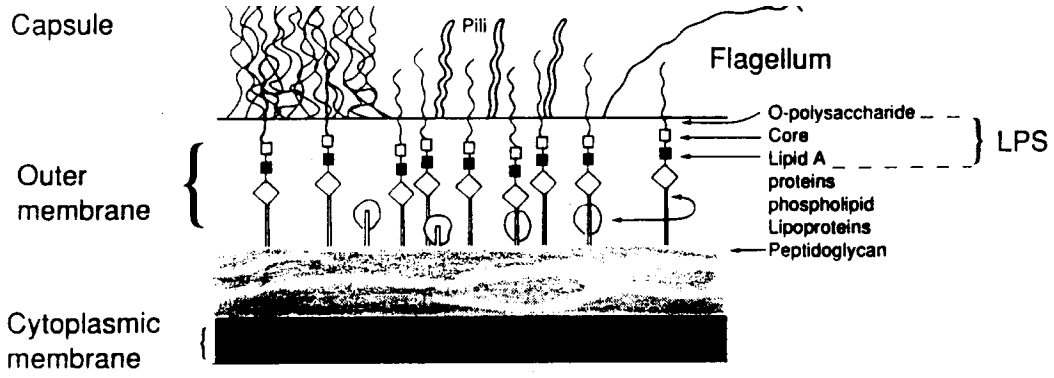

Figure 4: The Gram-negative bacterial cell wall.*

(K antigen). In addition, extruding protein structures, flagellae, pili, or fimbriae, may be present. These pili and fimbriae mediate the capacity of strains to adhere to and colonise mucosal surfaces.

Certain types of O an K antigens confer resistance to the bactericidal action of serum and phagocytosis by granulocytes. It has been suggested that the presence of large amounts of these polysaccharide structures may physically hinder the access of complement and/or antibodies to target structures on the bacterial surface, thereby either preventing activation of the complement pathways or yielding formation of the membrane attack complex at a site too distant from the bacterial cytoplasmic membrane (*Vermeulen* et al., 1988). Also, the anti-phagocytic effects of these structures may be related to interference with the hydrophobic nature of the bacterial surface.

Indeed, rough Gram-negative bacillary strains, defined by their deficiency of the polysaccharide side chain (O antigen), are sensitive to the bactericidal activity of the complement system and are readily ingested by granulocytes. Lipid A can bind the first complement factor (C1) directly, leading to an antibody-independent activation of the classical pathway. The polysaccharide region of LPS can activate the alternative pathway by a lipid A-independent, antibody-independent mechanism and has a modulating effect on the expression of lipid A binding and C1 activation. In addition to complement, specific antibodies to the surface structures are required for killing encapsulated (K+) and smooth (O+) bacilli in serum and for phagocytosis of such bacilli by PMN (*Vermeulen*, et al., 1988). Thus, Gram-negative bacilli that contain O and K antigens have an increased capacity of surviving in the bloodstream. Indeed, most episodes of Gram-negative bacteraemia are caused by smooth and/or encapsulated strains. Nevertheless, rough and unencapsulated strains of Gram-negative bacilli may also cause bacteraemia and septic shock. This is, however, rare and mostly seen in patients with severely diminished host defence. Once the imbalance between host defence and bacterial virulence has al-

*: Reproduced with permission from: Verhoef, J., and Visser, M.R.: Neutrophil phagocytosis and killing: Normal function and microbial evasion. In: The natural immune system: The neutrophil (Abramson, J.S., and Wheeler, J.G., Eds.). Oxford University Press, New York, 109-137 (1993).

lowed the microorganisms to invade and survive in the bloodstream, the ensuing cascade of events and ultimately the patient's outcome are dependent on the Gram-negative bacillary strain. All Gram-negative bacillary strains possess the capacity, upon invasion into the bloodstream, to cause septic shock syndrome.

Pseudomonas aeruginosa

Studies on the opsonic requirements of *P. aeruginosa* have shown that antibodies against mucoid exopolysaccharide (MEP; the primary constituent of the slime coat of mucoid strains) are important opsonins. These mucoid strains are the primary pathogens in cystic fibrosis patients. MEP promotes the adherence of *P. aeruginosa* strains to tracheal cells and respiratory mucins.

The heteropolymeric nature of MEP is explained by the presence of both common and type-specific epitopes; the common epitopes are divided into those that bind opsonising and those that bind non-opsonising antibodies. Most naturally occurring antibodies to MEP function poorly in in-vitro opsonophagocytosis assays with complement and are unable to protect host animals. In contrast, antibodies that are highly opsonic protect host animals against infection. and have been found in older CF patients who are not colonised with *P. aeruginosa*. Opsonising antibodies to MEP are usually not found in younger non-colonised or chronically colonised CF patients. These findings suggest a protective effect for the opsonising antibodies. MEP, therefore, has become a promising vaccine candidate for the prevention of *P. aeruginosa* infection in CF patients. Unfortunately, in humans MEP appears to be poorly immunogenic in inducing opsonic antibodies (*Schreiber* et al., 1991; *Garner* et al., 1990).

Neisseria

Specific antibodies and the complement system play key roles in host defence against *N. meningitides* and *N. gonorrhoea*. They can lyse bacteria, enhance phagocytosis and neutralise the effects mediated by endotoxin (*Jarvis* and *Vedros*, 1987; *Ross* et al., 1987). In *N. meningitides* the presence of a bactericidal antibody, however, is of utmost importance. Anti-capsular polysaccharide antibodies and anti-outer membrane protein antibodies in general facilitate phagocytosis and killing. Antibodies against meningococcal LPS also appear to contribute to opsonophagocytosis. However, for optimal phagocytosis complement should also be present.

Although the alternative pathway is not able to halt meningococcal dissemination in susceptible infants (indicating the importance of antibodies and the classical complement pathway), the relative importance of the alternative pathway is shown in families with a sex-linked properdin (alternative pathway) deficiency. Individuals belonging to these families experience multiple, sometimes fatal, episodes of fulminant group B, C, and Y meningococcal infections (*Jarvis* and *Vedros*, 1987). In contrast, individuals with a deficiency in early classical complement components show an unexpected lack of meningococcal disease. Some unknown compensatory mechanisms must be involved in the resistance against meningococci in these patients. Perhaps antibodies and the successful use of the alternative pathway may be responsible for this resistance mechanism.

Other microorganisms

The opsonic requirements of many other microorganisms (bacteria, fungi, parasites) have been studied. The general conclusion is that cell wall antigens

may determine whether the microorganisms is readily phagocytised. In some microorganisms the capsule is the determining factor, while in others proteins or lipopolysaccharides may be responsible for the resistance to opsonisation. For example, the expression of plasmid-encoded proteins is associated with resistance to complement-mediated opsonisation and neutrophil phagocytosis in the cell wall of *Yersinia enterocolitica*. PMN also play a role in eliminating virus particles (*van Strijp* et al., 1989, *Turner*, 1990). For example, interaction of herpes virions through PMN-complement CR1 and CR3 results solely in binding to PMN, but not in internalisation (*van Strijp* et al., 1989). For internalisation, interaction with FcR is mandatory. Recently, it has been shown that some parasites (e.g., *Trypanosoma cruzei*, the causative agent of Chagas' disease) produce a glycoprotein (gp160) that restricts complement activation by inhibiting C3 convertase formation. This glycoprotein is similar to a human complement regulatory protein, the decay-acceleration factor (*Joiner* et al., 1988).

CONCLUSION

Opsonins are of crucial importance for host defence against invading microorganisms. Many microbes have developed strategies to combat the interaction of bacteria with opsonins and thus evade recognition by phagocytic cells. Patients with low levels of opsonins, such as patients with hypogammmaglobulinaemia with complement deficiencies suffer from recurrent infection.

How important opsonins are in the containment of translocated bacteria from the gut into the mesenteric lymph nodes is not known. It is possible that at the site of the regional lymph node alternative opsonins and receptors are important, such as CD14 in the membrane of phagocytic cells, fibronectin binding receptors, binding via mannose-sensitive receptors or α-D-Gal(1-4)-ß-D-Gal.

It is also possible that in some tissues bacteria are fixed to cells and that phagocyte had to eliminate these fixed bacteria. This process appears not to involve opsonins and is mainly driven by surface charges (*Vandenbroucke*, 1988; *Pascual*, 1989). However, there is evidence that the phagocytic cell is damaged in the process of eating bacteria that are fixed to other cells (*Vandenbroucke*, 1988). This process does not appear to be very efficient.

In conclusion, opsonins are important; in some events phagocytosis can occur in the absence of opsonins. But it is likely that phagocytosis in the absence of opsonins is less efficient.

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IMMUNOMODULATION WITH LIPOSOMAL MTPPE AND IFN-γ IN GRAM-NEGATIVE SEPTICAEMIA

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INTRODUCTION

Severe infections represent a continuing threat to patients. The disappointing results with antibiotics are most prominent in the immunodeficient host. Factors considered important in the development of septicaemia include broad-spectrum antibiotic therapy, immunosuppressive treatments, invasive devices and surgery, penetrating wounds, burns or other trauma, anatomic obstruction, intestinal ulceration, the increased average age, and the very young, as well as progressive clinical conditions (malignancies, diabetes, AIDS, and other serious chronic diseases). Acquired and congenital immunodeficiencies as well as disease-associated host defence disorders add to the problem of increased fatal bacterial infections.

The major factor contributing to the failure of antibiotics to adequately combat bacterial infections in the immunodeficient host is probably the lack of support by the host defence system especially those who had persistent neutropenia. Different methods are available to improve treatment. One method is the intensification of antibiotic treatment, for instance the application of more drugs at the same. Another possible way to improve the therapeutic results might be the stimulation of the non-specific host defence.

Activation of the non-specific host defence has some advantages. An important advantage is that immunomodulation can be effective in different types of infection, and compared with antibiotic treatment there is no induction of tolerance of the microorganisms to the treatment. Especially the cells of the mononuclear phagocyte system (MPS) play a key function in the non-specific host defence. Activation of these cells will result first of all in enhanced killing of intracellular microorganisms infecting the MPS. However, it is expected that activation of the MPS can also enhance the resistance to more systemic (extracellular) infections. Activation of the non-specific host defence can be achieved with immunomodulators: biological or synthetic agents that influence or modify (parts of) the innate resistance in a direct or indirect way, independent of the challenge. Many different agents are tested for their immunomodulatory capacity. The immunomodulatory agents are from natural origin, for instance extracts of bacterial or herbal origin and cytokines, or synthetic (for instance some of the muramyl peptide derivatives). Here we focus in particular on muramyl tripeptide phosphatidylethanolamine (MTPPE),

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and the cytokine interferon-γ (IFN-γ). MTPPE is a derivative of muramyl dipeptide (MDP), the smallest fragment of the petidoglycan with adjuvant activity. Macrophage stimulating and antimicrobial activity of MDP (and derivatives) and IFN-γ has already been shown *in vitro* and *in vivo* to a broad spectrum of microorganisms. Thus far promising studies with IFN-γ in chronic granulomatous disease (CGD) patients has led to the approval of IFN-γ for prophylaxis against opportunistic infections in these patients (*The International Chronic Granulomatous Diseases Cooperative Study Group*, 1991).

MURAMYL PEPTIDES AND INTERFERON-γ

MDP can be produced synthetically, and many different modifications are made to reduce toxicity or improve activity and usability. The derivative discussed here is MTPPE, a synthetic hydrophobic derivative of MDP, in the liposome-encapsulated form (LE-MTPPE). MTPPE shows improved activity over MDP and due to its hydrophobicity a greatly increased association with liposomes. IFN-γ is a cytokine primarily produced by TH1 cells, CD8+ T cells and NK cells upon stimulation by for instance IL-1 and IL-12, and has stimulating activity on macrophages. IFN-γ influences all major macrophage functions including MHC II expression, antigen presentation, FcR1 receptor expression, uptake and intracellular killing of microorganisms, tumour cell cytotoxicity, and the production of monokines (*Baron* et al., 1991; *Biliau* and *Dijkmans*, 1990; *Czarniecki* and *Sonnenfeld*, 1993; *IJzermans* and *Marguet*, 1989; *Murray*, 1988, 1992; *Williams* et al., 1993).

Both muramyl peptides and IFN-γ were found to have potent antimicrobial enhancing effect on macrophages. The agents were found to induce the production of reactive oxygen and nitrogen intermediates (ROI and RNI), and other antimicrobial agents by macrophages, explaining the enhanced antimicrobial activity of the infected cells. Therefore, the rational to use these immunomodulators for the activation of the host defence to intracellular infections *in vivo* is easy to understand. However, macrophage activity can be enhanced by the immunomodulators also with respect to extracellular infections. *In vitro* results are some times in contradiction, however, increased uptake and killing of *Pseudomonas aeruginosa* by macrophages exposed to IFN-γ *in vitro* was noticed (*Pierangeli* and *Sonnenfeld*, 1993). Treatment of human peripheral blood monocytes with IFN-γ *in vitro* greatly enhanced both respiratory burst and microbicidal activity towards *P. aeruginosa* (*Kemmerich* et al., 1987). Others demonstrated that IFN-γ had no effect on macrophage phagocytic capacity of *Staphylococcus aureus* (*Quiroga* et al., 1992), or was even shown to have a negative effect on the uptake and killing of *P. aeruginosa* and *S. aureus* by macrophages (*Speert* and *Thorson*, 1991). Culture of human monocytes in the presence of IFN-γ enhanced the capacity to produce superoxide anion. However, the phagocytosis of *P. aeruginosa* was substantially depressed in a dose dependent fashion (*Speert* and *Thorson*, 1991). These findings are supported by *in vivo* incubation of resident or exudate peritoneal macrophages with i.p. injected IFN-γ. IFN-γ did not result in increased *in vitro* phagocytosis of *Salmonella typhimurium* as compared with untreated mice (*van Dissel* et al., 1987). Similar results were obtained after 18 h of *in*

vitro incubation of resident or exudate peritoneal macrophages with IFN-γ.

Others found that peritoneal macrophages incubated with IFN-γ for 12 h exhibited enhanced bactericidal activity against *S. typhimurium* (*Kagaya* et al., 1989) or *Salmonella enteritidis* (*Sasahara* et al., 1992), independent of oxygen metabolism. These results suggest that increased generation of ROI may not be primarily responsible for the observed ability to inhibit intracellular growth of bacteria. It was observed in our laboratory that peritoneal macrophages exposed LE-IFN-γ or LE-MTPPE or these agents combined (LE-MTPPE/lFN-γ) resulted in increased production of both ROI and RNI. However, the peritoneal macrophages did not exhibit increased phagocytic activity towards *K. pneumoniae* (*ten Hagen* et al., 1995). In contradiction it was demonstrated that MDP-lys(L18), an MDP analogue, stimulated alveolar macrophages to phagocytise *P. aeruginosa* (*Ozaki* et al., 1989). Also peritoneal macrophages isolated form mice treated with MDP showed marked augmentation of the phagocytosis of *Escherichia coli in vitro* (*Friedman* and *Warren*, 1984). These results clearly show that exposure of macrophages *in vitro* to IFN-γ or MDP derivatives results in a heterogeneous response with respect to bacterial killing. The discrepancy found is probably not only due to the different bacteria used but also depends on macrophage culture purity and condition.

Administration of MDP or MTPPE, as well as IFN-γ was shown to stimulate the host resistance in mice to *K. pneumoniae* infection in several models. The host resistance could be enhanced by prophylactic intravenous or subcutaneous administration of muramyl peptides to intravenous and intramuscular infections with *K. pneumoniae* (*Ausobsky* et al., 1984; *Chedid* et al., 1977; *Melissen* et al., 1992; *Parant* et al., 1978). Also intragastric administration of MDP in mice infected with *K. pneumoniae* resulted in enhanced resistance when administered 7 days before challenge (*Parant* and *Chedid*, 1985) or intramuscular infection with *K. pneumoniae* (*Chedid* et al., 1977). However, oral administration is less potent when compared with intravenous administration. Orally administered MDP analogues were not active towards intraperitoneal *P. aeruginosa* infection, whereas these compounds intravenously, intraperitoneally or subcutaneously injected protected mice from the infection (*Fraser-Smith* et al., 1983).

Protective activity of MDP (the derivative MDP-lys(L18)) was also demonstrated against *E. coli* infections and in lesser amount against *P. aeruginosa* (*Matsumoto* et al., 1983; *Osada* et al., 1982). There was a significant reduction in mortality in mice intraperitoneally infected with *E. coli* and treated with MDP compared to the controls (*Cheadle* et al., 1989), which was shown by others to correlate with increased WBC count (*Stellato* et al., 1988). MDP analogues administered intraperitoneally one day before infection with *P. aeruginosa* enhanced nonspecific host resistance (*Furuya* et al., 1989). A significant reduction in *E. coli* bacteraemia was observed in animals treated with a combination of MDP and clindamycin when compared to animals receiving placebo or either agent alone, indicating that immunomodulation might be beneficial in initially failing antibiotic treatment (*Lamont* et al., 1983). However, subcutaneous MDP pretreatment failed to enhance cytotoxic activity of the MPS towards intravenous infection with *E. coli*, which is most likely due to the intravenous route of infection (*Dunn* and *Horton*, 1990). Although good results are obtained with prophylactic administered MDP, mice

infected intramuscularly could even be protected by intravenous administration of MDP 1 h after infection (*Chedid* et al., 1977).

As stated before, due to the lack of sufficient host defence, especially immunocompromised patients are prone to severe infections, with often a bad prognosis. Important therefore is the potency of the immunomodulators to enhance host resistance adequately in the immunocompromised host. MDP was shown to enhance survival from subcutaneous infection with *K. pneumoniae* in 7-day old new-born mice, this in contradiction with LPS treatment (*Parant* et al., 1978). The results indicate that MDP does not only affect the macrophages directly, but must also have other activities, which are absent in LPS. It is claimed by the same authors that MDP is capable of enhancing the host defence to a *K. pneumoniae* infection in thymectomised, irradiated, and bone marrow reconstituted mice (*Parant* et al., 1976). Galland, Polk and colleagues showed that *K. pneumoniae* infected wounds can be treated to some extent with MDP in immunocompromised mice (*Galand* et al., 1983a; 1983b; *Galland* and *Polk*, 1982; *Polk* et al., 1982; 1990). Survival in mice starved for 48 h and treated prophylactically with 500 μg MDP before intramuscular infection with *K. pneumoniae* was increased to approximately 90% compared with 40% in the controls (*Galland* et al., 1983a). The MDP treatment resulted also in lower local and systemic bacterial spread and increased survival in mice immunosuppressed by cyclophosphamide (*Galland* et al., 1983b). However, immunosuppression with hydrocortisone was shown to have a deleterious effect in this wound infection model on the host defence following activation by MDP. Administration of MDP prior to inoculation of burn wound with *P. aeruginosa* had no beneficial effect on survival in mice (*Stinnett* et al., 1983). These results indicate that although host defence can be augmented towards infections in immunocompetent hosts, less favourable effects are observed in the immunocompromised host.

Hershman and colleagues (1988a) demonstrated that administration of IFN-γ can augment the host resistance to a *K. pneumoniae* infection. Mice infected intraperitoneally with *E. coli* after which the mice were secondarily infected intramuscularly with *K. pneumoniae* received a daily subcutaneous dose of 7500 U IFN-γ for 6 days experienced a 2-fold increased survival compared with the controls (63% and 35%, respectively). Prophylactic administration of IFN-γ to mice receiving *K. pneumoniae* intramuscular or as a wound infection resulted in significant increase in survival compared with the controls (*Hershman* et al., 1988a; 1988b; 1988c; 1989). In this model also therapeutic administered IFN-γ (1 h after intramuscular challenge) resulted in significant augmented host defence (*Hershman*, 1989). However no beneficial effect was seen when these mice were infected with *P. aeruginosa* (*Stinnett* et al., 1983). Mice infected in the right hind leg and receiving IFN-γ subcutaneously in the same leg showed the same improved survival compared with mice treated with IFN-γ in the other leg, indicating a systemic activity of the host defence by IFN-γ (*Hershman* et al., 1988b). These results indicate that host defence activation by IFN-γ is probably mediated by macrophages not necessary located at the site of infection, but resulting from a general strengthening of the host defence.

LIPOSOMAL MTPPE AND INTERFERON-γ

Although good macrophage activation and antimicrobial activity is shown after exposure to immunomodulators *in vitro*, *in vivo* results are often quite disappointing. Actually this is not surprising. The *in vivo* experiments are complicated by several factors, of which next to short half life, dilution, and lack of significant localisation at site of interest are the most important.

Important advantages of the use of liposomes as carriers is that by encapsulating of the agents in liposomes half life in the body is prolonged, high concentrations at specific sites can be reached, co-encapsulation of agents facilitates synergy *in vivo*, toxicity is reduced, an immunological reaction is prevented. Liposomes are microscopic vesicles consisting of one or more lipid bilayers surrounding an internal aqueous compartment. Liposomes are biodegradable, and non-immunogenic when composed of natural phospholipids. A variety of agents can be entrapped in liposomes: hydrophobic agents with high efficiency in the lipid bilayers, and hydrophilic agents in the inner aqueous space. As the macrophage is believed to be the most important target cell for immunomodulation the use of classical liposomes (ranging from 1 to 20 μm in diameter), which have a natural fate to localise in large numbers in these cells, is quite obvious.

The advantage of classical liposomes to rapidly accumulate in MPS cells, primarily the macrophages residing in the liver and spleen (*Melissen* et al., 1994a), results in an augmented localisation of the encapsulated agent in the macrophage. In mice LE-MTPPE was preferentially taken up by the liver and spleen (32% and 17% respectively after 60 min). Localisation in the lung however only reached 8.4% of the injected dose after 5 min, declining rapidly below 5% after 60 min (*Melissen* et al., 1994a).

Initial studies with liposomes of PC and PS (molar ratio, 7:3) encapsulating MDP demonstrated that MDP was poorly retained within liposomes after their preparation (50% released in 5 h at 37°C) (*Phillips* and *Chedid*, 1988). The lipophilic derivative MTPPE however, has been shown to associate more efficiently with the liposome (93%), and is also much more stable (*Dukor* and *Schumann*, 1987; *Gay* et al., 1993). The encapsulation of MTPPE and IFN-γ prolongs half life of these agents in the body. They are not excreted shortly after intravenous administration (*Fogler* and *Fidler*, 1984). The plasma levels of free MTPPE is very low when liposome-encapsulated (*Gay* et al., 1993). There was also no macrophage mediated release. The rapid clearance of the LE-MTPPE from the circulation is mediated by the tissue macrophages, and not via excretion in the urine. Intact liposomes can be observed in macrophages for several days (*Fidler* et al., 1988; *Raz* et al., 1981). These results indicate that LE-MTPPE forms a depot of immunomodulatory material within the macrophage and considerable time (up to days) is necessary to degrade the liposome to release the incorporated muramyl peptide.

The successful use of IFN-γ for *in vivo* immunotherapy is also limited by the rapid clearance of the cytokine from circulation, and the potential toxicity from high dosage regimens (*Goldbach* et al., 1995; *Bennet* et al., 1986; *Kurzrock* et al., 1985). Free IFN-γ has a serum half life of approximately 20 min, and is degraded and secreted from the body. Liposome-encapsulation of IFN-γ (LE-IFN-γ) increases half-life and the ability of this agent to stimulate the host defence.

The increased activity of LE-MTPPE and LE-IFN-γ over free immunomodulators was shown in an *in vivo* infection model using *Listeria monocytogenes* in our laboratory. Encapsulation of MTPPE or IFN-γ increased their efficacy 33- and 66-fold respectively in mice infected with *L. monocytogenes* (*Melissen* et al., 1993). An increased activity of LE-MTPPE over free MTPPE was also shown in a *K. pneumoniae* infection model (*Melissen* et al., 1994b). Moreover, MDP encapsulated in liposomes was 10- to 15-fold more active to *S. typhimurium* and *S. enteritidis* infection as free MDP (*Phillips* and *Chedid*; 1987).

Both MTPPE and IFN-γ induce unwanted side-effects such as fever, weight loss, liver and kidney toxicity, and MTPPE induces also histopathological changes in arteries. Liposomal encapsulation decreased toxicity of MTPPE, the liposomal formulation had a no-toxic-level of 0.1 mg/kg compared with 0.01 mg/kg for free MTPPE (*Schumann* et al., 1989). Reduction of toxicity of IFN-γ by liposomal encapsulation has also been demonstrated (*Hockertz* et al., 1991).

Together, these results demonstrate that liposomal encapsulation reduces toxicity of the agents by shielding them of from the body, and reducing localisation to sensitive sites (site avoiding delivery), but also increases localisation at the site of interest :the macrophage (site specific delivery). This means that lower dosages can be applied, and better results obtained *in vivo*. However, it was shown in our laboratory that LE-MTPPE administered after infection with *K. pneumoniae* could also have a dose depending negative effect on the host resistance (*Melissen* et al., 1994b). Most important observation is that in the *K. pneumoniae* infection models best antimicrobial effects were obtained when immunomodulators were administered 24 h or more before infection (*ten Hagen* et al., 1995; *Parant* and *Chedid*, 1985; *Melissen* et al., 1994b).

The possibility of co-encapsulation of agents into liposomes also provides an important tool for drug delivery *in vivo*. *In vivo* synergy is questionable since *in vivo* the simultaneous exposure of macrophages to additional immunomodulators after intravenous administration is expected to be minimal. With agents co-encapsulated in liposomes, simultaneous delivery of the agents to the macrophage is guaranteed. Synergy between MTPPE and IFN-γ in the free form was shown *in vitro* using *L. monocytogenes* infected peritoneal macrophages (*Melissen* et al., 1993). Co-encapsulation of MTPPE and IFN-γ also improved survival of mice suffering from a *K. pneumoniae* septicaemia compared with the agents in the free from (*ten Hagen* et al., 1995)

In a murine model mimicking a naturally acquired septicaemia with *K. pneumoniae* the effect of MTPPE and IFN-γ on the host defence was studied in our laboratory. In this model bacteria are injected intraperitoneally, allowing the bacteria to multiply and appear in the blood, resulting in a septicaemia followed by death of all animals within 5 days after challenge. A single prophylactic dose of 25 μg LE-MTPPE resulted in 30% survival (*ten Hagen* et al., 1995). However, repeated prophylactic administration of LE-MTPPE (5 dosages of 25 μg daily), resulted in a survival of 65%. These findings indicate that the MPS cells do not become refractory to treatment. The beneficial effect of multiple treatment was also shown with an MDP analogue in an intraperitoneal infection model with *P. aeruginosa*: increased survival form 45% in control mice or mice treated with a single dose of norMDP, to 90 % in mice receiving 4 dosages (*Fraser-Smith* and *Matthews*, 1981).

In the *K. pneumoniae* septicaemia model utilised in our laboratory also the effect of LE-IFN-γ and the liposome-encapsulated combination of IFN-γ with MTPPE was studied. Intravenous injection of a single dose of LE-IFN-γ 24 h before infection resulted in 15% survival (*ten Hagen* et al., 1995) whereas five dosages of LE-IFN-γ could further increase the survival of mice to 65%. Moreover, combination of MTPPE together with IFN-γ by co-encapsulation in liposomes resulted in 100% survival (*ten Hagen* et al., 1995).

A VIEW ON THE MECHANISM

From the discussed studies it can be concluded that muramyl peptides and IFN-γ are potent stimulators of macrophage function. Exposure of macrophages to these agents results in an enhanced metabolic activity, excretion of ROI and RNI, production of important host defence activating monokines, and an increased antimicrobial activity of the cells. However, *in vitro* studies also frequently show that only macrophage activation is not sufficient.

Studies with macrophages exposed to LE-MTPPE, LE-IFN-γ or LE-MTPPE/IFN-γ *in vitro* demonstrated an enhanced production of nitrogen or oxygen intermediates when stimulated with heat-killed Gram-negative bacteria (*ten Hagen* et al., 1995). However, an increased antibacterial activity to *K. pneumoniae* could not be found *in vitro* when isolated macrophages were exposed to the above mentioned immunomodulators. These results are very striking as macrophages are thought to be the primary target for the immunomodulatory agents, certainly when encapsulated into liposomes. Moreover, the *in vitro* results also indicate that the observed increase in survival in immunomodulator treated mice suffering from a *K. pneumoniae* septicaemia, can not be explained solely by the increased activity of the tissue macrophages themselves.

It has been demonstrated that administration of MDP or MTPPE (free or liposome encapsulated) resulted in an increased blood clearance capacity of the MPS cells (*Ausobsky* et al., 1984; *Melissen* et al., 1992; *Parant* et al., 1978; *Fraser-Smith* et al., 1982; *Izbicki* et al., 1991). It was therefore speculated that increased survival from Gram-negative infection in mice induced by these immunomodulators resulted in the first place from an augmented phagocytic activity of the tissue macrophage, and hence an increased clearance of bacteria from blood. Studies with IFN-γ also demonstrated host defence activation, which was speculated to be a result of increased bacterial clearance rather than prevention of systemic spread (*Izadkhah* et al., 1980; *Matsumara* et al., 1990).

Others demonstrated that also locally the access of bacteria to the bloodstream is restricted in a wound infection after treatment with MDP (*Polk* et al., 1982). They claim that the MPS cells are not significantly enhanced by MDP, because increase of bacterial concentration in the liver coincided with an increase in the degree of bacteraemia. However, when bacteria do progress to the blood, for instance in an intraperitoneal infection model, lymphatic filtration can not explain the improved resistance after immunomodulator treatment. Increased phagocytic activity of the MPS cells on the other hand is also not a likely explanation as was shown *in vitro* as discussed above. These findings indicate that direct activation of macrophages by the immunomodulator is not the only

explanation for the increased host defence observed *in vivo* in severe (Gram-negative) infections. The improvement of the host defence *in vivo* might result from improved macrophage activity as well as enhanced macrophage cell number. Therefore increased clearance by the MPS might still be one of the explanations.

Melissen et al. (1994a) demonstrated that no correlation existed between liposome uptake and phagocytosis of bacteria *in vivo*. Therefore we propose that immunomodulation results in a cascade of events resulting in direct and indirect activation of macrophages, of which the indirectly activated macrophage may be the most active. Certainly in an intracellular infection the direct or indirect activation of macrophages would explain the observed increase in microorganism killing.

A finding which also might explain the host defence activation is the observed increase in the number of granulocytes and monocytes in the blood as was shown after treatment with free MTPPE or LE- MTPPE (*ten Hagen* et al., submitted for publication [a]; *Melissen* et al; 1992). We found that LE-MTPPE/IFN-γ treatment resulted in the first place in strongly augmented haemopoietic cell numbers in liver and spleen (*ten Hagen* et al., submitted for publication [a]). Especially myeloid cell numbers (monocytes and macrophages) were increased in these organs, whereas strongly increased erythropoiesis was also observed in the spleen. Secondly, treatment with LE-MTPPE/IFN-γ induced a shift in the bone marrow haemopoiesis towards generation of myeloid cells, whereas erythropoiesis declined. These results indicate that immunomodulation results in a dramatic increase in the number of MPS cells, resulting in an increased phagocytic capacity of this system. Together these results suggest that 1) increased recruitment of macrophages and granulocytes from bone marrow, 2) local proliferation of myeloid cells, and 3) augmented haemopoiesis in bone marrow account for the observed host defence improvement. Another striking observation is the dramatic augmented erythroblast cell number in the spleen after immunomodulation. It might be that the often observed anaemia accompanying sepsis is counteracted by the LE-MTPPE/lFN-y induced enhanced erythropoiesis in the spleen.

Upon stimulation macrophage produce many different cytokines (i.e. IL-1, TNF-α, IL-12 etc.). Especially IL-1 and IL-12 have a stimulating effect on T cells, and NK cells, responding with production of IFN-γ, which has in turn a stimulating effect on macrophages. Studies with MDP, MTPPE or IFN-γ demonstrated increased colony stimulating activity (colony stimulating factors (CSF) which stimulate cell proliferation) in serum after treatment. TNF-α production by macrophages is also strongly increased, and is known to have stimulating activities on growth and development of lymphoid tissues (*De Togni* et al., 1994). This mechanism also includes an important role for T cells. It was shown that potentiation of resistance by IFN-γ is possible in *Leishmania donovani* infected euthymic mice, but not in nude mice (*Murray* et al., 1995). Transfer of CD4+ or CD8+ T cells permitted nude mice to respond to IFN-γ treatment, which on the other hand could not be compensated with T cell derived cytokines alone. NK cells or NK derived endogenous IFN-γ did not seem to play any apparent role. The anti-Leishmanial effect correlated with a markedly enhanced mononuclear cell recruitment to infected liver foci. It was demonstrated in our laboratory that T cells play a very important role in the LE-MTPPE, LE-IFN-γ or LE-MTPPE/IFN-γ increased host defence

to *K. pneumoniae* septicaemia (*ten Hagen* et al., submitted for publication [b]). Depletion of CD4+ T cells or CD8+ T cells dramatically inhibited antimicrobial potentiation by the immunomodulators. Moreover, blocking of IFN-γ *in vivo* demonstrated that especially the production of endogenous IFN-γ is important in the host defence activation by the immunomodulators (*ten Hagen* et al., submitted for publication [b]). It was shown that treatment with LE-MTPPE/IFN-γ preferentially induced a Th1 T cell response in the spleen, resulting a high numbers of IFN-γ producing (Th1) cells. It is tempting to speculate that Th1 T cells, CD8+ cells and NK cells (cells known to produce IFN-γ) play a key role in the cytokine network induced by macrophage targeted immunomodulators.

PROSPECTS

With the ongoing problems with severe infections, and the inability of antibiotics to provide adequate therapy in immunodeficient patients, selected patient groups must be tested for the beneficial activities of immunomodulation. As is shown above the most promising results can be expected with prophylactic treatment. Especially patients who are prone to opportunistic infections, the immunocompromised patients, are of interest. Good results can only be anticipated when formulations are used in patients, still in possession of a good deal of their immune system, but on the brink of becoming severely immunosuppressed. In these patients combination of the best possible antibiotics with the most promising of the immunomodulators: in our perspective liposome-co-encapsulated MTPPE and IFN-γ, must be tested.

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COLONY STIMULATING FACTORS (CSFs) TO PREVENT OPPORTUNISTIC INFECTIONS

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SUMMARY

The colony stimulating factors, i.e., granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF), stimulate the production of phagocytes both *in vitro* and *in vivo*. G-CSF selective stimulates neutrophil formation; GM-CSF stimulates the formation of neutrophils, monocytes and eosinophils. G-CSF levels increase with infections and reduced levels of G-CSF result in severe neutropenia. Administration of the CSFs cause a dose dependent rise in the blood neutrophil count attributed to increased production of these cells by the bone marrow. Randomised clinical trials have established that the CSFs are useful to accelerate marrow recovery in a variety of clinical settings including myelosuppression after chemotherapy and haematopoietic transplantation. G-CSF is also useful for treatment of severe chronic neutropenia due to congenital cyclic or idiopathic neutropenia. It is also used widely for patients with neutropenia due to HIV infection and autoimmune diseases. The potency and relative safety of these agents has prompted the exploration of their use in normal subjects to facilitate the collection of neutrophils and peripheral blood stem cells. In all of these applications, the underlying principle is to increase production of phagocytes because of the vital role of these cells in preventing and containing infections.

INTRODUCTION

The colony stimulating factors or CSFs are a family of glycoproteins which play a regulatory role in the production deployment and function of white blood cells. *Bradley* and *Metcalf* (1966) coined this term after they discovered that mouse bone marrow cells will form clusters and colonies of differentiating haematopoietic cells when grown in a semi-solid medium overlaying other cells capable of producing the CSFs. Concomitantly *Ishikawa* et al. (1966) made similar observations. Subsequent studies showed that the CSFs can be detected in the supernatants from cultures of many types of cells (*Metcalf*, 1991). The CSFs are also found in serum, plasma and urine, particularly with acute infections and after endotoxin administration (*Cebon* et al., 1994; *Selig* and *Nothdurft*, 1995; *Waring* et al., 1995).

The development of molecular biotechnology has greatly expanded our knowledge of the CSFs and related haematopoietic growth factors (HGFs)

and the interleukins (now IL-1 through IL-17). There are three CSFs which have been studied extensively including clinical investigations: granulocyte colony-stimulating factor (G-CSF); granulocyte-macrophage colony-stimulating factor (GM-CSF); and macrophage colony stimulating factor (M-CSF). A fourth factor, once called multi-CSF but now called IL-3, has also been widely studied. Many of the interleukins as well as the HGFs can affect haematopoietic colony formation *in vitro*, but the term CSF is currently not used for these factors. Only G-CSF and GM-CSF are approved for clinical use by governmental authorities in the USA and Europe.

Early studies of the CSFs focused on their effects on cell proliferation. It is now widely recognised that they have many other effects, including acceleration of differentiation and maturation, enhancement of function, modulation of cytokine production and inhibition of apoptosis of both mature and immature cells (*Hill* et al., 1995; *Dale* et al., 1995; *Price* et al., 1996). This diversity of effects has provided many opportunities for clinical applications of the CSFs (*Mertelsman* and *Hermann* 1990; *Morstyn* and *Dexter*, 1994).

COLONY-STIMULATING FACTORS AND THE PRODUCTION AND FUNCTION OF PHAGOCYTES

Clinical development of the CSFs has been based on our understanding of the role of phagocytes, i.e., neutrophils, monocytes, macrophages and eosinophils, in host defences and the physiological effects of these growth factors on the deployment and function of these critical cells. Phagocytes are derived from haematopoietic stem cells. Throughout life, normal production of phagocytes depends on a continual input from these precursor cells (*Quesenberry*, 1995), (*Babior* and *Golde*, 1995); (*Lehrer* and *Ganz*, 1995). Formation of phagocytes from early precursors ordinarily takes ten to fourteen days. For neutrophils there is a marrow storage pool of cells which can be readily released into the circulation in response to endotoxaemia or infection. For monocytes and other leukocytes there are no marrow storage pools, but there are large supplies of these cells and their progeny in many tissues. Blood neutrophils have no proliferative potential; they are all destined to die in the blood or tissues. By contrast, monocytes are released earlier in their development, have considerable proliferative potential and a relatively long tissue life span (*Lehrer* and *Ganz*, 1995). These overlapping and complementary features of leukocytes contribute to the strength of the host defence system.

Infection or tissue injury provokes an acute inflammatory response. Neutrophils are mobilised from the marrow reserve and transit through the blood to the site of invasion and injury. Classical studies of inflammation demonstrate the orderly process of fluid exudation, neutrophil then monocyte accumulation, fibroblast and epithelial cell proliferation and wound healing. In this response, it is the neutrophil supply which is often deficient, leading to enhanced susceptibility to serious infections (*Dale*, 1995).

There are many clinical examples of the critical role of neutrophils. For example, in the leukocyte adhesion deficiency syndromes, neutrophils and other leukocytes are produced abundantly (*Harlan*, 1993). However, the cells cannot adhere to the vascular en-

dothelium normally. Therefore they can not migrate to the tissues and repeated serious infections occur. Glucocorticosteriod therapy predisposes to infection in part by similar mechanisms, i.e., impairment of the cellular component of acute inflammatory response (*Dale*, 1974; *Goldstein*, 1992). Impairment of the neutrophil response is also induced by administration of monoclonal antibodies directed to the critical integrins on the neutrophil surface, e.g., CD11b/18, which are responsible for mediating this response (*Harlan*, 1993). Neutropenia predisposes also to infection because of a deficient acute inflammatory response. Tissue localisation of infection fails and bacteraemia and the sepsis syndrome follow because the invasion by microbes goes unchecked (*Dale*, 1995). In some other rare diseases such as the Chediak-Higashi syndrome and glycogen storage disease 1b, there are deficiencies of both the neutrophil supply and capacity of the neutrophils to kill microbes (*Smolen* and *Boxer*, 1995). Overall, however, it is the supply of cells rather than deficiency in their capacity to kill bacteria or fungi which is the predominant phagocyte disorder predisposing to infections.

With minor infections the body's supply of phagocytes in the marrow, blood and tissues is sufficient to protect the host from serious consequences. With more severe infections, the inflammatory response and the outcome for the patient depends upon the capacity of the host to increase the supply of these cells. Patients recently receiving myelotoxic chemotherapy or radiotherapy are vulnerable to infections because of low circulating neutrophils and a reduced proliferative capacity of their haematopoietic system (*Liles* and *Dale*, 1995). In a variety of haematological disorders causing severe chronic neutropenia, e.g., congenital, cyclic and idiopathic neutropenia, the severity of reduction in the neutrophil supply determines the frequency and severity of bacterial infections (*Dale*, 1979). Agranulocytosis occurring as a idiosyncratic reaction to drugs, nutritional deficiencies such as folate and vitamin B_2 deficiency, alcoholism and ageing are other causes of increased susceptibility to infections due to a reduced neutrophil response.

CLINICAL APPLICATIONS OF THE CSFs

Because phagocytes play such a critical role in preventing opportunistic infections, there have been many efforts to find ways to stimulate their production, including treatment with vitamins, lithium, androgens and the glucocorticosteroids. In general these agents proved to be relatively ineffective (*Dale*, 1995). In the late 1980's, granulocyte colony-stimulating factor (G-CSF) and granulocyte macrophage colony-stimulating factor (GM-CSF) became available for clinical investigations. They have proven to be very useful agents for preventing fever and infections by enhancing the phagocyte supply under a variety of circumstances (*Lieschke* and *Burgess*, 1992; *Petersdorf* and *Dale*, 1994).

G-CSF

G-CSF is a 174 amino acid glycoprotein produced *in vitro* and *in vivo* by macrophages, fibroblasts, endothelial cells and other types of cells (*Molineux* and *Dexter*, 1994). Endogenous levels of G-CSF are increased with infections and endotoxaemia (*Cebon* and *Layton*, 1994). This growth factor is essential for maintenance of normal blood neu-

trophil levels. Experimentally induced deficiency states result in reduction of blood neutrophils to about 20% of normal (*Hammond* et al., 1991; *Lieschke* et al., 1994).

Early clinical trials of G-CSF showed that it induces a dose dependent and selective increase in the blood neutrophil count (*Morstyn* et al., 1988; *Gabrilove* et al., 1988). In haematologically normal persons, blood neutrophils can readily be raised to 2 to 3 times the normal level within a few hours after injection of this agent (*Chatta* et al., 1994; *Liles* et al., 1997). With repeated administration, neutrophil levels rise to a dose dependent plateau. This occurs because G-CSF increases proliferation of marrow neutrophil precursors, stimulates the transit of neutrophils through the marrow and releases the maturing cells from the marrow to the blood (*Price* et al., 1996).

Randomised control trials have established that G-CSF can reduce the duration and severity of neutropenia and reduce the occurrence of fever and infection following cancer chemotherapy (*Lieschke* and *Burgess*, 1992). Two randomised control trials established this effect (*Crawford* et al., 1991; *Trillet-Lenoir* et al., 1993). They showed that the duration of severe neutropenia was decreased from approximately 6 days to 3 days when patients with small cell lung cancer treated with a combination of cyclophosphamide, doxorubicin and etoposide received G-CSF at a daily dose of 5 μg/kg/day beginning the day after completion of the six cycles of chemotherapy. The differences in the duration of neutropenia and the occurrence of fever with neutropenia (febrile neutropenia) were statistically highly significant. Although not a primary endpoint in this trial, the occurrence of documented infections was reduced by approximately 50% (*Crawford* et al., 1991). Roughly 1/3 of the febrile patients had a documented infection. There was one important difference between these two trials. In the US study patients were randomised at enrolment, but, if they developed febrile neutropenia, they were allowed to cross over from the placebo to the treatment arm in subsequent cycles. There was a more rapid attrition of patients from the placebo group than from the G-CSF group, complicating analysis of the data. The second randomised study, performed in Europe, did not allow for cross-overs (*Trillet-Lenoir* et al., 1993). It showed similar overall results to the first trial, but also better maintenance of on-schedule administration of chemotherapy. In these studies, antibiotic treatment was not standardised so many potential interrelationships of neutropenia, prophylactic and therapeutic antibiotics and G-CSF administration were not studied. The efficacy of antibiotics, singly or in combination, as an alternative to G-CSF also has not yet been studied carefully. In general, however, prophylactic and excessive use of antibiotics in this setting is to be avoided because of the well recognised frequency of bacterial and fungal superinfection in these highly susceptible hosts (*Goldmann* et al., 1996).

Subsequent to these two randomised trials, numerous studies have confirmed that G-CSF can accelerate marrow recovery after a wide variety of chemotherapeutic agents. The efficacy of G-CSF is largely determined by the capacity of the marrow to respond to treatment. Clinical benefit is reflected by the duration and severity of neutropenia which would occur if the CSFs were or were not given. Most studies suggest that the greatest clinical benefit occurs when this cytokine is administered soon after the completion of chemotherapy, usually the following day (*Ozer*, 1994). The benefit is attributed to stimulating marrow recovery in advance of the nat-

ural response. Because there are few side effects associated with G-CSF treatments and its benefits are so readily apparent, there has been much discussion about the appropriate patients for treatment with this relatively costly therapy (*Boogaerts* and *Demuynck*, 1996). Currently it appears if there is at least a 30% risk of febrile neutropenia in a patient receiving conventional chemotherapy, the cost is justified by the reduction in hospital and other medical expenses (*Smith*, 1996). This estimate does not take into account the patient's loss of income with the occurrence of fever, infection or hospitalisation after chemotherapy or the impact of this medical treatment on the patient's quality of life.

G-CSF is also proven to accelerate bone marrow recovery after intensive chemotherapy and haematopoietic transplantation (*Bierman* et al., 1996). Benefits are in general similar to those seen after chemotherapy but may be of greater magnitude because of the marked susceptibility to infection which follows transplantation. Despite optimal care there is still a period of severe susceptibility to infection after marrow ablation which cannot be overcome by G-CSF or any other growth stimulating factors.

Soon after the introduction of G-CSF it was recognised that the peripheral blood contains large quantities of haematopoietic precursor cells a few days after starting a daily schedule of administration of this cytokine (*Sheridan*, 1996). These cells, now called peripheral blood progenitor cells (PBPCs of PBSCs), can be frozen for later infusion and can be used as an alternative to bone marrow cells for haematopoietic transplantation. Most major transplant centres now use PBPCs for autologous and allogeneic transplantation because infusion of very large numbers of these cells reduces both the duration of neutropenia and thrombocytopenia after transplantation very substantially.

G-CSF is also effective for treatment of patients with severe chronic neutropenia due to congenial, cyclic and idiopathic neutropenia (*Dale* et al., 1993). Neutrophil production in these conditions is impaired because of intrinsic disorders of neutrophil formation. Production can be enhanced by daily subcutaneous injection of G-CSF doses of 1-10 μg/kg/day for most patients. Treatment reduces the occurrence of mouth ulcers and gingivitis as well as the occurrence of fever and requirement for antibiotic treatment and hospitalisation (*Dale* et al., 1993). Continued treatment is required, but it has been well tolerated and well accepted by hundreds of patients without loss of effectiveness due to the development of antibodies to the G-CSF (*Dale*, 1995).

G-CSF is also now used on a long term basis to prevent infections associated with neutropenia in patients with myelodysplasia, human immunodeficiency virus (HIV infection) and the neutropenia associated with autoimmune diseases. In patients with presumed autoimmune neutropenia the presence of antineutrophil antibodies does not appear to reduce the efficacy of G-CSF treatment. As in the chemotherapy setting, the cost of treatment as well as the severity of the susceptibility of infections of the individual patient are critical factors in selecting patients for treatment.

GM-CSF

GM-CSF is a 127 amino acid glycoprotein which also stimulates the proliferation of haematopoietic cells of the granulocytic series *in vitro* and *in vivo* (*Gasson*, 1991). It also stimulates formation of monocytes and eosinophils. In general, the time course for the neutrophil response for GM-CSF is similar

to that for G-CSF but the increase in the blood neutrophils after GM-CSF is less (*Lieschke* and *Burgess*, 1992).

There are now numerous trials of GM-CSF to accelerate marrow recovery after chemotherapy and haematopoietic transplantation utilising both bone marrow and peripheral blood progenitor cells (*Gerhartz* et al., 1993; *Advani* et al., 1992; *Hill* et al., 1995). In both of these circumstances, GM-CSF accelerates recovery of blood neutrophils with similar effects to those observed with G-CSF. GM-CSF has been approved to hasten marrow recovery with delayed engraftment and for treatment of patients whose marrow transplant appears to have failed. It is used for most of these applications somewhat less than G-CSF because of a somewhat more severe side effect profile (*Ozer* 1994; *Hill* et al., 1995).

The use of CSFs in the treatment of acute myelocytic leukaemia (AML) has undergone rapid evolution recently (*Geller*, 1996). Patients with AML are particularly prone to infections because their primary disease impairs neutrophil production and the recovery of haematopoiesis after chemotherapy is slower than for non-haematological malignancies. Several recent randomised control trials have examined the utility of GM-CSF of G-CSF in the treatment of AML. It is very important that neither GM-CSF or G-CSF treatment was found to be associated with an increase in relapse rates for leukaemia, despite the observation that leukaemic blast cells for these patients often will proliferate on exposure to these growth factors. Several studies have demonstrated a significant improvement in neutrophil recovery with associated reduction in severe bacterial infections in most patients (*Geller*, 1996). In one trial with GM-CSF there were fewer severe infections, fewer fatal infections, fewer fatal pneumonias and fewer deaths associated with fungal infections (*Rowe* et al., 1995). As in other clinical trials, most patients in these studies have received extensive antibiotic treatment. Few inferences about the specific relationships of neutropenia, antibiotics and CSFs can be derived from these studies.

OTHER APPLICATIONS OF THE CSFs

Because patients with infections associated with severe neutropenia often have marrow diseases preventing or blunting their responsiveness to the CSFs or because their infection is so severe that they do not have time to respond, there has been recently an awakening interest in neutrophil transfusion therapy for the prevention and treatment of infections in these patients (*Maakestad* et al., 1996). Considerable impetus to these studies came from investigations in normal subjects showing that G-CSF is well tolerated and serves to rapidly increase the blood neutrophil count and expand haematopoiesis without significant side effects (*Chatta* et al., 1994). Initial clinical trials of G-CSF to mobilise and facilitate neutrophil collection from normal subjects have been encouraging and further clinical trials are now underway (*Bensinger* et al., 1993; *Caspar* et al., 1993; *Griggs* et al., 1995). In the past, this type of transfusion support has been limited by the number of cells which can be collected. After G-CSF or G-CSF plus dexamethasone, 100×10^9 neutrophils can often be collected from normal donors. Studies of the efficacy of these cells in protecting the host or accelerating clearance of infections are underway.

OTHER HAEMATOPOIETIC GROWTH FACTORS

There are a number of other HGFs at various stages of clinical development including IL-3, a molecular hybrid of IL-3 and GM-CSF, M-CSF and several interleukins. Many of these factors are regarded as working at an earlier stage of haematopoiesis than G-CSF or GM-CSF. Most appear to have at least some side effects but several of these factors may prove to be useful as adjuncts to G-CSF and GM-CSF.

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GENOTYPE x ENVIRONMENT INTERACTIONS AS RELATED TO ANIMAL HEALTH IMPAIRMENT (WITH SPECIAL EMPHASIS ON METABOLIC AND IMMUNOLOGICAL FACTORS)

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SUMMARY

In intensive animal production systems, diseases are commonly infectious and multifactorial in nature. They are the negative reflection of the dynamic balance between host resistance factors and environmental stressors including microorganisms. Stressors in the animal's environment may be related to housing and microclimate conditions, nutritional factors, hygiene and infection burden, and managerial strategies such as transport. The different genetic make-up of the animals leads to different disease outcome too. The current paper gives an overview of research conducted in the area of nutrition and metabolism as related to health, and the area of immunology as a parameter of health, both under the influence of stress. It is hypothesised that research for adequately elucidating the relationships between nutrition and health should be focused on the interactions between environmental conditions, nutritional factors and health parameters. These interacting processes should lead to adaptational responses. This research should comprise a non-steady state and adaptational responses of animals under pressure. Research on antimicrobials as related to these interactions fits well in this design.

INTRODUCTION

Millions of people die each year due to combined effects of malnutrition and infectious diseases, particularly in developing countries and among infants and weanling children. Metabolic, hormonal and physiological responses to infection jointly accelerate the utilisation of nutrients and or body reserves. The multiple host responses to infection lead to substantial direct and indirect losses of nitrogen and other essential nutrients. Malnutrition shows an adverse effect on general host resistance, and on immunological and physiological mechanisms of host defence (*Suskind*, 1977; *Beisel*, 1982; *Sijtsma*, 1989). Basically, there are no large differences between human and food animal species in this respect. The phenomenon not only applies to a situation of malnutrition, but also or rather, to common practice conditions where infections and stress are involved.

Table 1: Results of a multivariate logistic regression analysis of factors associated with the prevalence of coccidiosis in broilers (after *Henken* et al., 1992)

Variable	Number of flocks	Odds ratio[1]
Breed:		
1	2	–
2	79	0.12
3	71	0.01
4	5	0.01
5	11	0.16
6 (reference)	21	
Lighting regimen:		
intermittent	91	7.53
continuous (reference)	98	
Ambient temperature:		
per °C	189	0.72
Aerial ammonia concentration:		
<14 ppm	100	0.29
>14 ppm (reference)	89	
Aerial carbon dioxide concentration:		
>0.4 vol%	112	0.41
<0.4 vol% (reference)	77	
Amount litter:		
per kg/m^3	189	2.08
House surface:		
600-800 m^2	34	8.18
<600 m^2 (reference)	34	

[1]: Odds ratio (OR) is the parameter that expresses increased (OR>1) or decreased (OR<1) risk in animals exposed to a certain variable, in comparison with exposure to the reference or with non-exposure (*Martin* et al., 1987). E.g., the more litter material is provided per m^3, the higher the probability of coccidiosis.

Well-controlled experimental studies in man to investigate effects of and possible interactions between nutrients, nutritional stress and infections are rather difficult to perform. Nutrition is just one area where stress may play a role. Experimental infection of man is not extensively done due e.g., to ethical and practical reasons. Therefore, it appears indicated that an animal model be used and, where feasible, this model should be operated in an *in vivo* setting. Rodent models are different from food animal (e.g. pig) models, specifically with respect to features such as fermentation patterns in the gut and to food intake behaviour. Additionally, a pig model would physiologically seen be more close to human physiology, and hence would be highly attractive for experimentation. At the same time, this model should be able to handle different stress conditions, be they nutritional, climatic, social, managerial, transport-related, or infectious in nature, as often occurs in animal production (*Henken*, 1982; *Verhagen*, 1987; *Schrama*, 1993; *van Diemen*, 1995; *Gorssen*, 1995).

The department of Animal Husbandry of the Wageningen Institute of Animal Sciences (WIAS) operates 6 climate-respiration chambers under full automation of climate control and parameter recording (*Verstegen* et al., 1987). This facility plays a key role in interdisciplinary research into genotype x environment interactions and adaptation processes in farm animals, associated with different stress conditions including infections.

Infections of farm animals lead to productivity loss, be it growth, yield of products or meat. The magnitude of production loss depends on the severity of infection (e.g. clinical versus subclinical or latent infection; acute versus chronic infection; generalised versus local; pathogenic versus apathogenic). The mode of action whereby productivity is affected differs between agents.

An example is presented by *Zwart* and co-authors (1991) and *van Dam* (1996) about trypanosomiasis, an exotic protozoic infection of ruminants in Africa caused by *Trypanosoma vivax*. Infection leads to severe anaemia, fever, reduced food intake, decreased productivity and high mortality in livestock. Productivity losses in goats appear to be associated with retarded food consumption and increased energy requirements for maintenance due to the infection. Food intake was decreased by 13-24% (*Verstegen* et al., 1991) and in other studies even by 20-62% (*van Dam*, 1996b). Energy balances as determined through climate-respiration research and expressed in metabolisability of energy appeared to be unaffected by the infection. Due to the infection, energy is spent in different directions as compared to non infected animals. The mechanisms underlying this apparent change in energy partitioning remain to be unravelled. Interactions between the animals and their environment, with or without pathogens, are complex.

This paper addresses the interactions between animal health and the environment with emphasis on effects of nutrition and stressors. It also puts forward the need for taking the system or animal under stress into account when studying new curative or preventive means.

STRESS, ANIMAL PRODUCTION, IMMUNE RESPONSE, NUTRIENTS

Stressors in animal production

In intensive animal production systems, such as for pigs and poultry, where relatively many animals are confined in relatively small areas, the environment of the animals is paramount with regard to the occurrence of stress. Accumulation of stressors in such systems will ultimately lead to decreased production and hence to loss of income to the farm manager. Stress might be climatic in nature or nutritional, managerial, social or associated with the infection burden in a house. Stressors are any environmental factor that provokes an adaptive response in the animal.

An example of the multifactorial nature of animal infections is given in Table 1 for coccidiosis in broilers (*Henken* et al., 1992). In this study it was found that apart from a breed effect, the risk of coccidiosis is influenced by several environmental factors (e.g., lighting regimen, ambient temperature and air quality). In general, diseases in animal production systems are multifactorial in nature. Environmental conditions are paramount for establishing good health, especially when interacting. The risk of coccidiosis in the example in Table 1 is many times higher when different environmental factors are operat-

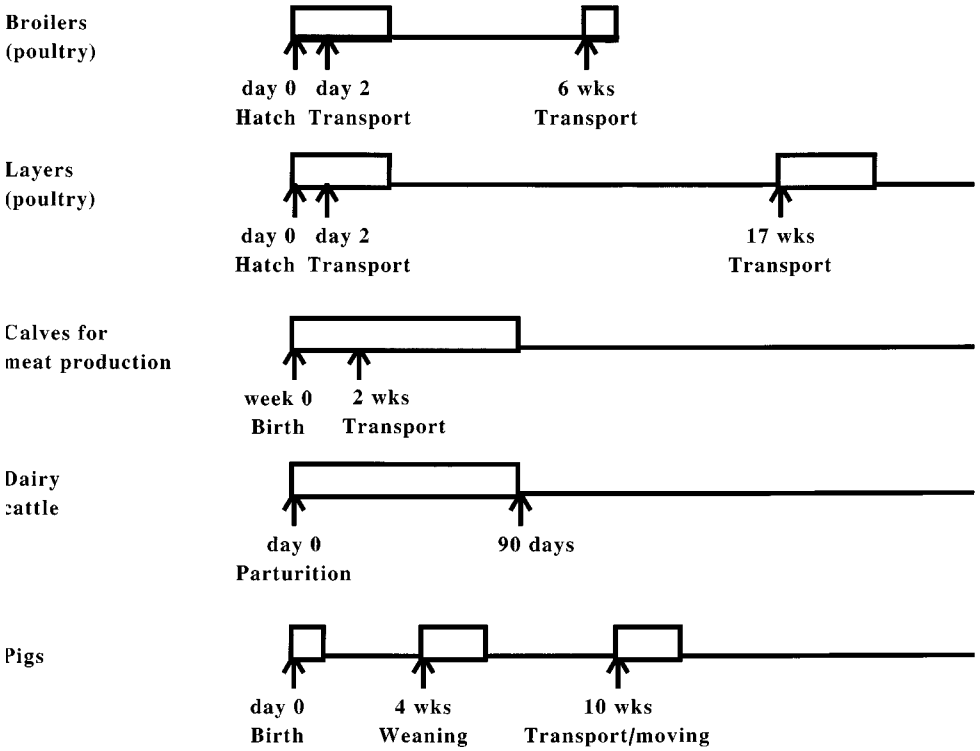

Figure 1: Specific periods of high risk for different species. Bars indicate periods of increased risk.

ing in the worst situation. A similar approach was handled for studying causes of variation in the use of antimicrobials among veterinary practitioners and their meat pig producers (*Noordhuizen* et al., 1995). In addition to technical features, also managerial strategies appeared to play a key role in use of antimicrobials.

During the life of farm animals the exposure to risk factors is not constant over time. There are time points where certain animals or animal groups are particularly at-risk when exposed to stressors including pathogens. These specific time periods of higher risk are commonly associated with the neuroendocrine status of the animal, or unpredictable changes in the environment. In Figure 1, examples are given of moment of an increased risk for the different farm animals. These examples (Figure 1), represent moments in time when animals are exposed to certain changes in the environment (e.g. transport in combination with changes in housing, climate and dietary composition) or in their physiological status (e.g., birth and parturition). These particular periods often show the highest incidence of both metabolic, nutritional and respiratory diseases.

Stressors and maintenance of homeostasis

Generally, animals (including farm animals) live under a wide range of environmental conditions, which can vary within a short time. Animals are often exposed to alteration in environmental conditions (e.g. regarding climate, social factors). Despite the variability in external conditions animals try to main-

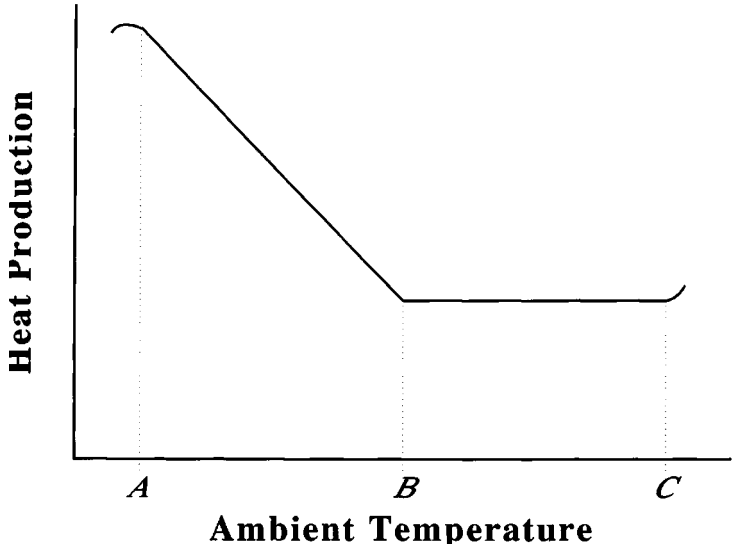

Figure 2: Thermoregulatory concept; the relation between ambient temperature and heat production in homeothermic animals (after *Mount*, 1979).

tain a steady state regarding their internal milieu, homeostasis (*Mount*, 1979; *Curtis*, 1983). Factors endangering the homeostasis (stressors) will elicit a wide range of biological responses, e.g. in behaviour, neuro-endocrine system and autonomic system (*Moberg*, 1985). The latter two comprise the activation of the sympatic-adrenomedullary system, which involves the immediate release of catecholamines, or the hypothalamic-pituitary-adrenocortical system, which involves the more gradual release of glucocorticoids (*Dantzer* and *Mormède*, 1983; *Moberg*, 1985; *Oliverio*, 1987). In general, the release of catecholamines and glucocorticoids in a stressed animal are directed to the rapid mobilisation of energy reserves for metabolic processes (*Dantzer* and *Mormède*, 1983) including immune responses (*Siegel*, 1980, 1995). Genetic selection in animals over the years has been directed to an improvement of productivity parameters but at the same time has been accompanied by a negative selection in physiological adaptiveness and immunological responsiveness of animals. It can thus be imagined, that selection has resulted in lowered responsiveness to stress as well.

An example of homeostasis is the relative constancy of body temperature in homeothermic animals. *Young* and co-authors (1989) extensively described the short-term (behaviour and metabolical) and long-term (metabolical and morphological) adaptation responses to cold climatic conditions. The thermoregulatory concept is depicted in Figure 2 representing the short-term response to the stressor temperature. In the zone AC (Figure 2), animals are able to cope with the thermal stressor (i.e., body temperature can be maintained). Outside that zone the animal is unable to cope and will enter a state of breakdown (hyperthermia and hypothermia, respectively). Zone AC is divided into a zone (BC) where heat loss is regulated and a zone (AB) where heat production is regulated. Within the zone BC, the zone of thermoneutrality, heat production is not affected by the environmental temperature. Thus a temperature in the thermoneutral zone does not elicit a

stress response. However below the temperature B, the lower critical temperature, the animal has to increase its heat production in order to maintain homeothermy.

Immunological implications of stress

It is well known that the immune system is integrated in the stress response together with the neural and endocrine systems. In this respect stress describes the defence responses to (abruptly) changing environmental conditions (*Donker*, 1989; *Siegel*, 1995). Stress responses are often, but not always, detrimental to efficient growth, skeletal integrity and disease resistance (*Siegel*, 1995). Calculation of the environmental effects on health, and production or 'performance' in case of food production animals, requires the possibility to control and condition the environment. At our department the climate-respiration chambers allow to create conditioned circumstances, and to measure various metabolic functions (*Verstegen* et al., 1987).

The important stressors in modern husbandry are heat-stress (poultry), transport (cattle; pigs) and weaning with subsequent mixing and changing housing (pigs) (see Figure 1). Therefore an important part of the studies is focused to the (in)direct effects of these stress situations.

A large part of poultry production is located in hot areas of the world. Acute thermal stress and prolonged stress can occur during transport (*Kettlewell* and *Mitchell*, 1993), ventilation failure, poor litter conditions, high densities of birds, and very hot summers such as recently in the Netherlands and Western Europe. It is well accepted that low and high environmental temperatures affect health and production characteristics of poultry (*Siegel*, 1980; *van der Hel* et al., 1991, 1992). Heat-stress may result in 20% reduction of feed intake and delayed growth (*van der Hel* et al., 1991). Immunosuppressive effects of (acute, short) heat stress in poultry are well known (reviewed by *Siegel*, 1995). These effects may be related with 'stress' hormones like adrenocorticotropin hormone (ACTH) and corticosteroids. It has been proposed that heat-stress at various ages has different effects on performance of birds at later ages. Stress at young ages may have a more profound effect on production and health characteristics than stress at older ages. On the other hand, stress at young ages may 'compensate' effects of stress at older ages (*Arjona* et al., 1987, *Gross* and *Siegel*, 1980), i.e. birds exposed to heat-stress at an early age showed increased body weights and improved feed efficiency at later age.

Negative effects of weaning on performance (*Leibrandt* et al., 1975) and immune responsiveness in pigs have been described (*Blecha* et al., 1983, *Blecha* and *Charley*, 1990). Decreased growth, and increasing incidence of diarrhoea after weaning may rest on rapid changing of the intake of dietary components (*Leibrandt* et al., 1975, *Le Dividich* and *Herpin*, 1994), and/or an impaired or immature immune system (*Blecha* et al., 1983, *Blecha* and *Charley*, 1990) rendering piglets unable to cope with infection after withdrawal of maternal protection. On the other hand, interaction between levels of food intake, temperature stressors and immune status hamper understanding of the (indirect) effects of weaning on health status (*Le Dividich* and *Herpin*, 1994). For instance, effects of weaning are often connected with effects of subsequent mixing (*Ekkel* et al., 1994).

The examples mentioned above probably share at least one important denominator: activation of the immune system by pathogens or ubiquitous microorganisms under 'stressful' situati-

ons. It is not always clear whether the detrimental effects of these stressors on resistance depend on neural or endocrine mediated suppression of the immune system, or deficiencies of nutrients due to (re)allocation or higher requirements. Our interests is focused on the contribution of additional nutritional components on the maintenance of health by either modulation of the immune system, or supplementation of deficiencies.

Partitioning of nutrients

Nutrition is an important factor in animal production because of its impact on productivity and because of being a main production cost element. In both pig and poultry production more than 50% of the total costs is made up by food cost. Energy, amino acids (≈ protein), fatty acids, carbohydrates, vitamins and minerals need to be consumed to facilitate production. Of the consumed nutrients by animals only part is deposited in products (eggs, milk or/and meat).

In Figure 3 a generalisation of the partitioning of nutrients by animals is given. The consumed nutrients become available for the animal after digestion which requires a good functioning of the gastrointestinal tract. Part of the consumed nutrients will be lost with excreta. The remaining part, the available nutrients, can be used by the organism for either maintenance or production processes.

In case insufficient nutrients are available, only part of the processes will be covered depending on their priority. This is mostly on the expense of production processes. The production (e.g., growth) of animals is dependent upon the amount of food consumed, the availability of the nutrients and the amount of nutrients required for maintenance. Sub-optimal conditions (e.g. infection and exposure to stressors) will affect production by influencing food consumption, availability and/or maintenance requirements of nutrients (which will be discussed below).

ENVIRONMENTAL PHYSIOLOGY

Climate-respiration chambers

The department of Animal Husbandry of the Wageningen Institute of Animal Sciences (WIAS) operates 3 pairs of climate-respiration chambers, which have a fully automated climate control and a fully automated data recording regarding thermogenesis. Historically, these facilities have primarily been used for mono-disciplinary research on energy metabolism: regarding either the influence of nutrition or the influence of climatic condition on energy metabolism (*Verstegen*, 1971). Nowadays, these facilities play a key-role in interdisciplinary research centred around genotype x environment interactions and adaptation processes in farm animals associated with different stress conditions (e.g., *Henken*, 1982 [poultry]; *Verhagen*, 1987 [pigs]; *Schrama*, 1993 [calves]; *Gorssen*, 1995 [racing pigeons]; *van Diemen*, 1995 [piglets]). The thermogenesis as such (heat production, energy metabolism) of animals is not the main aim in the current research but most of the times used as a parameter for the adaptive response in animals. In the next paragraph, this type of research using the climate-respiration chambers will be further elaborated regarding (1) the effect of stressors on nutrient utilisation and (2) the effect of infection on nutrient utilisation.

A detailed description of the chambers is given by *Verstegen* and co-

Table 2: Clustering of experimental options of the Wageningen climate-respiration chambers

Cluster	Options
Climate	Temperature; relative humidity (vapour pressure);air velocity/draught; floor temperature; circadian climate rhythms (including short-term changes); ...
Nutrition	Level (*ad libitum* vs. restricted); dietary composition; frequency; ...
Housing conditions	Group size (individual vs. group housing); stocking density; floor type (concrete, straw, etc.); ...
Air quality	Germ contents (SPF vs. "conventional conditions"); gas concentrations (e.g., NH_3); ...
Animal factors	Species; breed; genotype; age; immune status; ...

authors (1987). The 3 pairs of chambers differ in size. The inner space available for the animals are 6 x 3 x 2 m, 1 x 0.8 x 1 m, 0.8 x 0.5 x 0.45 m (length x width x height), respectively and enables housing of various (farm) animal species ranging from mouse to cow. Furthermore, a wide variety of experimental factors can be imposed on the animals, which are summarised in Table 2.

In addition to routine measurements such as weight changes, video recordings and blood sampling, the climate-respiration chambers enable the measuring of the complete energy and nitrogen balance. For the energy balance (the partitioning of energy) the heat production of the animal(s) is determined by measurements at 9 min intervals by measuring the gaseous exchange of oxygen, carbon dioxide and methane. The latter is also an indicator of level of fermentation in the gastrointestinal tract. The narrow measuring interval of 9 min facilitates the assessment of short-term reaction (within a day) of animals to various factors. Furthermore, the 9 min heat production measurements combined with the recorded physical activity during the same interval enables the partitioning of heat production into a part related and a part unrelated to activity (according to the method of *Wenk* and *van Es* [1976] using Burglar devices). In addition to physical activity measurements, body temperature can be related to heat production by the continuous recording using a telemetric system (*van der Hel* et al., 1993).

Involvement of above mentioned measurements in research is mainly related to mechanistic processes underlying adaptational features of animals exposed to stressors, and their immune and health responses, as well as reproductive responses.

Effect of stressors on nutrient utilisation

Stressors, such as climatic or social stress due to mixing of animals, may have a different impact on the utilisation of nutrients. Generally, there may be effects observed on feed intake, digestibility or availability, allocation and reallocation of nutrients (Figure 3).

Climate

The impact of climate on nutrient utilisation varies between cold stress and heat stress. *Kleiber* (1961) generalised the influence of ambient temperature on *ad libitum* food intake. For ex-

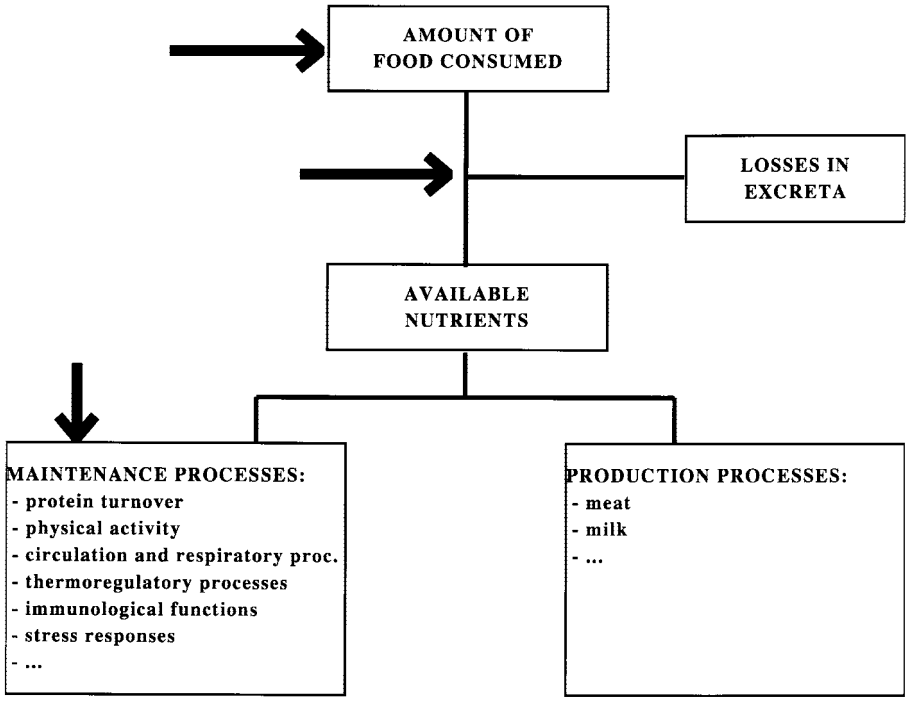

Figure 3: General scheme of partitioning of nutrients by animals. Arrows indicate the critical points where suboptimal conditions affect the different processes. *Live processes can be ordered into two groups: maintenance and production processes. Maintenance processes are those which are essential for sustaining the homeostasis of the animal and thus for survival. Processes which are part of the maintenance requirements are e.g. protein turnover, physical activity, circulation and respiratory function, thermoregulatory processes, immunological functions, combating/coping responses to stressors. Depending on the amount of available nutrients, the needs for the different processes will be covered.*

ample as reviewed by NRC (1981) for pigs the relationship is curvilinear. At low temperatures (cold stress) there is little effect. However, at high temperatures (heat stress) food intake is strongly reduced because this results in a lower heat production which enables the maintenance of homeothermia.

The results of climatic effects on availability of nutrients vary. As reviewed by *Christopherson* and *Kennedy* (1983) the digestibility of roughages in ruminants seems to decline with decreasing temperature, coinciding with an increased passage rate of digesta. However, this effect might be partially confounded with food intake which is also negatively related to temperature. In preruminant calves the data about the effect of temperature on the availability are conflicting. *Cockram* and *Rowan* (1989a) found lowered digestibility values, whereas in other studies no temperature effects were found on digestibility (*Williams* and *Innes*, 1982; *Cockram* and *Rowan*, 1989b; *Schrama* et al. 1993; *Arieli* et al., 1995). In our studies on young calves (*Schrama* et al., 1993 [Figure 4]; *Arieli* et al., 1995) also no effect of temperature on the metabolisability of energy was observed. Metabolisable energy is gross energy corrected for faecal and urine energy losses. Metabolisability is meta-

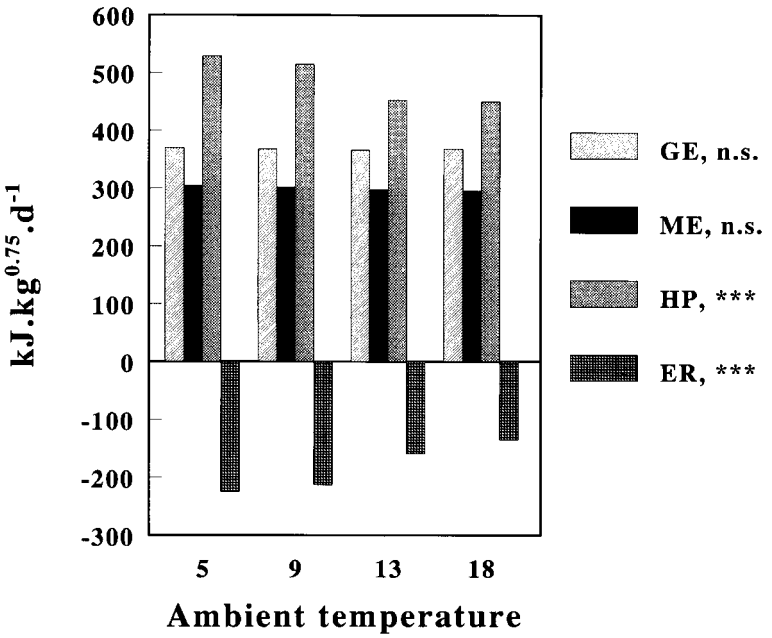

Figure 4: Effect of ambient temperature on partitioning of energy in young, restrictively milk-fed calves (*Schrama* et al., 1993) (GE = gross energy intake; ME = metabolisable energy intake; HP = heat production; ER = energy retention).

bolisable energy intake expressed as percentage of gross energy intake and thus a measure for energy availability. Similarly no climate effect on metabolisability was observed in young piglets (*van Diemen* et al., 1995b) and young broiler chickens (*Henken* et al., 1983).

Regarding energy partitioning, suboptimal climatic conditions will lead to an increased maintenance energy requirement due to energy expenditure on thermoregulatory processes (see Figure 3). According to the concept of thermoregulation (Figure 2) the increased maintenance requirement under cold stress is reflected in an increased heat production. This has been demonstrated in many studies in farm animal species (*NRC*, 1981; *Curtis*, 1983). When food intake is not altered, the increased energy requirements for maintenance under cold stress leads to a reallocation of spending of the available energy on the expense of production processes (*Close*, 1987). An example of this reallocation is given for calves in Figure 4, which indicates that energy retention (≈ energy growth) changes with ambient temperature. At high ambient temperatures (heat stress), the reduction in available nutrients (energy) spent on production processes is mainly related to the decline in food consumption (*Close*, 1987).

Chronic and acute stressors

The impact of chronic stress on energy partitioning in restrictively fed sows was studied by *Cronin* and co-authors (1986). The chronic stress was imposed on the sows by tethering, which resulted in the performance of behavioural stereotypes. The availability of energy was not affected by tethering, but the allocation of the available energy for different live processes was strongly affected by tethering. The maintenance requirement for energy was increased

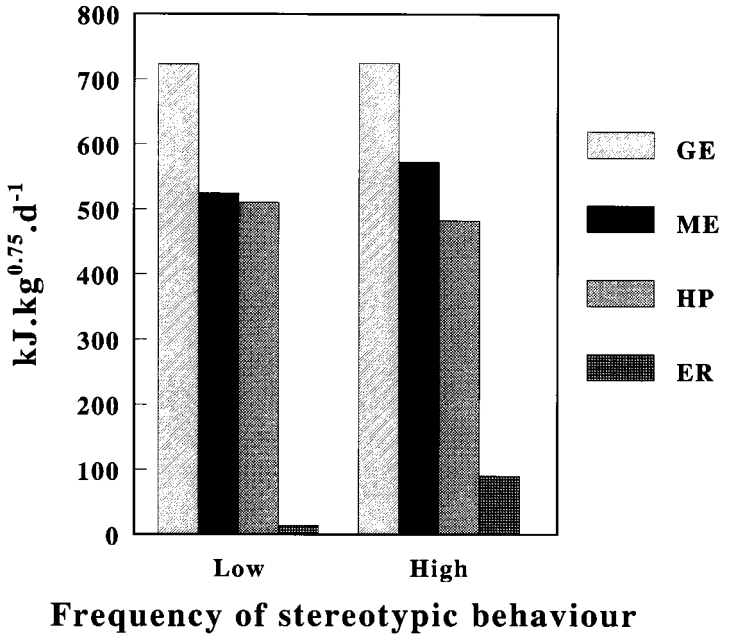

Figure 5: Impact of different coping strategies (low versus high frequency of stereotypic behaviour) in chronically stressed (= tethered) sows on partitioning of energy (*Schrama* and *Schouten*, 1996, unpublished data) (GE = gross energy intake; ME = metabolisable energy intake; HP = heat production; ER = energy retention).

by 16% in tethered sows compared to loose housed sows. The higher maintenance requirement was mainly accounted for by the higher physical activity in tethered sows. Animals can be different regarding their coping strategies (*Schouten* and *Wiepkema*, 1991). In a pilot study (*Schrama* and *Schouten*, unpublished data), the energy partitioning of two groups of tethered sows (low versus high frequency of stereotypic behaviour) was studied (Figure 5). Availability of energy was reduced in the low stereotype group reflected by the 28% higher energy losses in faeces plus urine compared with the high stereotype group. In addition to the lower availability, the energy requirement for maintenance was increased with 16% in the low stereotype group, which is also reflected in the higher heat production (Figure 5).

In most cases, the normal procedure in our climate-respiration chambers is that the experimental period is preceded by an adaptation period. At the start of the adaptation period, the animals are mostly exposed to a complex of stressors (resulting in acute stress), such as transportation, regrouping (in group housed animals), change in housing system, feeding level, dietary composition and climatic conditions. Energy partitioning is often measured during the adaptation period since it provides valuable information for practical animal husbandry. Under normal farm condition, animals are often exposed to such changes. The impact on voluntary food intake of such a complex of acute stressors is mostly a reduction. This effect of an decreased food intake (expressed per kg metabolic body weight [$W^{0.75}$]) has often been observed in *ad libitum* fed piglets (e.g., *van Diemen* et al., 1995b). However, overall results are conflicting. In the studies of *Verhagen* (1987) with 25 kg piglets, a clear re-

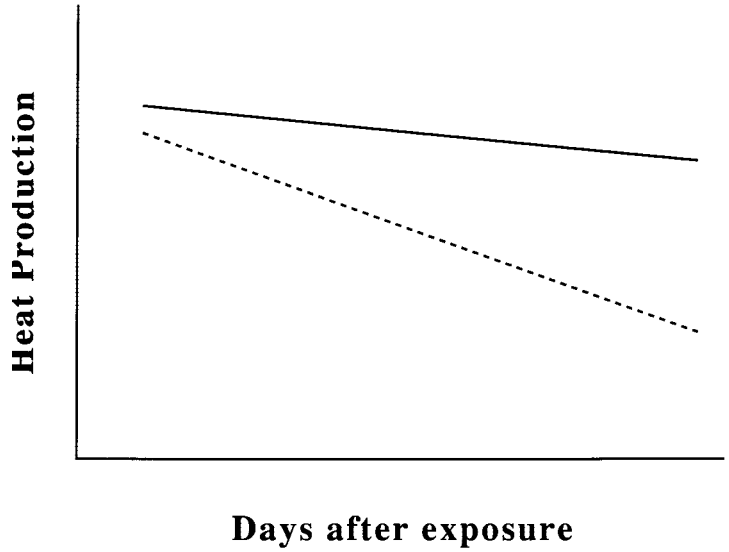

Figure 6: General pattern of the alteration in heat production with time in animals after exposure to a complex of stressors at low (----) respectively high (——) feeding level.

duction in food consumption was not always found, while in the study of *del Barrio* and co-authors (1993) even an increased food consumption during the first days after introduction into the new environment (chambers) was observed.

Also regarding the availability of energy the results are not consistent after exposure to a complex of stressors. Either a reduction in availability is observed during the first week after introduction into the chambers or no difference is observed. In pigs, e.g. van *Diemen* and co-authors (1995b) and *Heetkamp* and co-authors (1995) reported a decreased availability of energy (≈ metabolisability) of 2.5 and 1.6% during the first week, while *del Barrio* and co-authors (1993) found no difference. In young milk-fed calves, *Arieli* and co-authors (1995) observed an 8.3% lower availability of energy in the first compared with the second week after introduction into the chambers. However, in most of those studies the recovery from the exposure to a complex of stressors was confounded with a possible age-effect.

Regarding the allocation of available energy to maintenance and production processes, the impact of the complex of stressors imposed on animals at the time of introduction into the chambers is rather consistent. In most studies an increased energy requirement for maintenance is observed in the first compared with the second week. In pigs, *del Barrio* and co-authors (1993), *Heetkamp* and co-authors (1995) and *van Diemen* and co-authors (1995b) found, respectively, a 13, 18, and 4% higher energy requirement for maintenance over the first compared with the second week. In calves, *Arieli* and co-authors (1995) found a 17% higher energy maintenance requirement. The reallocation of the available energy over maintenance and production processes is also reflected in the often observed alteration (decline) in heat production with day after introduction into the chambers in many studies on piglets (e.g., *del Barrio*, 1993; *Heetkamp* et al., 1995) and young milk-fed calves (e.g., *Schrama* et al., 1992, 1993; *Arieli* et al., 1995). The generalised picture of the time related alter-

ation in heat production after exposure to a complex of stressors in animals fed at two different but constant feeding levels is given in Figure 6. With time the heat production declines.

In conclusion, exposure to stressors has an negative impact on production of animals due to alteration in food consumption, the availability of nutrients and/or allocation of absorbed nutrients to different processes. The way of interference strongly varies between different types of stressors, but also between studies on the same stressors. In general, stressors can cause a reallocation of absorbed nutrients toward maintenance processes on the expense of production processes.

Effect of infection on nutrient utilisation

In general, infectious diseases lead to an impaired productivity in farm animals. In this paragraph, some examples of the impact of infectious diseases on the nutrient utilisation are given from the energetic point of view.

Trypanosomiasis

Trypanosomiasis is a protozoan disease characterised by severe anaemia, fever and reduced food intake. It leads to high mortality and depressed productivity in livestock. The impairment of production is mainly related to the reduced food consumption and to the increased energy requirement for maintenance. Measured energy balances (*Verstegen* et al., 1991; *van Dam* et al., 1996b) showed that the availability of energy (≈ metabolisability) was unaffected by the infection.

In addition to the reduced feed intake it was found that less available energy was spent on production processes because the energy requirements for maintenance were higher. Compared with control goats the energy requirements for maintenance were increased in the infected goats by 22% to 25% (*Verstegen* et al., 1991; *van Dam* et al., 1996b). Part of the increased energy requirements for maintenance was accounted for by higher body temperature (fever) in infected goats by 1°C (*Zwart* et al., 1991; *Verstegen* et al., 1991) and 1.3°C (*van Dam* et al., 1996a). In the latter study it was demonstrated that per °C rise in body temperature the heat production increased by 21 $kJ.kg^{-0.75}.d^{-1}$ (≈ 6.3% of the energy requirement for maintenance). Thus in the studies of *van Dam* and co-authors (1996a,b) the increase in maintenance energy requirement was for approximately 38% related to the occurrence of fever. It is tempting to speculate about the energy requirements of an immune response directed towards the parasite. As will be discussed later, such an energy requirement may be an inevitable, indirect consequence of activation of the cytokine network causing anorexia. This speculation might also be true for the examples following later in this paragraph.

Apart from the reallocation of available nutrients between maintenance and production processes, *van Dam* and co-authors (1996a) demonstrated that trypanosomiasis also results in a reallocation between the maintenance processes. In that study total daily heat production was divided into daily energy expenditure spent on standing and daily energy expenditure corrected for standing cost (Table 3). In infected goats the time spent standing was reduced leading to a reduced daily energy expenditure spent on standing (Table 3). Thus part of the energy cost of infection was masked by the reduction in time spent standing.

Atrophic rhinitis

Atrophic rhinitis is a progressive infection of the upper respiratory tract in piglets caused by toxin producing *Pas-*

Table 3: Total daily heat production (HP_{tot}), daily energy expenditure on standing ($HP_{standing}$) and daily energy expenditure corrected for standing (HP_{cor}) as affected by *Trypanosoma vivax* infection in West African dwarf goats (*van Dam* et al., 1996a)

	Control	Infected
HP_{tot}, $kJ.kg^{-0.75}.d^{-1}$	306	342
$HP_{standing}$, $kJ.kg^{-0.75}.d^{-1}$	36	27
HP_{cor}, $kJ.kg^{-0.75}.d^{-1}$	270	315

teurella multocida strains. An experimental infection model with *Pasteurella multocida* derived toxin was developed to mimic subclinical atrophic rhinitis symptoms (*van Diemen* et al., 1994). An energy metabolism study on piglets challenged with a toxin doses causing subclinical disease symptoms (*van Diemen* et al., 1995b) suggested that the altered utilisation of nutrients (energy) was mainly related to the decreased food consumption. Both availability of energy and the maintenance energy requirement were unaffected by the toxin challenge. Although the total amount of energy spent on maintenance processes was unaffected by the toxin challenge, the allocation to the different maintenance processes was altered (*van Diemen* et al., 1995a). This was indicated by the reduction in energy expenditure on physical activity by 10% in toxin challenged piglets compared with non-challenged piglets.

Gastrointestinal nematodes

Kloosterman and *Henken* (1987) reviewed the impact of gastrointestinal nematodes on energy partitioning in calves. Impaired productivity regarding energy partitioning in infected calves is related to (a) reduced food consumption, lower digestibility and availability of energy and to (b) an increased energy spending on maintenance processes. However, anorexia was the major factor involved. In contrast to the examples of atrophic rhinitis and trypanosomiasis, gastro-intestinal nematodes have a negative impact on the availability of nutrients. This is quite logic since such gastro-intestinal infection may result in dysfunction of the gastro-intestinal tract.

As reviewed by *Lunn* and *Northrop-Clewes* (1993), the reduced availability of nutrients in animals with gastro-intestinal parasitic infections can be due to maldigestion, malabsorption and(or) gastro-intestinal losses (such as enhanced mucus production). In addition to the reduced availability of nutrients, the dysfunction of the gastro-intestinal tract caused by gastro-intestinal infections, may also lead to enhanced maintenance requirement, energetically spoken. Energetically the gastro-intestinal tract is a highly metabolic active organ. In ruminants the portal-drained viscera represent 5-11% of the total body but contribute for about 16-29% to the total energy expenditure (*Ortigues* and *Visseiche*, 1995). In general, information about the impact of infectious diseases on the energy expenditure of different organs is lacking for farm animals. Apart from the direct effects of infectious diseases due to dysfunction, the energy expenditure of organs/tissues might be altered due to a reallocation of nutrients/energy between the various organs/tissues.

In conclusion it can be stated that infection leads to a change in usable nutrients, but at the same time to a realloca-

tion of the spending of these nutrients. One could state that the priorities shift between certain body processes.

IMMUNOLOGICAL IMPLICATIONS OF NUTRITIONAL FACTORS AND STRESS

It has long been suspected that during disease or during a status of enhanced immune responsiveness towards the infectious agent (and vaccines), a reallocation of nutrient resources occurs. For instance, (frequent) vaccination of young chickens leads to a temporary retardation in growth, and pigs reared under 'clean' conditions grow faster than those raised under natural conditions. Activation of the immune system also leads to changes in metabolism and allocation of various essential elements such as amino-acids, vitamins, unsaturated fatty acids and trace elements. Sometimes a reallocation of nutrients, such as iron and zinc (*Brock*, 1994, *Klasing*, 1984) is suspected to act as a non-specific defence system (deprivation of these nutrients for microorganisms), sometimes activation of the immune system is accompanied by enhanced requirements of these elements. With respect to the former, in essence there is no deficiency, and additional administration of elements may even lead to toxic effects or support of microbial growth (as indicated for iron). Chronic shortage of essential elements (vitamins, metals) on the other hand may lead to a deficient or inadequate immune response. This example shows that supporting the immune system by nutrition is often a matter of finding the optimum.

The energy requirements for an immune (antibody) response to a single antigen in 'normal and unselected individuals' under normal conditions are generally small (*Donker*, 1989). The small effects on total energy expenditure in the study of *Donker* (1989) may be explained by a masking due to a reduction in energy expenditure on activity similar as described above in this paper regarding trypanosomiasis and atrophic rhinitis. A reduction in energy expenditure on activity in piglets immunised with an antigen cocktail was found to compensate fully for the increased energy expenditure elicited by the humoral immune response (*Gentry* et al., 1997). Moreover, animal models in the form of specifically selected lines indicate the existence of direct relationships between the immune system and the allocation and use of nutrients. In chickens, experimental selection for decreased antibody responsiveness resulted in significantly enhanced body weight (and growth), whereas birds with high immune responses remained smaller and grew slower (*Parmentier* et al., 1996). Also other poultry lines divergently selected for immune responses (*Martin* et al., 1990), and comparison of stocks of commercial (fast growing) broilers (*Sacco* et al., 1994) revealed a negative relationship between body weight and, for instance, antibody titres. Although an additive genetical (co-selection or pleiotrophic) relationship between genes associated with immune responsiveness and genes affecting growth cannot be excluded, all these experiments suggested an allocation of resources either towards the immune system or towards growth/performance.

The physiological mechanisms underlying the reallocation of nutrients during infection are as yet unknown. A direct relation between immune response and metabolism was illustrated by the increased fat deposition during

Table 4: Body weights at 5 weeks of age of H(igh) antibody, L(ow) antibody producing, or C(ontrol) chicks at one day before (d-1) and one day after (d+1) intraperitoneal treatment with either 1 mg *E. coli* lipopolysaccharide (LPS), or phosphate buffered saline (PBS)

Line[1]	Treatment[2]	Weight at d-1 (g)	Weight at d+1 (g)	Growth[3] (g)	Growth[3] (%)
H	PBS	519	563	44	8.6
H	LPS	522	544	23	4.6
C	PBS	612	670	58	9.4
C	LPS	601	631	30	5.2
L	PBS	587	640	53	9.1
L	LPS	596	633	37	6.9

1: H = high line, the chicken line selected for a high antibody production against sheep red blood cells; L = low line, the chicken line selected for a low antibody production against sheep red blood cells; C = control line, the chicken line which has been randomly mated.

2: PBS = 1 ml of phosphate buffered saline injected i.m.; LPS = 1 mg of *E. coli* lipopolysaccharide injected i.p.

3: Increase in body weight during 48 hr (grams).

the first days after immunisation of birds with SRBC, sheep red blood cells (*Henken* et al., 1982). Protein deposition was depressed during the period in which antibody titres were increasing and a relative high amount of energy was deposited as fat tissue. This was followed by a period with increased protein deposition (*Henken* et al., 1982). Immune responses may enhance corticosteroid levels in the blood (*Besedovsky* et al., 1975) which on their turn may favour fat deposition and increased protein catabolism (*Brown* et al., 1985, *Siegel*, 1980). Low immune responsiveness of individual birds selected for decreased immune responsiveness may account for low levels of corticosterone, which may lead to relatively higher protein (and water) deposition. Alternatively, the relation between body weight (nutrient utilisation/allocation) and 'immune responsiveness' may rest on the 'cachetin' characteristics of interleukines (IL-) 1 and 6, and/or tumour necrosis factor α (TNF-α), and other acute phase proteins, that are produced shortly after infection by macrophages, antigen-presenting cells and lymphocytes. Infection induces a change in behaviour of the individual (e.g. decreased food intake) directly or indirectly due to the binding of IL-1 to hypothalamus receptors. Immune stimulation of chickens with compounds that induce IL-1/TNF-α release by macrophages (e.g. LPS, bacterial lipopolysaccharides and SRBC) resulted in reduced growth and feed intake (*Klasing* et al., 1987; *Klasing* and *Johnstone*, 1991). This phenomenon is acute and probably temporal. Injection of growing chicks lead to a 50% growth retardation during 24 hours (Table 4), which, however, was followed by a period of increased growth during the subsequent 2 weeks (unpublished results).

In general, the continuous activation of the immune system leads to accelerated use of protein (muscle catabolism), later on followed by utilisation of nutrients derived from the visceral organs. The degree of environmental sanitation may thus affect growth rate and feed efficiency in animals (reviewed by *Klasing* and *Johnstone*, 1991). Continuous stimulation with pathogens, but also ubiquitous micro-organisms, may

lead to enhanced or chronic 'cachetin' release. Also in pigs increased growth performance was accompanied by increased disease susceptibility (*Dritz* et al., 1995). Increased growth performance in segregated rearing production systems may be the result of decreased stimulation of the immune system. As a consequence however, pigs raised in herds free from most common porcine pathogens are more susceptible to clinical epidemics of disease caused by uncommon porcine pathogens (*Harding*, 1993), which may be due to the absence of a low pathogen burden that non-specifically stimulates the animal's immune system.

The former data with respect to the 'costs' of immune mediated resistance to microbes indicates that much more needs to be known about the relationships between the immune system, nutrients and the environment. If activation of the immune system has its price, this is most pronounced in situations in which the individual is forced to react with a specific immune response, most probably based on its genetic make up. Next, the persistence/chronicity of the infectious agent determines the magnitude of this response which may lead to trauma.

CONCLUDING REMARKS

Much research has been conducted in the area of nutrition (e.g., suppletion or depletion studies) and its impact on health and immune responsiveness. Similarly, much research is performed regarding the relation between stress (e.g., different stressors or different stress levels) and health or immune responsiveness. Most investigations in these areas are commonly performed in well controlled and steady state situations (e.g., using inbred animal lines and standardised laboratory conditions). Historically, the execution of experiments under such conditions is done to guarantee reproducibility and repeatability. In common practice, animals have a certain diverse genetic background, and differences exists between and within lines and/or breeds. Moreover, environmental conditions imposed on animals are continuously variable between different situations, but also over time. Both variation in genetics and in environment should be considered when studying the relationship between nutrition and health. When looking at practical animal husbandry (but also in human medicine) most problems regarding health arise after (sudden) exposure of subjects to changes in environmental conditions (Figure 1). Exposure to such change requires adaptational responses. Thus individuals are in a non steady-state. In general, information on animals that are adapting and on adaptational responses is scarce.

Exposure to pathogens often coincides with exposure to stressors (changes in environmental condition). It is reasonable to assume that the adaptive response of the individual to stressors affects the resistance against these pathogens, opportunistic pathogens and apathogens. It is hypothesised by us that studying the impact of nutrition on health is only of interest when animals (including humans) are exposed to stressors causing adaptational responses. In those situations the subject must make choices (if possible) in the allocation of nutrients, which are scarce (limited supplied) over different vital live processes (e.g., immune responses, maintenance of body temperature, protein turnover, adaptational responses). The true component in this

research area that is largely lacking is knowledge of the interactions between stressors from the environment, nutrient partitioning and utilisation patterns, and health elements such as immune responsiveness. Stressors from the environment can be highly diverse and complex: from housing to climate, social conditions, management practices. Nutritional aspects are also manifold: from energy to proteins, trace elements, amino acids, to specific immune modulators. Health elements could be clinical or subclinical disease state or rather pathophysiological and immune responsiveness parameters. Even more important than the animal model as such, is the creation of a non-steady state for this type of research. Exposure to stressors (changes in environmental conditions) should lead to animals which are under pressure whereby the "weakest link in the chain" might be revealed. Moreover, these experimental conditions should be reproducible and thus well controlled.

Only then, the interactions named above can be studied in the proper way, and only then products such as antimicrobials or feed additives can be evaluated properly in this system under pressure.

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INACTIVATION OF ANTIMICROBIAL AGENTS INSIDE THE DIGESTIVE TRACT

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SUMMARY

Inactivation of antimicrobial agents in the human intestinal tract is a frequent event and may significantly influence the impact of the antimicrobial agent on the normal microflora. Inactivation by bacterial enzymes, or specific or unspecific binding to bacteria and faecal material are common phenomena which lowers the active *in vivo* concentration in the intestines. The most common and thoroughly studied mechanism is the irreversible enzymatic inactivation of beta-lactam agents by beta-lactamases. Treatment with beta-lactam agents is often associated with an increase in the amount of beta-lactamase producing strains in the normal intestinal microflora. An inverse correlation between beta-lactamase activities in faeces, compared with the concentration of drug in the intestines and the level of ecological disturbance in the normal intestinal microflora, have been shown during administration of oral cephalosporins. Highly erythromycin resistant enterobacteria occurs frequently during treatment with erythromycin. The mechanism of this resistance is enzymatic inactivation of erythromycin, but it is uncertain whether the activities of these enzymes are sufficient to significantly reduce the high intestinal levels of erythromycin obtained after peroral administration. Oral administration of quinolones causes minor selective alterations in the intestinal microflora despite very high drug levels in faeces. Quinolones like norfloxacin have been shown to be reversibly inactivated in the intestinal tract by binding to bacteria and faecal material. Metronidazole have been reported to be significantly inactivated, probably by binding, by several susceptible and resistant organisms commonly found in the normal microflora. Sonicated cell extracts of *Enterococcus faecalis* have also been found to inactivate metronidazole *in vitro*. Intestinal inactivation of antimicrobial agents is probably of great importance with respect to reducing the ecological disturbances and maintaining the colonisation resistance of the normal intestinal microflora.

INTRODUCTION

It is well known that besides invaluable clinical achievements, treatment with antimicrobial agents against bacterial infections also may cause unwanted effects on the normal gastrointestinal microflora (*Edlund* and *Nord*, 1993). The ecological disturbances in the normal intestinal microflora after antimi-

crobial administration can be described from different aspects. Quantitative changes in the microflora are common, such as repression of susceptible bacterial groups, which may lead to a decreased colonisation resistance. Overgrowth of potentially pathogenic microorganisms belonging to the normal microflora like enterococci or yeasts may occur, as well as superinfections with exogenic potentially pathogenic bacteria like *Clostridium difficile*, which may cause life-threatening infections. Establishment of resistant strains, either by selection of intrinsically resistant microorganisms, or by selection of bacteria with acquired resistance, is also a common finding after antimicrobial therapy. Resistant factors may further be spread among other bacteria and bacterial groups by transposons, plasmids or other genetic material (*Hawkey*, 1986; *Quintiliani* and *Courvalin*, 1995). Several antimicrobial agents have also the ability to act as inducers and promote a temporary increase in levels of bacterial produced drug-inactivating enzymes.

The human microflora is a huge potential reservoir of potentially pathogenic microorganisms which under certain circumstances may translocate and cause infections in other sites of the body. Bacteria from the intestinal microflora dominate as causative agents in post-operative infections, and resistance among them may seriously complicate the prophylaxis and treatment of intra-abdominal infections (*Nord* et al., 1986; *Hawkey*, 1986).

The potential of an antimicrobial agent to disturb the normal intestinal microflora is related to the antibacterial spectrum of the agent, the route of administration, dose and pharmacokinetic properties (*Bergan*, 1986). The microflora can be influenced by high intestinal drug concentrations due to incomplete absorption of orally administered agents and by secretion of the antimicrobial agent in the bile or from the intestinal mucosa. Inactivation of the agent in the intestinal tract by enzymes or by binding of the drug to intestinal content may also significantly influence the impact of the antimicrobial agent on the normal microflora. In the present paper, different mechanisms of inactivation of antimicrobial agents in the digestive tract will be discussed.

ENZYMATIC INACTIVATION OF ANTIMICROBIAL AGENTS

Enzymes that inactivate or modify antimicrobial agents were detected almost in parallel with the discovery of antimicrobial agents. Primarily, these enzymes protect the enzyme-producing bacteria themselves, or co-infecting bacteria in mixed infections. Members of the normal microflora that produce large amounts of these enzymes, may also inactivate antimicrobial agents at the site of infection, and thereby protecting the infecting bacteria against the killing activity of the antibiotic (*Neu*, 1994; *Quintiliani* and *Courvalin*, 1995).

Beta-lactam agents

The most common and thoroughly studied mechanism is enzymatic inactivation of beta-lactam agents by beta-lactamases. Beta-lactamases are enzymes which irreversibly hydrolyse the beta-lactam ring of penicillins, cephalosporins, monobactams and carbapenems (*Livermore*, 1995; *Quintiliani* and *Courvalin*, 1995). The ability to produce beta-lactamases is widely spread among microorganisms. The beta-lactamases of Gram-positive bacteria are mainly extracellularly excreted, while

Gram-negative bacteria accumulate their beta-lactamases in the periplastic space, although extracellular release does occur. The enzyme production may be either chromosomally or plasmid mediated and may be constitutive or inducible. Nearly 200 beta-lactamases of various bacteria differing in substrate profiles, potential for inhibition, and physical characteristics have been described. Several classification schemes have been proposed, the most recent and comprehensive of which was developed by *Bush* (1995). Enzymes from aerobic microorganisms are well recognised, but beta-lactamases from various anaerobic bacteria such as *Bacteroides, Fusobacterium, Porphyromonas, Prevotella* and *Clostridium* strains are now commonly reported (*Aldridge,* 1995; *Finegold,* 1995). Bacteria belonging to the *Bacteroides fragilis* group are dominating in the intestinal microflora. Recent studies have shown that at least 90% of all strains in the *B. fragilis* group produce beta-lactamases. Twenty-five percent of these strains produce high levels of enzymes (*Cornick* et al., 1990). Most beta-lactamases from the *B. fragilis* group are constitutive chromosomal cephalosporinases, although penicillinases also have been described, as well as enzymes capable of hydrolysing the most stable beta-lactams such as cefoxitin and imipenem (*Cuchural* et al., 1986; *Hedberg* et al., 1992). Chromosomal beta-lactamases are widely present in enterobacteria and are inducible, with the exception of enzymes produced by *Escherichia coli*. Very low levels of the inducible enzyme are present in the absence of antibiotics, but temporary high levels can be observed in the presence of an inducing compound. Beta-lactamase derepressed mutants can be selected from inducible populations during therapy with weak inducers. Such compounds kill the inducible cells but allow survival and overgrowth of derepressed mutants, which arise at frequencies as high as 10^{-5} in inducible bacterial populations (*Livermore*, 1995). Cefoxitin, imipenem and meropenem are examples of beta-lactamase-stable strong inducers, while ampicillin, amoxicillin and narrow-spectrum cephalosporins are beta-lactamase-labile strong inducers. Plasmid-mediated beta-lactamase production genes are widely spread in bacteria belonging to the normal intestinal microflora, and are responsible for most of the ampicillin resistance now seen in 50% of clinical *E. coli* isolates (*Livermore*, 1995). Plasmid-mediated beta-lactamases are also widespread among staphylococci, and high-level ampicillin resistance in enterococci has recently occurred, and is now reported world-wide, as a result of genes encoding for beta-lactamases acquired from staphylococci (*Shlaes*, 1993). Extended spectrum beta-lactamases which attack many of the newer "beta-lactamase-stable" cephalosporins and penicillins, are now increasingly being reported. To overcome the clinical problem of beta-lactamase resistance, a combination of a labile beta-lactam agent with a beta-lactamase inhibitor like clavulanic acid, sulbactam or tazobactam can be used. The success of this strategy depends on how efficient the inhibitor is, how much enzyme the bacteria produce, the stability of the protected drug, the permeability and intrinsic susceptibility of the organisms, and surrounding conditions, especially the pH (*Livermore*, 1995).

Treatment with beta-lactam agents is often associated with an increase in the number of beta-lactamase producing strains in the normal intestinal microflora. In a study where amoxicillin was given perorally to 10 healthy volunteers, the colonisation of beta-lactamase producing amoxicillin resistant enterobacteria were strongly induced

Table 1: Relationship between the concentration of cephalosporins in faeces, beta-lactamase activities and ecological disturbances in the intestinal microflora, in 34 subjects receiving peroral cephalosporins

Number of patients	Drug concentration in faeces	β-lactamase activity	Overgrowth of: Enterococci	Yeasts	*C. difficile*
7	- [a]	++	-	-	-
10	-	+	+	(+)	-
4	+ [b]	+	+	+	-
5	-	-	+	(+)	+
2	+	-	+	-	+
6	++[c]	-	++	+	++

[a] -: negative; [b] +: positive; [c] ++: strongly positive

during administration (*Brismar* et al., 1993). In another study where 14 patients with *Helicobacter pylori* infection were treated with omeprazole plus amoxycillin for two weeks, a significant selection of resistant enterobacteria and enterococci were observed, and 10 patients increased their intestinal beta-lactamase production during treatment (*Stark* et al., 1996). The faecal levels of amoxicillin were under the detection limit in all samples from these studies, probably partly due to enzyme inactivation. The ecological effects in the intestinal microflora by 7 to 10 days administration of three oral cephalosporins - cefuroxime axetil (n=10), cefpodoxime proxetil (n=10) and ceftibuten (n=14) - to healthy volunteers lead to significant increases in beta-lactamase production during administration (Table 1). There was an inverse correlation between enzyme activity in faeces during administration compared with the concentration of drug in the intestines and the level of ecological disturbances in the normal intestinal microflora (*Edlund* et al., 1994). The results of these studies imply that from an ecological point of view, the increase in beta-lactamase activity during beta-lactam administration is probably an advantage because it helps to preserve the colonisation resistance and thus, protects against invading pathogens.

In another study, the induction ability of cefoxitin on beta-lactamase production was studied in aerobic and anaerobic microorganisms isolated from the intestinal microflora (*Stark* et al., 1995). It was shown that the enzyme production was highly enhanced in the presence of sub-inhibitory concentrations of the inducer, in pure bacterial broth cultures. However, when the induction assay was performed with the inducible bacterial strains *(Citrobacter freundii, Bacteroides ovatus* and *Clostridium butyricum)* incubated in faecal suspension, the induction ability was strongly reduced. Thus, it seems that faeces inhibits the beta-lactamase induction of aerobic and anaerobic bacteria.

Macrolides

Macrolide antimicrobial agents are generally bacteriostatic agents that inhibit bacterial RNA-dependent protein synthesis (*Yao* and *Moellering,* 1995). Erythromycin is the prototype of this group; newer semisynthetic agents are clarithromycin, azithromycin, dirithromycin and roxithromycin. Macrolides are mainly active against Gram-positive bacteria. By contrast, enterobacteria are

naturally resistant to low levels of these drugs, due to cell impermeability (MIC of erythromycin against most enterobacteria ranges between 2 and 256 mg/l) (*Quintiliani* and *Courvalin*, 1995). High drug concentrations are obtained in the intestinal tract after peroral administration of clinical recommended doses, and oral administration of erythromycin, clarithromycin and roxithromycin has been reported to markedly alter the aerobic and anaerobic intestinal microflora (*Brismar* et al., 1992; *Edlund*, 1993). However, highly resistant enterobacteria (MIC ≥500 mg/l) occur frequently during treatment with erythromycin. The mechanism for this high-level resistance to erythromycin in enterobacteria has been shown to be enzymatic inactivation of erythromycin, either by production of erythromycin esterases which destroy the lactone rings of 14-membered macrolides, or by a phosphorylation catalysed by a 2'-phosphotransferase (*Quintiliani* and *Courvalin*, 1995). These highly resistant enterobacteria are often isolated from the intestinal microflora after peroral erythromycin administration (*Andremont*, 1986), but it is uncertain whether the activities of these enzymes are sufficient to significantly reduce the high intestinal levels of erythromycin obtained after peroral administration.

BINDING OF ANTIMICROBIAL AGENTS TO FAECAL MATERIAL

Quinolones

Quinolone antimicrobial agents (ciprofloxacin, enoxacin, fleroxacin, norfloxacin, ofloxacin, pefloxacin and sparfloxacin) exhibit a bactericidal antibacterial activity resulting from inhibition of the essential enzyme DNA gyrase. The function of this enzyme is required for DNA replication, transcription, recombination, DNA repair and transposition (*Yao* and *Moellering*, 1995). The *in vitro* activity of quinolones is primarily directed against aerobic and anaerobic facultative Gram-negative bacteria. Peroral administration of quinolones results in elimination or strong suppression of intestinal enterobacteria (*Edlund* et al., 1987; *Edlund* and *Nord*, 1993). Ciprofloxacin and ofloxacin also affect intestinal enterococci and anaerobic microorganisms to a minor degree (*Bergan* et al., 1986; *Edlund* et al., 1988). Resistance to quinolones can be mediated in two different ways; alterations in the A subunit of the DNA gyrase or alterations in the bacterial outer membrane protein, thus affecting uptake of the drug into the bacterial cell. The latter case seems to be associated with reduced susceptibility to other groups of antimicrobial agents such as chloramphenicol, cefoxitin and tetracycline. Resistance against quinolones is still rather uncommon, although findings of resistant *Pseudomonas*, staphylococci and enterobacteria are increasingly reported (*Quintiliani* and *Courvalin*, 1995). Several clinical studies on the newer quinolones have shown that peroral administration results in very high concentrations in the intestinal tract. Peak faecal concentrations of norfloxacin of 120 to 2,700 mg/kg after therapeutic doses have been reported, which is, with few exceptions, far exceeding the MIC's for all intestinal microorganisms (*Edlund* et al., 1987). Thus, despite high drug levels in faeces, the main part of the aerobic Gram-positive and the anaerobic flora remains unaffected after norfloxacin administration. The same pattern is true for other quinolone agents (*Edlund* and *Nord*, 1993).

It has earlier been shown by using ^{14}C labelled norfloxacin, that nor-

floxacin is reversibly inactivated in the intestinal tract by binding to faecal material (*Edlund* et al., 1988). The specific binding was reversible, saturated after 90 min of incubation at 37°C, and increased linearly with faecal concentration. The maximal binding capacity (B_{max}) of the specific binding was 0.12 μmol/g and of the unspecific binding 11.8 μmol/g faeces. The binding capacity of unlabelled ciprofloxacin, norfloxacin, enoxacin, ofloxacin and pefloxacin to faeces was studied by competitive assays, and was shown to be in the same range as that of ^{14}C norfloxacin. The results of norfloxacin binding to pure bacterial suspensions, suggest that the main part of the binding is to the bacterial fraction of faeces.

Nitro-imidazoles

Nitro-imidazoles (metronidazole, tinidazole and ornidazole) have their bactericidal activity inside the cell. The nitro group is reduced by a nitroreductase enzyme in the cytoplasm, generating highly cytotoxic intermediate compounds of free radicals which disrupt the DNA of the cell (*Yao* and *Moellering*, 1995). Nitro-imidazoles exhibits an excellent activity against all anaerobic bacteria but are inactive against all aerobic and facultative anaerobic bacteria. Orally administered nitro-imidazoles are well absorbed and have been shown to cause only minor alterations in the oral and intestinal microflora (*Heimdahl* et al., 1980; *Edlund* and *Nord*, 1993). The main mechanisms involved in resistance against metronidazole is slower penetration of the drug into the cell. It has been shown that metronidazole can be inactivated by aerobic bacteria, not susceptible to metronidazole (*Edwards* et al., 1979; *Quintiliani* and *Courvalin*, 1995). The phenomenon that metronidazole treatment of anaerobic infections occasionally results in clinical failure, although the pathogenic strain isolated is sensitive to the drug *in vitro*, has been studied by several groups. *Edwards* et al. reported that several organisms commonly found in the normal microflora, such as *E. coli* and *Klebsiella aerogenes*, were capable of absorbing and inactivating significant amounts of metronidazole *in vivo*, and suggested that metronidazole binds to particular fractions of the cells in a chemically unmodified form (*Edwards* et al., 1979). Time-kill curves and radioisotope experiments with ^{14}C-metronidazole have also revealed that the drug is taken up by both susceptible and resistant bacteria (*Ralph* and *Clarke*, 1978; *Tally* et al., 1981). *Nagy* and coworkers have studied the *in vitro* inactivation of metronidazole by 20 different clinical isolates of *Enterococcus faecalis* (*Nagy* and *Földes*, 1991). In this study, susceptible *B. fragilis* strains and metronidazole were cultured in broth medium, with or without *E. faecalis* strains. All of the tested *E. faecalis* strains were able to protect the *B. fragilis* group strains against the killing effect of metronidazole at a concentration 4-8 times higher than normal MIC. When *E. faecalis* strains were cultured anaerobically for 24 h in the presence of 4 mg metronidazole/l, no metronidazole could be detected subsequently in the culture supernatants by HPLC. Sonicated cell extracts of *E. faecalis* were found to inactivate metronidazole to the same extent, whereas culture supernatants had no such effect. Due to the findings that cell free extracts of the *E. faecalis* strain were able to inactivate metronidazole, the authors suggest that this mode of metronidazole inactivation differs to the binding mode of inactivation described earlier, and may be enzymatic. Further studies are needed to investigate whether these phenomena of metronidazole inactivation by microorganisms commonly found in the normal intestinal microflora contribute to the

fact that very low or undetectable intestinal levels of metronidazole are found after peroral administration.

CONCLUSION

Antimicrobial agents may be inactivated in the human intestinal tract by several mechanisms. Inactivation by bacterial enzymes, or specific or unspecific binding to bacteria and faecal material are common phenomena which lowers the active *in vivo* concentration in the intestines. Intestinal inactivation of antimicrobial agents is probably of great importance with respect to reducing the ecological disturbances and maintaining the colonisation resistance of the normal intestinal microflora.

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ANTIMICROBIAL PEPTIDES OF VERTEBRATES

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SUMMARY

Antimicrobial peptides have been found in animals as diverse as insects and man. Interest in this class of antibiotics has greatly increased recently as bacterial resistance to existing antibiotics has become a serious clinical problem. These peptides are membrane active agents with a broad spectrum of microbicidal activity. Their hallmark physical features are a highly positive net charge and the ability to adopt amphipathic conformations. Their broad spectrum of antimicrobial activity, unique mechanism of action, and ability in many cases to kill organisms that are resistant to traditional antibiotics makes them attractive for pharmaceutical development. One such peptide, an analogue of a frog magainin, is in phase III clinical trials for infected diabetic foot ulcers. Increasingly the search for these peptides in Nature is focused on higher vertebrates, including humans. Recent developments highlight the importance of vertebrate epithelial cells as sites of antibiotic peptide production and suggest a critical role for this system in human health and disease.

INTRODUCTION

Antibiotics have historically been derived from lower organisms such as bacteria or fungi. These creatures presumably produce such substances to gain competitive advantage in the microbial ecology. However, higher eucaryotes also produce antibiotic substances as part of their immune defences. Gene-encoded antimicrobial peptides are found widely in the animal kingdom and salient examples include the cecropins from insects (*Boman*, 1995), magainins from amphibians (*Zasloff*, 1987), and defensins from mammals (*Ganz* and *Lehrer*, 1995). These molecules tend to exhibit intrinsic specificity for microbial invaders and are relatively much less toxic for the metazoan host's cells. This specificity endows the animal with an "innate" immunity, in contrast to the better studied acquired immunity conferred by the clonal expansion of B and T cells. The possible importance of this system as a check on infection is evident when one considers that most bacteria have generation times of 20-30 minutes whereas the mounting of a specific immune response, dependent on the growth of mammalian cells, may take days or weeks.

STRUCTURE AND MECHANISM OF ACTION

Known antibiotic peptides derived from animals fall into several distinct structural categories. One major structural class of peptides is linear, α-helical, linear, and contains no disulphide bonds. Included in this group are the magainins, insect cecropins, and mammalian cecropins. A second important class are the defensins. In contrast to the α-helical molecules just described defensins have a conserved pattern of disulphide pairing that holds them in a β-sheet conformation. Other structural motifs have been observed for vertebrate antimicrobial peptides but they are in general less well studied.

Despite their structural diversity the activity and specificity for microbes of antimicrobial peptides is likely due to shared features. These characteristics include a highly positive net charge and the ability to adopt ordered amphipathic conformations. Antimicrobial peptides are typically surface and membrane active agents that kill by inserting into the membrane and forming pores, thereby disrupting the structural integrity and homeostatic mechanisms of the target cell (*Cruciani* et al., 1992; *White* et al. 1995). The positive charges serve to attract the antibiotic to the surface of the target microbe. The remarkable selectivity of these peptides is thought to be due to the relative abundance of negative charges in target cell membranes compared to those of the metazoan host (*Matsuzaki* et al., 1995; *White* et al., 1995). These membrane charges are due primarily to anionic phospholipids (*Matsuzaki* et al., 1995). Such charges act as the "receptors" for the highly cationic peptides. The amphipathic nature of the antibiotics is also a critical feature and allows them to insert into the membrane and form pores, holes, or ion channels. Formation of these holes is believed to involve the assembly of higher order structures containing multiple peptide molecules, but the exact nature of these complexes is unclear (*Vaz Gomes* et al., 1993; *White* et al., 1995). The presence of cholesterol in host cell membranes may serve to protect them from the disruptive effects of the peptides (*Tytler* et al., 1995). Since microbes lack cholesterol in their membranes this phenomenon probably further increases the selectivity of these peptides.

In many instances cells with a higher transmembrane potential are more susceptible to the killing effects of cationic antimicrobial peptides (*Matsuzaki* et al. 1995; *White* et al., 1995). Such a potential, being negative on the inside of the cell, may provide an electromotive force that aids in driving the integration of the positively charged peptide into the target membrane (*White* et al., 1995). The fact that synthetic magainins consisting of all D amino acids are as biologically active as their L amino acid containing counterparts indicates that their interaction with the target does not involve chiral interactions (*Wade* et al., 1990). This observation underscores the uniqueness of the mechanism of action of antimicrobial peptides as compared to traditional antibiotics which, like most pharmaceuticals, are direct inhibitors of enzymes with chiral active sites.

MAGAININS

The magainins are linear, cationic, amphipathic peptides of 21-27 amino acids originally isolated from the skin of the African clawed frog, *Xenopus laevis*

(*Zasloff*, 1987). Zasloff isolated two peptides, magainin 1 and 2, from *Xenopus* skin based on their antimicrobial activity after noting the remarkable resistance of the frogs to infection despite their lack of an advanced cellular immune system. The magainins are structurally similar to the insect cecropins in their α-helical secondary structure and lack of cysteine residues or disulphide bridges. As such they were the first known representatives of this class of antimicrobial peptides observed in vertebrates.

A broad range of pathogens are susceptible to magainins, including Gram-positive and Gram-negative bacteria, fungi, protozoa, and viruses (*Zasloff*, 1987; *Aboudy* et al., 1994). Magainins are stored in specialised secretory granule containing skin cells called granular glands. Subsequent studies showed that magainins are also produced by granular glands in the frog's digestive tract including stomach, small intestine, and colon (*Reilly* et al., 1994). They are produced as multimers in pro-proteins and the active form is liberated, after secretion, by a unique protease that recognises secondary rather than primary structure (*Resnick* et al., 1991; *Jacob* and *Zasloff*, 1994). Magainins exhibit a near random coil conformation in aqueous solution but adopt an amphipathic, α-helical structure in hydrophobic solvents and also upon interaction with target cell membranes (*Jacob* and *Zasloff*, 1994; *Maloy* and *Kari*, 1995).

Many other antibiotic peptides, distinct from the magainins, are also produced by *Xenopus*. These include PGLa, CPF (10 species), LPF, XPF and PGQ (*Soravia* et al., 1988, *Maloy* and *Kari*, 1995). Interestingly, magainin 2 and PGLa act synergistically against microbes (*Westerhoff* et al., 1995). This diversity in a single animal presages the even greater diversity across frog species. Other examples of antibiotic peptides isolated from frogs include ranalexin from the American bullfrog (*Clark* et al., 1995) and dermaseptin from *P. sauvagii* (*Mor* and *Nicolas*, 1994). These compounds differ from the magainins in sequence and structure and further reflect what is apparently enormous genetic diversity in amphibian antibiotic peptides.

CLASSICAL OR α-DEFENSINS OF GRANULOCYTES

Mammalian granulocytes kill ingested pathogens via two distinct mechanisms. One involves an "oxidative burst" that produces reactive oxygen species that react with and kill the microbe. The other mechanism is non-oxidative and requires antimicrobial peptides known as defensins (*Ganz* and *Lehrer*, 1995). Defensins were first discovered in the macrophages and neutrophils of rabbits and subsequently have been found in a variety of mammalian neutrophils. They are cationic, amphipathic peptides of 29-45 amino acids and contain a highly conserved pattern of six cysteine residues. These cysteines form three disulphide bridges which hold the peptide in a β-sheet conformation (*Martin* et al., 1995). The crystal structure of one human granulocyte defensin has been determined (*Hill* et al., 1991).

Defensins are stored in the azurophil granules of neutrophils and represent a significant proportion of the granule and total cell protein content. Upon phagocytosis a vesicle containing the engulfed microbe is fused with the granules, delivering a local high concentration of antibiotic peptide. Defensins kill a wide

spectrum of pathogens, including Gram-positive and Gram-negative bacteria, fungi, and enveloped viruses (*Ganz* and *Lehrer*, 1995; *Martin* et al., 1995).

Defensins are produced as precursors and are cleaved by proteases to release the active, mature antimicrobial peptide. The pro-piece is typically negatively charged and may serve to neutralise the cationic peptide. This mechanism may help to prevent cytotoxicity to the host cell (*Valore* et al., 1996).

Many defensins have been characterised from a variety species. Tissue and cell type expression vary surprisingly amongst species. Defensins are present in rabbit pulmonary macrophages and circulating neutrophils. However, they are absent from rabbit peritoneal macrophages and monocytes. Humans produce four α-defensins in their neutrophils; HNP-1, 2, 3, and 4. These molecules have not yet been observed in human monocytes. Rats produce neutrophil α-defensins but interestingly they are completely absent in mouse granulocytes (*Eisenhauer* and *Lehrer*, 1992; *Ganz* and *Lehrer*, 1995; *Martin* et al., 1995).

ENTERIC DEFENSINS

In addition to being present in mammalian phagocytic cells, where they were first discovered, classical or α-defensins are also produced by Paneth cells in the small intestines of mice and humans (*Selsted*, 1992, *Jones* and *Bevins*, 1992). Paneth cells are specialised epithelial cells residing in the base of structures called crypts in the intestinal mucosa. Consequently the murine molecules have been termed cryptdins. Although they are epithelial cells, Paneth cells differ in important ways from the cells lining the rest of the intestine. Perhaps most strikingly they contain secretory granules reminiscent of those found in neutrophils. They are known to be active secretory cells and are thought to play a defensive role since they produce lysozyme as well as defensins (*Jones* and *Bevins*, 1992).

The existence of an extensive family of sixteen mouse cryptdins has been reported (*Huttner* et al., 1994). In contrast, human Paneth cells are known to produce only two defensins, HD-5 and HD-6 (*Jones* and *Bevins*, 1992; *Jones* and *Bevins*, 1993). The production of these molecules is developmentally regulated and coincides temporally with the appearance of Paneth cells in the gut (*Mallow* et al., 1996). In humans, expression of HD-5 is first detectable at 13.5 weeks of gestation and HD-6 at 13.5-17 weeks. Levels of mRNA measured by northern blotting at 19-24 weeks ranged from 40-250 fold less than those seen in adult small intestine. Mallow et al. (1966) hypothesise that low levels of Paneth cell defensin expression in the immature gut of pre-term infants may in part be responsible for the prevalence of necrotising enterocolitis in these children.

Cryptdins have been detected in the contents of the small intestine, suggesting that they are actively secreted by Paneth cells into the lumen (*Selsted* et al., 1992). This is in contrast to the situation in myeloid granulocytes where defensins are thought to contact microbes inside the cell after fusion of phagocytic vacuoles with the granules. Detection of constitutive defensin mRNA production in Paneth cells by *in situ* hybridisation (*Jones* and *Bevins*, 1992) indicates a further important difference compared to the regulation of circulating granulocyte defensin production wherein the protein is stored

and transcription ceases in the mature neutrophil (*Martin* et al., 1995).

The secretory nature of the Paneth cell and constitutive nature of defensin synthesis suggests that this system may play a role in maintaining the near sterile environment of the small intestine rather than strictly responding to acute challenges. Experiments in gnotobiotic mice demonstrate that intestinal defensin production is normal and is therefore not dependent on colonisation of the gut by bacteria (*Lehrer* et al. 1993). However, the presence of consensus binding sequences for transcription factors AP2 and NF-IL-6 in the 5' flanking region of the HD-5 and HD-6 (*Mallow* et al., 1996) genes implies that production of these defensins may be inducible in response to inflammatory stimuli. In fact, increased defensin expression in human necrotising colitis was recently reported (*Salzmann* et al., 1996).

β-DEFENSINS

The discovery of the magainins in frog skin suggested the epithelium of other vertebrates, including mammals, as a logical place to search for antibiotic peptides. Previous to that discovery antibiotic peptides had been found only in more traditional immune tissues such as the haemolymph of invertebrates, blood (granulocytes) of vertebrates and the defensively specialised Paneth cell. However, epithelial cell layers represent an important barrier between the animal and the microbial flora in the environment and are increasingly recognised as active immune tissues. These observations led to a search for antibiotic expression in the bovine airway and the discovery of tracheal antimicrobial peptide or TAP (*Diamond* et al. 1991).

TAP was the first of a new, structurally distinct family of mammalian defensins and the first found to be broadly expressed in the epithelial sheet. A search for antibiotic peptides in bovine neutrophils led to the discovery of thirteen peptides homologous to TAP (*Selsted* et al., 1993). Comparison of the amino acid sequences of these peptides and TAP resulted in a consensus sequence and showed clearly that the group represented a family. This observation led to the term β-defensins (*Selsted* et al., 1993).

β-defensins contain six conserved cysteines which form three disulphide bonds forming a molecule that is dominated by β-sheet just like their classical counterparts, but the pattern of these residues and bridges is substantially different (*Selsted* et al., 1993). The three-dimensional structure of one β-defensin, bovine neutrophil-defensin 12, has been determined by NMR and it is similar to that established for α-defensins (*Zimmerman* et al., 1995). They are also cationic, amphipathic, and lysine rich.

TAP was originally isolated from bovine tracheal mucosa based on its antibiotic activity. It is translated as a 63-amino acid propeptide. The mature peptide consists of 38 amino acids and is active against Gram-negative bacteria, Gram-positive bacteria, and fungi. Southern blotting of bovine genomic DNA with a TAP probe resulted in multiple hybridising bands implying the existence of a family of related genes. *In situ* hybridisation studies showed that TAP is expressed throughout the airway but not at other epithelial surfaces of the body (*Diamond* et al., 1993).

The TAP gene has been cloned and analysis of the 5' flanking sequence revealed the presence of a binding site for the transcription factor NF-KB. This

factor has been associated with numerous genes that are induced in immune response and inflammation (*Nolan* and *Baltimore*, 1992). *Diamond* et al. have reported that exposure of primary bovine tracheal epithelial cells to LPS in culture results in induction of TAP RNA (*Diamond* and *Bevins*, 1994; *Diamond* et al, 1996). The induction was a robust thirteen fold at sixteen hours post exposure. In these cells the LPS effect appears to be mediated, at least in part, by the CD14 antigen on the surface of the epithelial cells (*Diamond* and *Bevins*, 1994; *Diamond* et al., 1996).

The digestive tract represents another system that, like the airway, is exposed to the outside environment and is protected by an epithelial barrier. The tongue is covered by perhaps the most exposed and vulnerable epithelium in this system yet is rarely observed to become infected. This epithelium therefore seemed likely to be protected by antibiotic peptides as well. Prompted by these observations *Zasloff* and colleagues (1987) isolated another β-defensin from the mucosa of the bovine tongue. Reflecting its site of expression, this molecule was designated lingual antimicrobial peptide or LAP. A cDNA for LAP was also isolated which predicted a 64 amino acid pro-peptide (*Schonwetter* et al., 1995).

In situ hybridisation of the LAP cDNA with bovine tongue tissue demonstrated that LAP is expressed in the middle layers of the stratified epithelium of the normal tongue. Interestingly, similar experiments using tissue from cows with tongue lesions showed a dramatic induction of LAP mRNA synthesis associated with the wounds and accompanying areas of inflammation. This result served to generalise observations made with TAP in the airway to other exposed epithelia in mammals and most importantly showed that epithelial antibiotics are induced in response to insult. Induction may be the result of exposure to microbial products such as LPS, to inflammatory cytokines, or both.

A survey of bovine tissues by northern blotting showed expression of LAP or closely related molecules in many epithelial sites throughout the body, including conjuctiva, airway, and the digestive and urinary tracts (*Schonwetter* et al., 1995). Interestingly, expression in the tongue also appears to be developmentally regulated, being absent in the foetus and expressed robustly postnatally. Whether this is genetically programmed or due to induction after exposure to environmental microbes remains to be determined.

Antimicrobial peptides homologous to the β-defensins have been isolated from chicken leukocytes (*Harwig* et al., 1994). These three peptides were named gallinacins to reflect their source, *Gallus gallus.* Of the 36-39 residues in the gallinacins, 9, including 6 cysteines, are invariant amongst the gallinacins and known bovine β-defensins. The presence of β-defensins in birds suggests an ancient origin for these molecules, before the evolutionary divergence of birds and mammals.

Recently the amino acid sequence for a human peptide with homology to TAP was reported (*Bensch* et al., 1995). This molecule was isolated from human haemodialysis fluid in a general search for peptides. A computer homology search revealed a similarity to TAP. The activities, sites of expression, and regulation of this molecule remain to be addressed.

OTHER ANTIMICROBIAL PEPTIDES OF VERTEBRATES

The classes of vertebrate antimicrobial peptides discussed above represent the best studied and best understood to date, but the family of known peptides is considerably more diverse. A search for antibiotic activities in pig small intestine by Hans Boman's group at the University of Stockholm led to the discovery of cecropin P1, a molecule with surprising similarity to the insect cecropins (*Lee* et al., 1989). Cecropin P1 is 31 amino acids long and contains several amino acids which are conserved amongst the insect cecropins. It is linear, cationic, amphipathic and α-helical like the magainins and cecropins and as such represents the first of this structural class of antibiotic to be described in a mammal.

Boman's group also isolated PR39 from pig intestine. PR39 is a proline rich, 39 amino acid peptide which lacks cysteine. Subsequent cDNA cloning demonstrated that this peptide is produced in bone marrow rather than intestine, suggesting the source in intestine may be resident lymphocytes (*Boman*, 1995). FALL-39 is a peptide derived from a human bone marrow cDNA coding for a larger putative precursor. One 39 residue portion is similar to PR-39, and synthesis of this peptide yielded a compound with antibiotic activity (*Agerberth* et al., 1995). If this peptide indeed exists physiologically it represents the first human cysteine free peptide antibiotic, distinguishing it from the human defensins.

At least two other proline rich antibiotics have been isolated from mammals. These are the bactenecins Bac 5 and Bac 7 which were isolated from bovine neutrophils (*Frank* et al., 1990). Also from bovine neutrophils comes a tryptophan rich molecule known as indolicidin (*Boman*, 1995). Pig immune cells contain molecules called protegrins. Protegrins have two disulphide bridges rather than the three seen in defensins. They are also substantially smaller, consisting of only 16-18 amino acids (*Boman*, 1995). In addition to the variety described here from mammals is the great diversity in sequence and structure mentioned earlier for the antibiotic peptides of frogs. It appears that the surface has only been scratched in the search for vertebrate antimicrobial peptides.

PEPTIDE ANTIBIOTICS IN HUMAN HEALTH AND DISEASE

Most of the data discussed above are derived from *in vitro* or cell culture experiments. However, several observations support a critical role for antimicrobial peptides in the *in vivo* immune response. For example, a human genetic disease known as specific granule deficiency results in an almost total absence of defensin containing granules in neutrophils. Patients with this disease produce little or no defensin in their leukocytes and are prone to repeated and severe infection (*Ganz* et al. 1988). This unfortunate natural experiment lends support to the important role that the classical defensins play as antibiotics used to kill pathogens phagocytised by PMN. The genetic lesion causing this deficiency has not been elucidated, but decreased defensin mRNA levels in some cases suggest that the defect may be due to a transcriptional defect (*Tamura* et al. 1994).

It has also been observed that the level of circulating defensins in the blood increases dramatically in patients

suffering from septicaemia (*Panyutich* et al., 1993). Since most defensins are found as inactive complexes with proteins such as a_2-macroglobulin in the blood (*Panyutich* and *Ganz*, 1991) this probably represents a marker for increased neutrophil activity rather than a systemic antimicrobial host response.

It has recently come to light that a defect in epithelial defensin activity may be responsible for the devastating lung infections suffered by cystic fibrosis (CF) patients. Although the defective gene leading to CF has been identified and characterised the genesis of these often fatal airway infections, with organisms such as *Pseudomonas aeruginosa*, has remained a mystery. Numerous hypotheses have been forwarded including poor clearance of microbes due to abnormal mucous (*Engelhardt*, 1992), defective phagocytosis by epithelial cells (*Pier* et al., 1996), as well as altered antimicrobial peptide metabolism or activity (*Zasloff*,1987).

Smith et al. (1996) have now reported the presence of an antibiotic activity on the surface of human airway epithelial cells in culture. They found that lung epithelial cells from healthy individuals were able to directly kill *Pseudomonas aeruginosa* and *Staphylococcus aureus* placed on their apical surfaces but that similar cells from CF patients could not. One obvious explanation for this result would be that the antibiotic activity is not produced or is otherwise absent from the CF cell's surface. However, antibiotic activity was recoverable from the fluid on the surface of both normal and CF epithelial cells. This activity is heat stable and less than 10 kD in size, consistent with it being due to a defensin.

It is known that the Na^+ and Cl^- concentrations in CF lung secretions are increased (*Joris* et al., 1993) and that the activity of defensins is inhibited in higher salt (*Martin* et al., 1995). Together these facts may offer an explanation for the lack of natural antibiotic activity observed on CF cells. Indeed, a series of experiments by *Smith* et al. (1996) demonstrated that lowering the salt concentration revealed antibiotic activity produced by CF cells and raising the concentration masked the activity described in normal lung epithelial cells. The identity of the antibiotic as a defensin has yet to be conclusively proven, but these results may represent the first evidence that epithelial defensins are critical for human health and may lead to new therapeutic approaches to CF lung pathology.

NON-ANTIBIOTIC ACTIVITIES OF DEFENSIVE PEPTIDES

In addition to being antibiotic, other activities have been ascribed to defensive peptides. For example, *Territo* et al. (1989) have demonstrated that two human defensins are chemotactic for monocytes. HNP-1 and HNP-2 were active at nanomolar concentrations. The activity was specific for monocytes as no effect was seen with any defensin on human neutrophils. This activity is of obvious utility in a wound or infection setting where defensins are released and attracting immune cells is desirable. These authors point out that patients with specific granule deficiency, who are deficient in neutrophil defensins, fail to recruit monocytes to wounds as immunologically normal patients do. Similarly, *Chertov* et al. (1996) have shown that HNP-1 and HNP-2 are chemotactic for T-cells. These observations, coupled with the impressive potency of the chemotactic activity, suggest an important physiological role for defensins in

potentiating the cellular immune response at appropriate sites.

Kudryashov et al. (1990) reported that defensins promoted wound healing. *Murphy* et al. (1993) pursued this observation by testing the ability of rabbit and human α-defensins to stimulate the growth of cultured epithelial cells and fibroblasts. They demonstrated that rabbit NP-1 and NP-5 as well as HNP-1, 2, and 3 stimulated DNA synthesis at physiologic concentrations. Additionally, each of these defensins acted synergistically with insulin. HNP-1 was also tested against fibroblasts and found to promote DNA synthesis. These workers suggest that the effects observed are receptor independent or due to co-operative allosteric interactions based on sharp dose response curves. The physiologic relevance of these findings has not yet been proven but seems an interesting area for further work. Such activities, in the setting of a wound, could obviously be of great importance.

Some defensins have also been called corticostatins because they potently inhibit corticosteroid production by cultured adrenal cells (*Zhu* et al., 1988). This activity occurs in the concentration range of 5-500 nanomolar and is thought to be due to competitive inhibition of ACTH binding to its receptor. One study extended these studies *in vivo* and showed that rabbit defensins could indeed reduce ACTH and stress induced corticosterone production in mice and rats (*Shamova* et al., 1993). Since glucocorticoids are known to be immunosuppressive, this activity may serve to globally bolster the immune response.

Other unknown functions may reside in the diverse classes of vertebrate antimicrobial peptides. Methods such as yeast two hybrid systems may in the future aid in revealing them.

CLINICAL APPLICATIONS

Antibiotic peptides of animal origin are currently under development as pharmaceuticals. The most advanced is MSI-78 produced by Magainin Pharmaceuticals. It is in phase III clinical trials as a topical treatment for infected diabetic foot ulcers. MSI-78 is a modified version of magainin 2, one of the original magainins discovered in *Xenopus* skin. It contains 22 amino acids and differs from the natural product only by 6 substitutions and one deletion. These changes result in a molecule that is substantially more potent than magainin 2. MSI-78 exhibits a number of additional attractive features, including the broad spectrum of microbicidal activity typical of defensive peptides and the ability to kill drug resistant bacteria such as methicillin resistant *Staphylococcus aureus* (*Jacob* and *Zasloff*, 1994). An interim analysis of an ongoing phase III trial showed that a 1 % cream of MSI-78 was equivalent to oral ofloxacin treatment for the clinical endpoints measured.

Another peptide antibiotic, MSI-843, is not derived from a naturally occurring peptide but was designed based on principles learned from antimicrobials observed in Nature. It is α-helical, cationic, and amphipathic. MSI-843 is active against many multiple drug resistant strains of *Pseudomonas aeruginosa* isolated from the lungs of cystic fibrosis patients. It may therefore be useful as an inhaled therapeutic in CF patients. MSI-843 is in pre-clinical development for this indication. *In vitro* studies suggest that the potency of this compound is less sensitive to fluctuations in ionic strength than known defensins.

Other possibilities include the use of magainins or similar compounds in cancer treatment. Many cancer cells exhibit membrane differences relative to non-transformed cells that make them significantly more susceptible to these peptides. Magainins and their derivatives have been used successfully in treating tumours in animal models of leukaemia, ovarian cancer, and malignant melanoma (*Baker* et al., 1993, *Soballe* et al., 1995). These compounds are currently in pre-clinical development for oncological applications.

CONCLUSIONS

Understanding of the role that antimicrobial peptides play in vertebrates, and especially humans, is in its infancy. Only recently have the β-defensins been discovered and shown to be expressed and induced in mammalian epithelial cells. The number and types of these peptides, their tissue specific expression and genetic regulation all remain to be addressed. Understanding these aspects may ultimately allow manipulation of this arm of the immune system for the benefit of human health, analogous to stimulation of the cellular and humoral immune system by vaccination. Derivatives of vertebrate antimicrobial peptides are already in clinical trials and observation suggests that many more remain undiscovered. This class of compounds may soon represent a new weapon against human pathogens.

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NONLINEAR DYNAMICS, CHAOS-THEORY, AND THE "SCIENCES OF COMPLEXITY": THEIR RELEVANCE TO THE STUDY OF THE INTERACTION BETWEEN HOST AND MICROFLORA

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SUMMARY

Theoretical and experimental studies of various biomedical systems, including heart, brain, immune system, and many ecosystems, have shown that many of these systems may be described in terms of non-linear dynamics. Two important consequences of this non-linear dynamical behaviour are irreversibility and unpredictability, due to the chaotic behaviour this type of system may exhibit. Another trend in modern science (often named "the sciences of complexity") deals with the often complex or chaotic collective behaviour of systems made up of large numbers of relatively simple entities. Very often such systems exhibit non-linear dynamical behaviour. Many such complex and non-linear systems have been studied successfully using computer simulation techniques.

It is proposed that, as has been demonstrated for the immune system, the intestinal microbial ecosystem may he viewed as such a complex system governed by non-linear dynamical equations. A discussion of techniques available for the study of such systems is given, with a special emphasis on computer simulation. Finally, the results of a pilot study using computer simulation of the interaction between the anaerobic and aerobic compartments of the microflora within a simple geometric model of the small and large intestine are presented.

INTRODUCTION

In recent years there has been a great deal of interest (and indeed a great deal of hype) concerning three catch-phrases: non-linear dynamics, chaos, and complexity. This interest (and hype) has led to a large number of popular-science articles decorated with very fancy graphics (fractals and the like). Naturally, a sceptical backlash from certain serious scientists (*Horgan*, 1995) has occurred. Some scepticism is of course always in place when a group of scientists claims to have opened up a new field of study which will (a) revolutionise science, and (b) explain virtually anything under the sun and beyond. Some scientists working in the fields of non-linear dynamics and complexity have indeed made such claims. Such claims abound throughout the history of modern science from Newton down to the present day (see *Prirogine*

and *Stengers*, 1984). Each time some breakthrough was reached, far-fetched claims about the general applicability of the new theory or model cropped up. Similarly, objections by serious scientists against such claims have been heard as often as the claims themselves. Even the critics must however concede that non-linear dynamics, chaos theory and studies of complex systems have been making solid contributions to fields of physics (e.g. *Ott* et al., 1994), meteorology (e.g. *Lorenz*, 1963), and ecology (*Bulmer*, 1994; *Lindgren* and *Nordahl*, 1994) to name but a few.

Leaving aside both the exaggerated claims and the often acrimonious responses, the aim of this paper is to explore the possible implications which techniques and insights gleaned from non-linear dynamics, chaos theory and studies of complex systems may have for the study of the intestinal microbial ecosystem and its interaction with the host. To achieve this, the meaning of the phrases "non-linear", "chaos" and "complex" within this context will be defined. The discussion of these topics is presented without any attempt at mathematical rigour. Those interested in a more rigorous discussion are referred to *Ott* et al. (1994), or for the more philosophically minded *Prirogine* and *Stengers* (1984) and *Kauffman* (1995). It will then be shown that both the microbial ecosystem and the host's immune and digestive system all meet the necessary conditions to be called complex non-linear dynamical systems. The types of behaviour which such systems may exhibit and the means to study them are explored. Two approaches to study the intestinal microflora and its interaction with the host follow naturally from this discussion: (i) computer simulation of the system, and (ii) time series analysis of series of measurements to measure degrees of chaos and (un)predictability. There have been some attempts at the first approach already, notably by *Freter* et al. (1983), who made a mathematical model of the competition for food substrate and binding sites in a continuous flow model of the intestine. Many other types of interactions (both antagonistic and mutualistic) exist within the intestinal microflora, and it should be possible to model many of these. In this paper a pilot study, using computer simulation of the interaction between the aerobic and anaerobic compartments of the microflora, is presented. This simulation lends further support to the idea that a qualitative and quantitative theoretical understanding of a number of features of the intestinal microflora can be obtained through computer simulation. Finally, an outline of a research programme to explore the interaction between microflora and host with techniques from non-linear dynamics and complexity studies is sketched.

THEORY

What are non-linear dynamical systems?

Probably the most important contribution of Newton and Leibnitz to science is the introduction of the concept of dynamical systems. In physics almost any system under study, whether planetary orbits, semiconductor electronics, or the Earth's atmosphere, may be considered a dynamical system. A dynamical system is a simply system which can be characterised by (a) a set of parameters the values of which define its *state* at a given point in time, and (b) a set of mathematically specified rules defining the change of state of the

system in time. These rules are generally specified as differential equations, defining the rate of change of each of the parameters describing the system, as a function of the current state of the system.

This definition is very broad indeed, and many systems in biology, medicine, economics and the social sciences may be described and studied as dynamical systems (e.g. *Prirogine* and *Stengers*, l984; *Kauffman*, 1994). A well known example of this is the Lotka-Volterra predator-prey model ecosystem. The set of numbers describing this systems consists of (i) the number of predators, and (ii) the number of prey. The rules specify that the number of prey increase at a rate proportional to the number present (exponential growth) in the absence of predators. When predators are present the number of prey caught, which is proportional to both the number of predators and the number of prey, must be subtracted. The predators starve in the absence of prey (exponential decay) and grow proportionally to the number of prey caught (again proportional to the product of prey and predator numbers). This system may show damped, undamped and increasing predator-prey oscillations. By specifying the initial conditions (e.g. from observation) and solving the differential equations involved, it is in principle possible to model or predict the future behaviour of the ecosystem.

The sequence of states the system passes through in time is called its *orbit.* If the system is dissipative, i.e. it loses energy in some way (and most systems do), the orbits converge to one of a small subset of all possible states called an *attractor.* The simplest kind of attractor is a single point: the system becomes stationary. The system is said to be at rest or in dynamical equilibrium. Another type of attractor is called a *limit cycle:* the system oscillates at a stable frequency and amplitude. A system may have numerous attractors, and the initial conditions determine to which attractor the system will converge. The set of initial states for which the system converges to a particular attractor is called the *basin of attraction* of that attractor. The Lotka-Volterra type ecosystem may have either a point attractor, i.e. the populations become stable, or a limit cycle attractor, i.e. predator-prey oscillations remain stable (e.g. *Bulmer*, 1994, pp. 39-45).

Depending on the kind of rules specified, dynamical systems are either linear or non-linear. In a linear dynamical system the differential equations are linear, which means that the effects of changes in the state of the system are additive and proportional to the magnitude of the changes. The result of changing multiple parameters simultaneously is simply a superposition of the change in each individual parameter. The additive nature of changes to the system means that different parameters of the system may each be studied separately. Furthermore, the linear nature of the of the equations ensures that, given an initial condition, the orbit of the system is uniquely defined. This means the system is time-reversible and predictable: past and future may be deduced with arbitrary precision from the present state. Furthermore, the attractors are guaranteed to be simple, and the equations can be solved quite readily (even with paper and pencil in small systems).

Because of all these features, linear systems have been studied most. Before the advent of electronic computers, mathematical simplicity was one overriding reason to study linear systems, but a more subtle reason may have been equally important (*Prirogine* and *Stengers*, 1984). The uniqueness of the orbit lent credibility to the idea of a

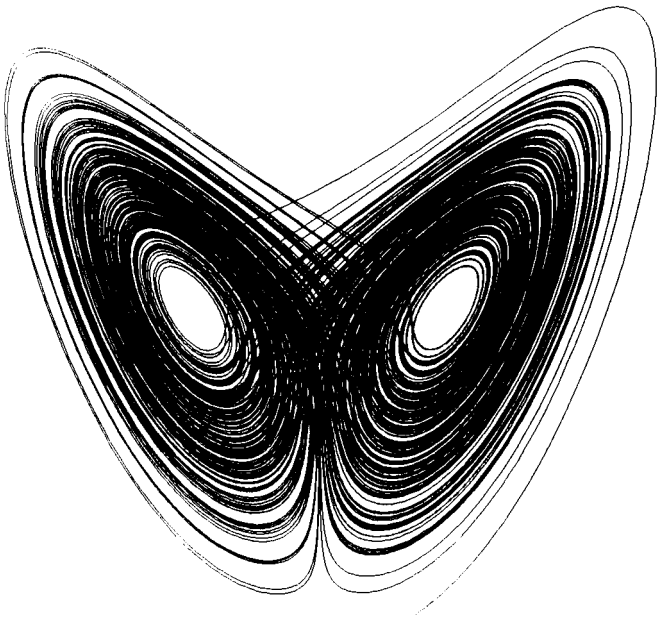

Figure 1: An example of the fractal shape of a strange attractor, the Lorenz-attractor, which may (roughly) he considered as consisting of an infinite set of different oscillations which the system may go through.

Cartesian, clockwork universe. All conditions were set at the time of creation, and the clockwork mechanism of Newtonian mechanics would automatically see to the rest. Unique orbits also provide complete determinism, which is not guaranteed to exist for non-linear dynamical systems. Besides, it was (and is) argued that many non-linear systems (such as the simple pendulum) can be approximated by linear systems to such a degree that there is no need to solve the more complicated non-linear form.

By contrast, in non-linear systems, changes in multiple parameter need neither be additive, nor proportional to the magnitude of the changes. The effects of changing individual parameters cannot in general be studied separately as in the linear case. Furthermore, the orbit of a system need not be unique for a given initial condition. In such cases bifurcations occur: places in the orbit were two possible future paths are open to the system, and no deterministic means exists to choose between the two paths. An element of randomness creeps back into the mechanics (*Prirogine* and *Stengers*, 1984).

In many non-linear systems with more than three parameters which can be set freely (or *degrees of freedom),* an effect called *chaos* may occur. Probably the most famous example of chaos has been found in meteorology, where *Lorenz* (1963) has shown that deterministic, but highly irregular flow patterns exist within weather systems. When chaos occurs the attractor cannot be described by simple forms such as limit cycles, straight lines or points; the attractor has a *fractal* shape (Figure 1). A fractal shape shows detail at every possible magnification. Attractors with this peculiar property are usually called *strange attractors.* These attractors can be thought of as (roughly) the union of

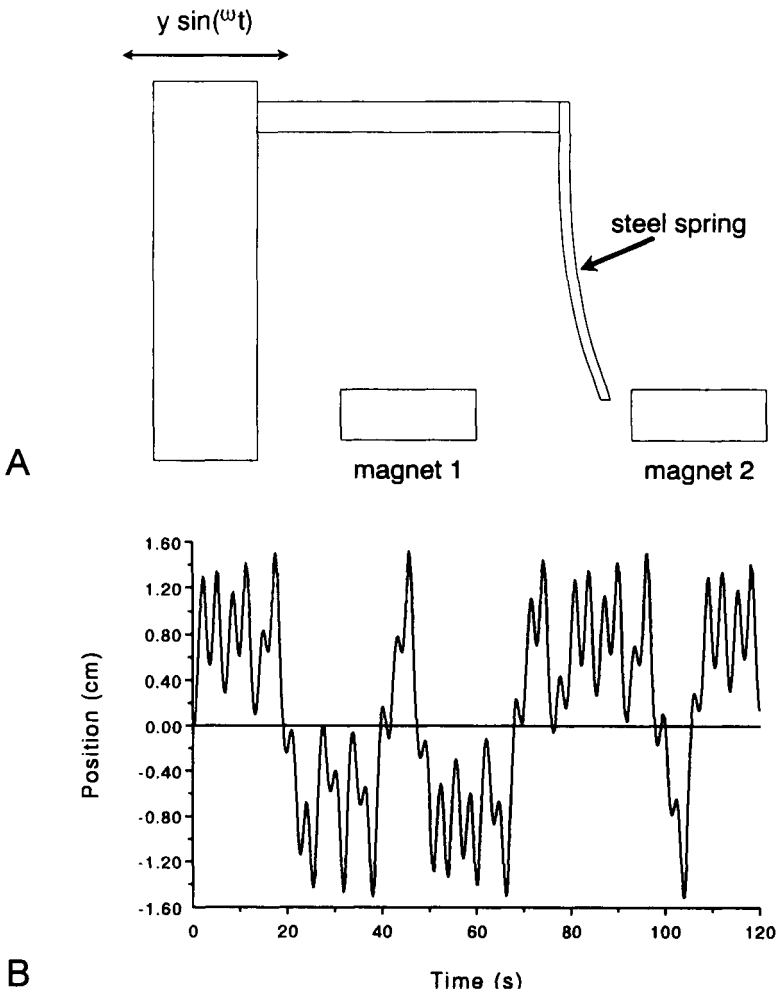

Figure 2: A simple system showing chaos (after Moon and Holmes, 1979): (A) diagram of apparatus, showing a steel spring suspended between two magnets; the top of the spring is forced to oscillate sinusoidally, (B) the graph shows the chaotic motion of the lower end of the spring .

an infinite collection of limit cycles, with the system switching very rapidly between them. The corresponding motion (or orbit) may appear to be random and look something like Figure 2, which shows the motion of a simple spring and magnet system (*Moon* and *Holmes*, 1979). The base of the spring is forced to oscillate at some frequency ω. The displacement of the end of the spring as a result of all forces is highly irregular, and yet it is not noise. The system is still deterministic. In fact, Figure 2B is not a series of measurements, but the result of a computer simulation using the set differential equations describing the system, so it cannot contain truly random noise. This type of seemingly random, yet fully deterministic behaviour is one of the hallmarks of chaos.

Another hallmark is the so called "butterfly effect": Change the initial conditions of wind speed in the global weather by an amount corresponding to the beat of a wing of a butterfly in Peking, and the path of a Caribbean hurricane is altered, because the change

introduced increases exponentially. In chaotic systems, infinitesimal changes in initial conditions propagate exponentially in time, resulting in drastically different outcomes from infinitesimally different initial conditions. This means that future and past cannot be deduced with arbitrary precision or for arbitrary periods in time from measured data, which always contain some finite error. It is possible to determine a degree of chaos: the Lyapunov exponent. This is a number which determines the doubling rate of the error in the prediction. If it is low the system is not very chaotic, and medium to long term predictions remain accurate over considerable periods of time. If it is high, errors increase rapidly, and only very short term prediction is possible. Other measures of degree of chaos exist, most notably the *fractal dimension* of the attractor, which is a measure of the complexity of the shape of the attractor. The more complicated the attractor, the higher the degree of chaos.

Can microbial ecosystems be described as non-linear dynamical systems?

Growth of bacteria, either single species (*Monod*, 1950), mixed cultures (*Gerritse* et al., 1992), or complete ecosystems (*de Wit* et al., 1995) can be described in terms of dynamical systems. The key feature of the dynamics of these systems is that they show autocatalytic or inhibitory loops: the presence of a bacterium is needed to make more of that kind bacterium (obviously). Furthermore, species A may inhibit species B by secretion of toxins. Species A might also enhance growth by production of metabolites which serve as food for B, or may remove substances toxic to B form the ecosystem. In systems which are far from thermodynamical equilibrium, such autocatalytic and inhibitory loops produce just the type of non-linear dynamics which can produce highly complicated and chaotic behaviour (*Prirogine* and *Stengers*, 1984). In practice, all ecosystems are far from thermodynamical equilibrium, since large fluxes of energy or food pass through them; only death (a point attractor of any ecosystem) corresponds to thermodynamical equilibrium.

For these reasons it may be assumed that techniques for analysis and modelling of non-linear dynamical systems in general are appropriate tools for the study of bacterial ecosystems, including the gut microflora.

Chaos and control systems

As odd as it may seem, the presence of chaos may be an advantage in control systems, if rapid responses are required. Chaotic systems would seem to be utterly unreliable, given their extreme sensitivity to initial conditions. Yet, as, e.g., *Ott* et al. (1990) have noted, that same sensitivity allows a control mechanism to control the system with very small corrective signals, provided the developing chaos can be analysed rapidly, i.e. proper feedback is available. Very small adjustments have large effects.

This may be of particular importance to biological control systems. Changing the mode of operation of, e.g. heart, nervous system or immune system rapidly, and without the expenditure of large amounts of energy is literally of vital importance to practically any organism. The constant feedback and small corrective steps to keep a such systems in the correct mode are probably not such a drawback, since the expenditure of energy can be small for chaotic systems. Chaotic dynamics have indeed been observed in, e.g., heart rate variations (*Goldberger* et al., l984), though there is still some debate about the significance and meaning of these

findings (*Kaplan* and *Talajic*, l991). It has been observed that a reduction in variability (and possibly chaos) of heart rate may indicate heart disease (*Kaplan* et al., 1991; *Skinner* et al., 1991). On the other hand, fibrillations seem to be highly chaotic in nature, with high fractal dimension (*Garfinkel* et al., 1992). Too much chaos is uncontrollable.

It has also been claimed that chaos is present in electroencephalograms (EEG). Here too, there is quite a lively debate about the reality and relevance of chaos (*Bullock* et al., 1995; *Pritchard* et al., 1995). Nonetheless, the fractal dimension of the attractor has been used as a measure of complexity of the EEG patterns. *Stam* et al. (1994) found that normal controls had significantly ($p<0.001$) more complex EEG patterns than patients with Parkinson's disease. Theirs in turn was significantly ($p<0.001$) more complex than EEGs of patients with Alzheimer's disease.

What are complex systems?

The "Sciences of Complexity" deal with systems which may show complicated behaviour, stemming from the behaviour of a large number of entities which themselves show a simple behaviour. The complexity does not stem from complex rules, but rather from the large number of entities or subsystems the system is made of. An objection which has been raised is that the term complexity has not been defined particularly strictly (*Horgan*, 1995). Indeed a number of (more or less conflicting) definitions have been given, yet these definitions are mainly aimed at measurement of complexity, i.e. assigning a number to it. Whatever the conflict about how to measure complexity, the basic premise that complex systems are systems which are made up of large numbers of simpler objects is agreed on by all those working in the field. Any ecosystem can of course be considered as such a system, being built up of large numbers of individual organisms, each of which may show a far simpler behaviour than the whole system. Similarly, the immune system may also be considered to be a complex system in this sense, since it is comprised of many cells which themselves exhibit rather simpler behaviour than the whole.

Having said this, what can actually be gained by calling ecosystems or the immune system "complex"? Do complex systems share certain properties which may be exploited to give extra insight into the behaviour of, e.g., the gut microflora and its interaction with the immune system? Several studies indicate that such common properties do exist (*Langton*, 1989; 1992; *Kauffman*, 1995). The most important feature is probably that such systems show global, co-ordinated behaviour, without the presence of any distinct "global controller": self-organisation. Though an ecosystem might show Lotka-Volterra type predator-prey oscillations, there is no external driving force which creates this; no "invisible hand". Similarly, the immune system has no "chief lymphocyte" which directs an immune response, neither has the brain a "chief neurone" in which central control of all behaviour is located. The behaviour of all such systems is collective, but not under any "Stalinist" rule, nor need any of the entities involved be aware of the nature of the collective behaviour. Secondly almost all such systems are non-linear system: given the large number of interactions in such systems, some are bound to non-linear. Given that, and the large number of entities (and therefore degrees of freedom), such systems are almost certain to show chaotic behaviour under a wide range of conditions.

Complex systems may show roughly four types of behaviour (*Langton*,

1989, 1992): (i) steady state, (ii) periodic, (iii) "complex", and (iv) highly chaotic. Steady state is the simplest: the system is frozen into a particular state. Though there may be some initial oscillations, these die out and the system settles down into its final state. There is a gradual transition into the periodic regime: initial oscillations persisting for longer and longer times until they become effectively infinite. Even though the system is oscillating, the spatio-temporal structure may be thought of as fixed, non-adaptive. Both the steady state and periodic classes of behaviour may be thought of as solid. Conversely, in the highly chaotic regime, no oscillations persist, and no structure is apparent at all. Though determinism might be present, the degree of chaos is so high it is indistinguishable from stochastic behaviour. The system might be thought of as being in a gaseous phases. As such, the system is not adaptive either, it is just a constant mess.

The most interesting behaviour is seen at the borderline between order and chaos, which might be thought of as a phase-transition between the solid and the gaseous phase. At this borderline, periodic oscillations may persist for long periods of time, or may vanish almost instantly. Definite structures may propagate through space and time, and produce complex interactions where they meet. It has also been shown that such systems, balanced on the "edge of chaos" can perform computing tasks: manipulation, storage and transmission of data. On the edge of chaos they are neither so rigid that manipulation or transmission is impossible, nor so chaotic that stored and transmitted data are scrambled. The systems can become truly adaptive. It is an attractive, but as yet unproven conjecture of many workers in this field that all living systems (single organisms and ecosystems alike) are balanced on the edge between order and chaos, since it is only on this edge that sufficient order is present for homeostasis, along with sufficient chaos for adaptive behaviour (*Langton*, 1992). There is a number of theoretical studies which suggest that evolution indeed drives the evolving entities to this edge (*Kauffman* and *Johnsen*, 1992; *Kaneko* and *Suzuki*, 1994).

Self organised criticality and power-law spectra in complex systems

It has been claimed that complex systems may show what has been called "self-organised criticality": a situation in which the slightest disturbance may cause either large or small cascades of events. The classical example of this is a large pile of sand, each grain on the surface of which is *just* held in place. Toss an extra grain of sand on the pile and you may see anything may happen from just a trickle to a huge avalanche (*Bak* et al., 1988). Similarly, in an ecosystem, the introduction of a new species (or a mutation in an existing one) may cause mass extinction or no effect whatsoever. In fact, if many species are present in the ecosystem, it becomes very hard to introduce new species. Usually, they fail to colonise. Occasionally however an intruder may wipe practically all others.

According to *Bak* et al. (1988), self-organised, critical systems may show shifts in behaviour at all scales, but not all magnitudes of shifts are equally likely. Small changes (small trickles) are more likely than large ones (avalanches). The likelihood (p) that a shift of a given magnitude (A) occurs is given by a power law:

$$p(A) \propto A^{-v} \qquad (1)$$

This equation implies two things: (i)

catastrophes cannot be prevented in such a system, and (ii) the rate of occurrence of catastrophes at given magnitudes can be predicted from small scale events. This power law is seen by some as a hallmark of self-organised criticality (*Bak* et al., 1988), others claim such systems do not actually show a power law (*Horgan*, 1995), but that very large scale events occur at lower rates than predicted by equation (1). Whatever the final outcome of that discussion, if some law may be postulated which, like equation (1), can predict the frequency of occurrence of large magnitude shifts in a dynamic system from the rate of occurrence of small magnitude events, this may become a diagnostic tool. If certain large scale shifts in the microbial ecology of the intestine are associated with disease, their rate of occurrence might be predictable from the normal, non-pathological population dynamics. If this is the case, modulating the dynamical behaviour of the flora, rather than its mean composition might become a goal of therapy. At this point in time, this idea is still very much speculation, yet there are ways to verify it. If we can determine the short to medium term (and therefore small to medium scale) fluctuations in the gut microflora of healthy volunteers, and we find a power law distribution, we can then try to predict the rate of occurrence of large scale shifts relating to certain well defined pathological situations, for which good epidemiological data are available, and in which the gut microflora is assumed to be involved in its aetiology. A good agreement between predicted and measured data would lend support to the thesis that the population dynamics of the gut microflora are involved causally.

Which techniques have been developed to study complex, non-linear dynamical systems?

A number of different tools to study complex, non-linear dynamical systems has been developed in the last decades. All rely on the availability of moderate to large amounts of computing power. The methods can be divided into two categories: (i) (time-series) analysis of observations, and (ii) computer simulations: science on the edge between theory and experiment.

The first set of techniques attempts to detect the "fingerprint" of non-linear, deterministic behaviour in measured time-series. If the data are of sufficient quality, it is possible to distinguish chaotic from stochastic behaviour (*Theiler* et al., 1992). The degree of chaos may be determined be measuring Lyapunov exponents (*Eckmann* et al., 1986; *Parlitz* 1992), or fractal dimensions of the attractor (*Grassberger* and *Procaccia*, 1983; *Brandstater* and *Swinney*, 1987). With lower grade data, spectral analysis, to measure the frequencies of shifts of different magnitudes can be performed, to see whether power law relationships are evident (e.g. *Bracewell*, 1986). All kinds of time series analysis described here do need larger numbers of points than are usually obtained in e.g. patient studies of the microbiology of the intestinal microflora. Some tens of sample points should be available per patient. This precludes the use of classical culturing for these types of analysis, for all but the wealthiest researchers.

The other set of tools consists of computer simulation techniques or "experimentation *in silico*". Computer simulations allow theorists to visualise

what should happen if their theories concerning complex systems are correct, or which parameter settings have the most profound influence on the system's behaviour. Computer simulations by themselves do not tell us anything about the real system, they tell us something about our theories concerning the system. Without computer simulations, theories of all but the simplest systems are hard to interpret in a quantitative way. Especially in the case of complex, non-linear systems, it is virtually impossible to say how the system will behave, given a set of experimental conditions. However, if quantitative models of each of the system's components are available, it is possible to create a computer program which could mimic the behaviour of the real system. By running such programs many times with many different settings of experimental parameters, it is possible to gain a great deal of insight into the behaviour of the system. Comparison with *in vivo* and *in vitro* experimental data must of course be performed to see whether the behaviour of the model system is anything like the real system.

Computer models come in two different basic types: *tactical* and *strategic* (*Levins*, 1968). A tactical model strives to explain as much detail as possible of a specific system for prediction or control purposes. The results of simulations of such a model can be highly accurate, but are not widely applicable. By contrast, strategic models are more or less qualitative. They cannot predict the behaviour of a specific system in detail, but they can explain the kinds of behaviour a class of systems sharing certain features may show. Insight, rather than prediction and control is the ultimate goal of such models. The results of these kinds of simulations are not at all numerically accurate, but they are widely applicable. Most modelling in theoretical biology is of the strategic type (*Bulmer*, 1994). A number of tactical models have been used within the field of microbiology (*Jahnke* et al., 1982; *Gerritse* et al., 1992; *de Wit* et al., 1995), usually applying to ecosystems of limited complexity. An example of more complex modelling is Cybermouse, a model murine immune system (*Sieburg*, 1990; 1993).

The "spatial vs. chemical detail" trade-off

When modelling an ecosystem it is of course impossible to capture all detail. Tracing every single cell's interaction with every chemical is beyond the power of any computer on earth. Some intelligent simplifications are needed. When designing such a simplified model, the most important trade-off is that between the spatial resolution required and the number of (chemical or microbial) species in the model. Models can in fact be classified based on the spatial/species resolution ratio.

At one end of the spectrum are those models which model "chemistry" in high detail, but do not show any spatial detail. Usually these models are *connectionist* models, using complicated graphs (food-webs) to define the interactions between various species within the system. Such models may be used for well mixed chemostats (e.g. *Gerritse* et al., 1993), and can be used to model complex chemistry (*Bagley* and *Farmer*, 1992) or food webs (*Lindgren* and *Nordahl*, 1994). Leaving out spatial detail may be safe enough if the ecosystem is fairly homogenous, yet there is one caveat. In a study of gypsy moth population dynamics, *Wilder* et al. (1995) found that chaos occurred when spatial detail was omitted. If spatial detail (and consequently diffusion) was included, highly regular travelling waves were seen instead. Diffusion was capable of damping out chaos, and changing it to regular behaviour.

Table 1: Parameters describing a bacterial metabolism and numerical values for the three "species" used (derived from *Gerritse* et al., 1992)

Symbol	Meaning	Strict anaerobes	Facultative anaerobes	Strict aerobes	Units
μ_{O_2}	maximum growth rate by aerobic metabolism (inhibition rate if negative)	-1.0 x 10^{-4}	4 x 10^{-4}	6 x 10^{-4}	/s
μ_{an}	maximum growth rate by anaerobic metabolism	1.0 x 10^{-4}	0.75 x 10^{-4}	0	/s
μ_{basal}	basal metabolism (minimum metabolic requirement)	1 x 10^{-5}	1 x 10^{-5}	1 x 10^{-5}	/s
K_F	half saturation uptake rate food concentration	2 x 10^{-2}	2 x 10^{-2}	2 x 10^{-2}	mol/l
K_{R,O_2}	half saturation respiration rate oxygen concentration	1 x 10^{-6}	1 x 10^{-5}	1 x 10^{-5}	mol/l
K_{T,O_2}	half saturation kill rate oxygen concentration*	1 x 10^{-6}	1 x 10^{-5}	1 x 10^{-5}	mol/l
κ_{O_2}	maximum oxygen kill rate	1 x 10^{-6}	0	0	/s
α_{O_2}	efficiency factor of aerobic metabolism	1	1	1	
α_{an}	efficiency factor of anaerobic metabolism	1	1	1	
α_{κ}	fraction of oxygen killed bacteria returned as food*	0.5	1	1	
β_{μ}	maximum oxygen uptake rate due to aerobic metabolism	1.10^{-9} - 1.10^{-7}	1.5 x 10^{-4}	1.5 x 10^{-4}	/s
β_{κ}	maximum oxygen uptake rate due to toxic effect on anaerobes or microaerophiles	1.10^{-9} - 1.10^{-7}	0.0	0.0	/s

*: Value has no influence on outcome if oxygen kill rate and uptake rate are zero, but causes divide by zero errors if set to zero itself.

On the other side of the scale are cellular automata: (usually rectangular) grids of simple "chemostats" of limited complexity, each interacting only with its nearest neighbours through simple rules. Such systems can show high spatial detail, at the expense of biochemical realism. Nonetheless, as extremely abstract systems they lend themselves to strategic modelling of spatial self-organisation processes, such as can occur in reaction diffusion systems (*Markus* and *Hess*, 1990). As microbiological systems can often be seen as reaction diffusion systems (e.g. *Blackburn* and *Blackburn*, 1993), it is reasonable to assume that some spatial detail must be included.

Many biological models are somewhere in between the two extremes, e.g. showing one (vertical) spatial dimension for microbial mat communities yet show a great deal of biochemical realism (*de Wit* et al., 1995). *De Wit* et al. (1995) could predict the vertical spatial distributions and diurnal cycles of coexisting cyanobacteria, purple sulphur bacteria and chemotrophic sulphur bacteria in a microbial mat community, based on detailed knowledge of metabolisms, light absorption, division rates, etc. The computations could be carried out on a simple personal computer. The success of such work strongly suggests that at least a strategic model could be made for the intestinal microflora. With considerably more computing power, and considerable input from *in vitro* measurements of microbial physiology, a tactical model could possibly be made.

AN EXPERIMENT *IN SILICO*

A computer simulation has been run, using a program developed as a pilot study within the ISGNAS research program. A full description of the computer program, its capabilities and the simulations run on it is in preparation. The model intestine consists of a 6 m long axisymmetric tube of varying diameter. The first 4.98 m are the small intestine, with a radius of 1 cm; the next 18 cm are the "caecum" (radius 5 cm), followed by a "colon" of 84 cm long and 3 cm radius. The lengths and radii may be varied at will. The intestine is subdivided axially into 100 sections and radially into l0 concentric shells. Each of the 1000 volume elements may be considered a separate "chemostat" coupled to its neighbours by transport mechanisms. Continuous laminar flow and diffusion are the transport mechanisms modelled to date. Extensions for peristaltic motion may be included later. Apart from up to 6 "species of bacteria", 2 "chemical substances" are included in the model: food and oxygen. Though I will use the phrase species, each type of bacterium represents a whole category of bacteria, all of which share an aerobic or anaerobic metabolism. This means that each "species" can metabolise a far wider set of food substrates (lumped together as one substance "food"), than a single species in reality. Within each category, mutualisms, such as the use of metabolites of the one species as substrate by others means that the effective yield of biomass per unit of substrate should be higher than in a true single species.

The metabolism of each species was modelled using Monod equations with modifications for (i) a basal metabolism, and (ii) mutual hindrance at high population densities. The model metabolism of each species is determined by 12 parameters, the meanings and values of which are summarised in Table l.

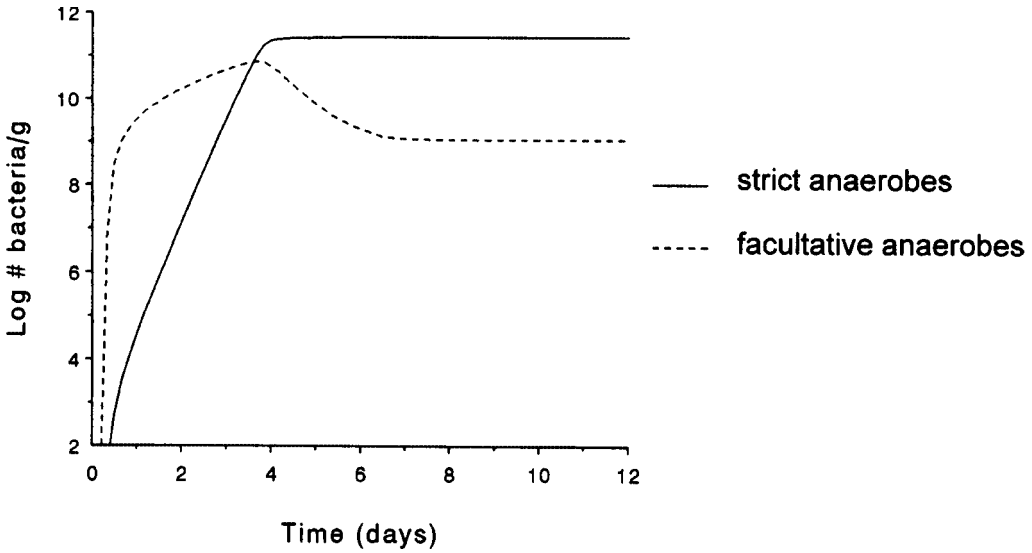

Figure 3: Colonization process in a di-associated sterile intestine modelled by computer simulation. Equal numbers of two species of bacteria (one strict and one facultative anaerobe) are fed into the sterile intestine, which contains an initial oxygen concentration of 0.1 mmol/l. Initially, the facultatives colonise. Later, as oxygen levels drop, the strict anaerobes outcompete the facultatives.

The model metabolism is a slight variation of that used by *Gerritse* et al. (1990). All concentrations are given in mol/l: food and all bacteria in moles of organic carbon, oxygen simply in moles of molecular oxygen (O_2). To convert to numbers of bacteria, it was assumed that the volume of a single bacterium was 10^{-15} l (i.e. a maximum of 10^{12}/g), and that they contained roughly 10% w/w of organic C. This yields a conversion factor from mol/l to bacteria/g of about $1.2x10^{11}$.

Using the above model, experiments were done to simulate colonisation in a sterile intestine. One or two species of bacteria, selected from three available types (strict aerobe, facultative anaerobe and strict anaerobe), were introduced into a sterile intestine, in which the oxygen concentration of the lumen was in equilibrium with the walls (0.1 mmol/l). The input of food, oxygen, and bacteria was in block waves with a 40% duty cycle. Food concentration at maximum was 7 mol/l, oxygen concentration 0.l mmol/l, and in most experiments the food inflow contained a maximum of $1.2x10^3$ bacteria/g of each species. Though this may be a bit high, runs with only 12 bacteria/g showed virtually identical results, so evidently this parameter is relatively unimportant in the initial colonisation phase.

Figure 3 and Table 2 summarise the results of the simulations. When strict anaerobes were introduced simultaneously with either facultative anaerobes or aerobes, the latter colonised within 1 day, reaching a maximum at day 4. After this, they were replaced by the anaerobes, which only appeared in any numbers at day 3. After 5 to 6 days a stable equilibrium was reached with strict anaerobes outnumbering facultatives or aerobes by 2.4-2.7 ^{10}log steps. Small oscillations caused by the periodic input of food remained visible. Once stabilised, the population did not change if the influx of bacteria from the "stomach" reduced to zero, thus they had colonised the lumen.

Table 2: Mean numbers of bacteria per gram at equilibrium, 12 days after colonization, for a mono- or di-associated model intestine

	Small intestine (^{10}log)	Large intestine (^{10}log)
Mono-associated with:		
strict anaerobe*	4.42 x 10^2 (2.65)	6.77 x 10^2 (2.83)
strict aerobe	1.04 x 10^8 (8.02)	1.10 x 10^9 (9.04)
facultativc anaerobe	7.73 x 10^8 (8.86)	2.22 x 10^{11} (11.35)
Di-associated with:		
strict anaerobe +	1.65 x 10^9 (9.22)	2.8 x 10^{11} (11.45)
strict aerobe	1.02 x 10^8 (8.01)	4.42 x 10^8 (8.65)
strict anacrobe +	1.66 x 10^9 (9.22)	2.8 x 10^{11} (11.45)
facultative aerobe	9.871 x 10^7 (7.99)	9.89 x 10^8 (8.99)

*: Does not represent colonisation, as the maximum input density of bacteria was 1.2 x 10^3/g (mean 4.8 x 10^2/g), and when the input density was reduced to zero, all anaerobes were washed out of the intestine with 3-4 days.

Facultative anaerobes by themselves could colonise in high numbers in the absence of strict anaerobes ($2.2x10^{11}$/g). Strict aerobes could colonise by themselves, but only in modest numbers compared to facultatives ($l.lx10^9$/g). By contrast, none of the strict anaerobes tested could colonise in the absence of bacteria with an aerobic metabolic ability.

A second experiment started with the stable mixed populations at t=12 days found with the first experiment. At that point, the aerobic fraction of the microflora was eliminated and the influx of aerobes halted, as a (crude) simulation of selective decontamination of the digestive tract. Depending on the oxygen uptake rate of the anaerobes (both β-parameters in Table 1), the populations could remain stable, even in the total absence of aerobes. Only if the inhibition of growth and destruction of bacteria required less than $1.2x10^{-8}$ mol O_2 per mol C bacterial biomass did the population become unstable and die out due to the increased oxygen concentration. The extreme sensitivity to the value of both β-parameters is shown in Figure 4. When both are set at $1.2x10^{-8}$, the flora remains stable, but at $1.1x10^{-8}$ a steady decline does set in after 4 or 5 days, and at $1.0x10^{-8}$ the decline starts 2 days earlier.

To test the stability of the ecosystem to perturbations around this critical point, the supply of food was altered in two ways: (i) above the stability threshold the period was increased while retaining the same total food supply (i.e. a few large amounts of food in stead of many small amounts), and (ii) below the stability threshold increasing the production of mucus.

Figure 5a shows the results of the first perturbation. As the period between meals increases, the oscillations in the population density increase, which is expected many types of damping systems. When food is supplied only once a day, the oscillations become so large that the population becomes unstable and dies out. Figure 5b shows the results of the second experiment. With increasing food supply, the survival increases, though in this experiment no permanent survival was observed.

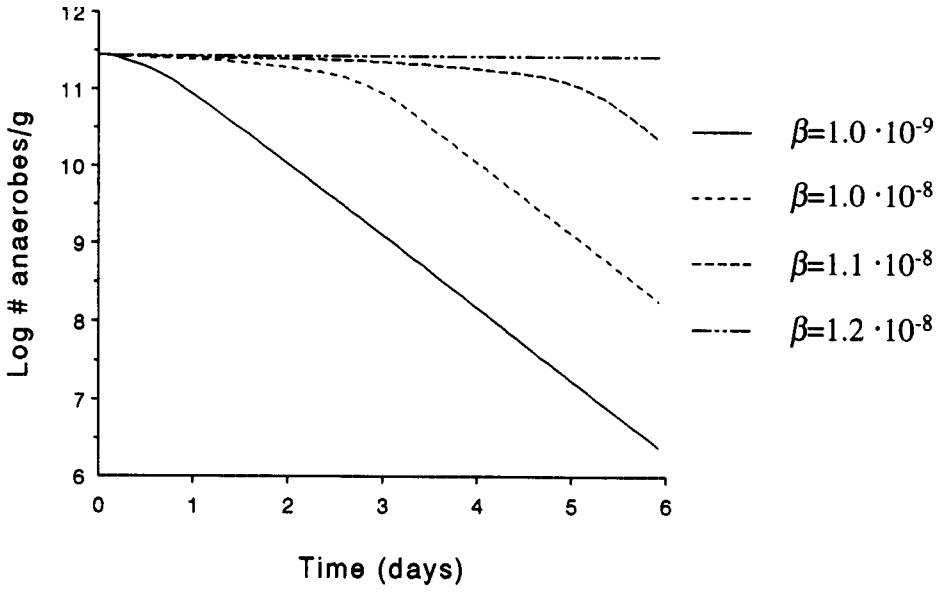

Figure 4: The survival of strict anaerobes when all facultative anaerobes and aerobes have been removed: Depending on the oxygen uptake parameters $\beta=\beta_\mu=\beta_K$ (Table 1) the bacteria survive or die out.

DISCUSSION

In my view, no "leap of faith" is needed to describe the intestinal microflora and the immune system as complex, non-linear dynamical systems. In fact, it is merely a generalisation of the modelling work of, e.g. *Freter* et al. (1983). Once this is accepted, it is a logical step to use non-linear time series analysis techniques and computer simulation as tools to study these systems. Computer simulation is probably the only way to verify that certain models work, i.e. explain observed data, in any system with more than 3 interacting objects when it is not in an equilibrium state. Computer simulation can distinguish the essential from the accidental parameters. Used properly non-linear dynamics may tell us both how to interpret our data within the framework of a complex model (i.e. a lot of simple interacting objects), and which parameters should be observed to distinguish between competing models.

What might we learn from non-linear dynamics in the intestinal microflora?

Here we enter the realm of speculation. Leaving aside a number of "ifs", computer simulation and time-series analysis might give us insight into the following issues:

- Under which conditions does the microflora become more or less self-regulating?
- If we extrapolate the power law spectrum of the population dynamics (if it exists), could we explain the occurrence of certain intestinal disorders as a consequence of this power law? If so, this could lead to preventive therapy: can we modulate the flora to change the power of the power law?
- What is the link between the power or fractal dimension of the time series and the number of species in the flora? Does this conform to the conventional notion of some 400 species?

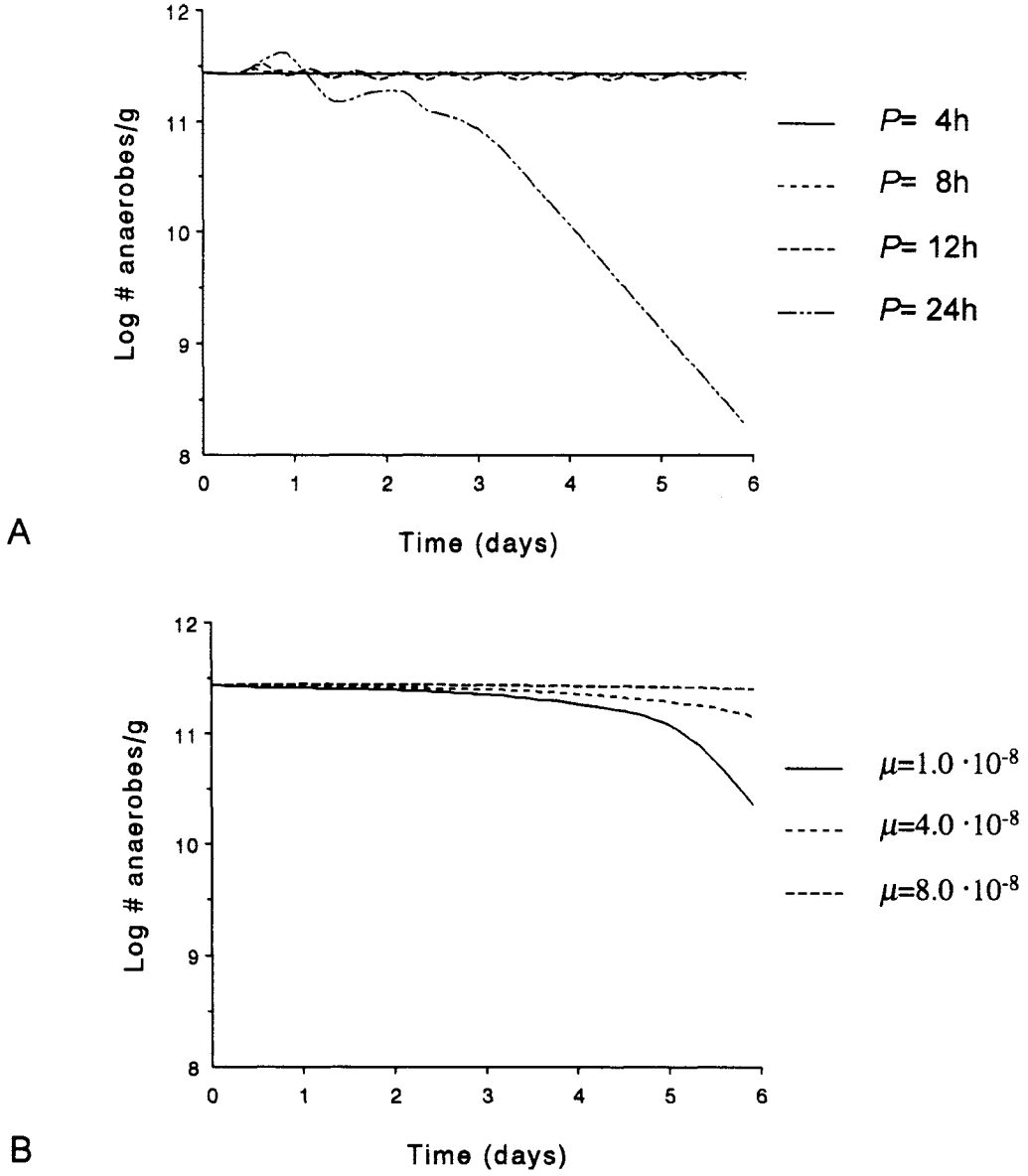

Figure 5: Modulating the survival of strict anaerobes when all facultative anaerobes and aerobes have been removed: (A) increasing the period P of the food supply cycle for the survivors in figure 5 (β=1.2x10^8) causes increasing oscillations which destabilise the population; (B) increasing food supply through mucus production increases survival for bacteria with β=1.1x10^8.

- how does all this influence colonisation resistance, i.e. can we predict colonisation resistance from population dynamics?
- What role do bacteriophages play?
- What attributes does a bacterium need to survive in the intestines?
- How do the mechanics (intestinal motility, lumen viscosity, etc.) influence the spatial and species distribution?
- What role does the immune system

(e.g., modelled using Cybermouse) play in modulating the flora?

These issues (and probably a lot more) can of course not be resolved by computer modelling work alone, but should be addressed by a concerted effort, incorporating the development of new theories and more accurate methods of observation. High quality data will be essential for the non-linear analysis approaches to work. The problems with cultural counts can however be surmounted with a number of techniques, such as measurement of microflora associated characteristics (MACs) (*Midtvedt*, 1985) and digital image analysis (*Meijer* et al., 1991; *Wilkinson* et al., 1994) especially in combination with 16S rRNA targeted fluorescence *in situ* hybridisation (*Langendijk* et al., 1995) and measurements of metabolic activity (*Nwoguh* et al., 1995; *Gribbon* and *Barer*, 1995). Such techniques promise to deliver both the data quality and achievable sampling rates needed for the kind of analysis envisaged.

What has been learned from the pilot study?

First of all it should be stated that no true chaos was observed in any of the simulations. Secondly, a number of things may be learned from the omissions in the model. Adherence sites on the epithelium were not modelled, yet in the absence of a true mucosal flora attached to the wall, a luminal flora could become perfectly stable. Evidently, bacteria *can* colonise the lumen without colonising the mucosa. Without an immune system reasonable ratios of aerobes to anaerobes were found. Thus, it is reasonable to assume that the immune system does not in fact regulate this ratio, but that the reduction of oxygen by aerobes creates an anoxic environment, in which they are outcompeted for food by strict anaerobes. Far from being a new idea, this has already been suggested by (e.g.) *Meynell* (1963), *Schaedler* et al. (1965) and *Schaedler* (1973) on the basis of experimental data. However, none of these authors could give a estimate of the magnitude of the effect on theoretical grounds.

Apart from the final numbers and ratios, sequence of the colonisation in Figure 3 is very reminiscent of the colonisation of the gut of germ free and new-born mice (*Schaedler* et al., 1965; *Schaedler* 1973), where the "normal" flora (fusiforms, *Bacteroides*, etc.) are preceded by the coliform facultatives. For about 2 days, the facultatives dominate the strict anaerobes, after which the anaerobes outcompete the coliforms. The difference between these observations and the simulation lies in the lactobacilli and lactococci, which are the first to appear in new-born mice. However, many lactobacilli grow readily at high oxygen levels (even in air, *Schut*, personal communication), and do not lower redox potential (Eh) (*Meynell*, 1963). Furthermore, they are not thought to enter into direct substrate competition with the coliforms, fusiforms, *Bacteroides*, etc. (*Schaedler* et al., 1965). Thus, they may not interfere with the type of interaction modelled in this experiment.

Selective decontamination could lead to a destruction of highly oxygen sensitive anaerobes, even when totally resistant to the antibiotics used. This effect should be larger in patients with reduced mucus production due to epithelial damage than in healthy volunteers. On the other hand, the oxygen uptake by anaerobes need not be unrealistically high (*Gerritse* et al., 1992) to retain a perfectly stable anaerobic flora when all aerobes have been removed. Even in these cases it is likely that the anaerobes become more sensitive to extra stress factors, such as a residual antibiotic resistance.

Since the main supply of oxygen in

the large intestine is the diffusion through the mucosa, oxygen availability should not change with food supply, to a first order approximation. Therefore, if the host is starved or if little or no fibre is contained in the diet, a shift in ecological balance towards a more aerobic flora is expected. This may suggest that an increased risk of intestinal overgrowth by aerobic pathogens during malnutrition can exist, even before the immune system is affected. Similarly, if the mucosa is damaged the diffusion of oxygen may increase, causing an increase in the numbers of aerobes, which in turn may result in more damage. This type of vicious circle may be considered an attractor in dynamical terms. Viewed in this way, an aerobic infection may contain a form of self organisation, the bacteria creating the conditions for their own success. Furthermore, if the epithelium is damaged by irradiation or chemotherapy, both the production of mucus and the oxygen uptake by the epithelium may be impaired. Both effects should contribute to an increase in aerobic bacteria. Both in man and in mice such an increase has been observed after irradiation (*van der Waaij*, 1978).

Concluding remarks

More work, *in vivo, in vitro* and *in silico*, is needed to show whether the tentative conclusions drawn from this pilot study hold up. A more complicated model, taking more microbial and chemical species into account, and the inclusion of receptors on the intestinal epithelium, an immune system, etc., are needed for the *in silico* part of the work. Simultaneously, the data analysis techniques reviewed here should be used to examine data from *in vivo* measurements.

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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.

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