Old Herborn University Seminar Monograph 16:
Host microflora crosstalk.
Editors: Peter J. Heidt, Tore Midtvedt, Volker Rusch, and Dirk van der Waaij.
Herborn Litterae, Herborn-Dill, Germany: 95-102 (2003).

OLD HERBORN UNIVERSITY SEMINAR ON HOST MICROFLORA CROSSTALK

MINUTES AND OVERVIEW OF THE DISCUSSIONS

DIRK VAN DER WAAIJ

Hoge Hereweg 50, 9756 TJ Glimmen, The Netherlands

DISCUSSION PARTICIPANTS (in alphabetical order):
Lars-Göran Axelsson, Stig Bengmark, Vanya E. Grant, Peter J. Heidt, Barbara H. Iglewski, Tore Midtvedt, Stefan Meuerer, Simon Murch, Elisabeth Norin, Alexandr Parlesak, Volker Rusch, Hilde Uvatne, Dirk van der Waaij, Elaine Vaughan, Agnes Wold

Elaine Vaughan: Approaches to investigate the diversity and functionality of intestinal microbes.

Some advantages and disadvantages of various molecular methods for investigating the microflora diversity were discussed. The microbial diversity and community behaviour over time of GI-tract faecal samples has been studied using fingerprinting techniques, such as DGGE or TGGE (denaturing or temperature gradient gel electrophoresis). TGGE/DGGE of 16S rRNA PCR products are especially suited to study diversity in samples with largely unknown microbial content, like the GI-tract, without culturing. The intensity of a band in D-TGGE is a semi-quantitative measure for the relative abundance of this sequence in the population. Bands can be excised from the D/TGGE gels, and advantage of this technique over other fingerprinting methods, and sequenced, and the identity determined by comparison to the databases. D/TGGE can also be done on gut mucosa biopsies. Fluorescent in situ hybridisation (FISH) with image or flow cytometry (rapid) analysis are excellent methods to identify and quantify bacterial groups in microflora without culturing, but do require prior knowledge of the microflora for 16S ribosomal RNA fluorescent probe design.

The question arose as to how the retrieval of mucosal biopsies affects the microflora. (The best material for mucosa-associated microflora comes from patients with abdominal pain who are biopsied in the colon. The best biopsy material for mucosa-associated microflora comes from patients who do not suffer infectious bowel diseases. Very early in the disease of Chronic Infectious Bowel Disease, changes in the histologic appearance and inflammation can be seen. The question was also raised as to how soon after onset of the disease does the microflora change? In man this cannot be studied because the colon needs to be flushed prior to scopy and biopsy. The benefit of scopy however, is the histology. Histologic appearance is the same in all patients while the microflora differs between individuals. Bacteria adhere to specific places (glycocalyx) which differ between individuals. In the crypts there are bacteria, which are perhaps not susceptible to oxygen; the way to isolate them is to make microtome (delete?) sections of the mucosa from the lamina propria up to the villi until the crypts are reached.

Using high throughput genomics methods involving DNA microar-

rays/chips, the diversity as well as the functionality of the microflora may be studied. Besides sequencing genomes of specific microflora commensals, the construction of metagenomic libraries consisting of the microflora DNA allows access to the genetic and functional diversity of the microflora in the absence of culturing. Samples from quite a number of people are required to study the influence of factors such as age and diseases. Thanks to the computer, molecular analyses as described above can be considered these days.

Vanya E. Grant: Flow cytometry: Can it help to analyse complex biosystems?

1. Validation by hybridisation as only a variable percentage is culturable.
2. Why do it?
3. High dimensionally; data handling i.e. an explosion of computer intensive data are generated.
4. Where is the money?

The reason why hybridisation is often difficult is because bacteria in the gut must be "stressed". This is because of lack of nutrients etc. The majority of the crypts are loaded with microbes, which are as yet not determined, even with 10-12 probes. Crypts may form a site for translocation. Gram-staining easily misses crypt colonisation by bacteria.

Questions that can be answered: Percentage of bacteria with intact barrier function that can be identified with probes for prokaryotics is relatively low, because only sick and dead bacteria can pick up the probe. Treatment with "Dibac C4" opens up the barrier. Some fluorescent stains act on Gram-positive bacteria only. This makes differentiation between Gram-positive and Gram-negative fractions in mixtures easy in flow-cytometry.

The 'money' (benefit) is in:

1. well cut biopsies (these should be least artificially changed).
2. allows analysis of many intestinal/faecal samples.

Note: In case bacteria have been exposed to antibiotics such as penicillins, cephalosporins, aminoglycosides etc. they may have become "leaky".

Hilde Ulvatne: Defensins and defensin-like molecules: Antibacterial Mode of Action.

Antimicrobial peptides (AMP) can be classified by structure, and include:

1. α -helices,
2. ß -sheets stabilised by disulphide bridges,
3. extended structures, and
4. loop-structures.

AMP are present in every organism so far investigated, and are located in epithelia, leukocytes, and mucosal secretions. Any organism may possess AMP from more than one structural class of AMP and many AMP within the same group (e.g. frogs have many different AMP of the α-helical group). AMP may act in synergy with each other and with other antimicrobial effector molecules in the host. AMP have different antimicrobial spectra, and some are more efficient against Gram-positive than Gram-negative bacteria, while others again are more efficient against Gram-negative than Gram-positive bacteria. Most AMP are gene-encoded, but some are generated by proteolytic cleavage of a native protein (e.g. lactoferricin and LL-37).

In humans, defensins are produced throughout the GI-tract, including the mouth, epithelia in the oesophagus, the stomach, and the small intestine and in colon. In the human gut, there are at least 9 different defensins (HNP 1- 4 in the neutrophils, HD-5 and 6 in the Paneth cells, HBD-1 in the small intestine and colon, HBD-2 in the gastric mucosa and colon, and HBD-3 in colon)

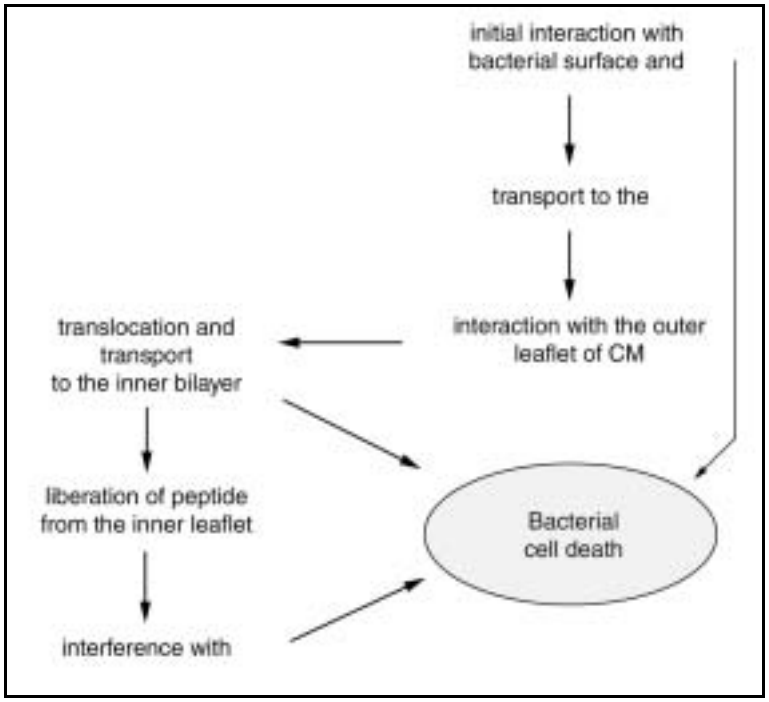

Figure 1: The interactions of antimicrobial peptides and the bacterial cell

The role of defensins in modulation of the intestinal microflora is difficult to elucidate, because of the high variability of bacteria in the intestine. There are also differences in the susceptibility pattern, and no clear-cut correlation between susceptibility and pathogenicity. Furthermore, bacterial proteases may destroy AMPs. Only little research has been done on development of resistance, but resistance mechanisms exist.

Lactoferrin (LF) is a multifunctional, iron-binding protein of 70 kDa. LF is present in secretions like milk and tears, in saliva, semen and colustrum, in addition to being a part of the secondary granules of polymorphonuclear leukocytes. Its strategic appearances on the mucosal surfaces and in leukocytes indicate a role in the primary defence of the organism. It has been shown to exert a broad antimicrobial activity, and the antimicrobial activity is not iron-dependent. Lactoferricin B, a 25 residue cationic antimicrobial peptide, is generated upon gastric pepsin cleavage of bovine lactoferrin.

The modes of action of AMP: Due to the amphipathic, cationic structure of most of these peptides, an effect on the cytoplasmic membrane of susceptible bacteria has traditionally been postulated as the main mode of action. Although an effect on the cytoplasmic membrane has been shown for most peptides, other mechanisms have also been reported, including interference with intracellular processes.

The mode(s) of action of AMP is illustrated in Figure 1. At several points, the interaction between peptide and bacteria may lead to the bacterial cell death, as indicated with arrows.

Lactoferrin B causes only minor leakage from liposomes, no general collapse of membrane integrity (measured by uptake of propidium iodide), minor effect on bacterial respiration

(measured by CTC), but causes concentration dependent depolarisation of the cytoplasmic membrane in *E. coli* (measured by JC-1). However, even though the membrane of *E. coli* gets depolarised, it does not necessarily cause death of these bacteria. This leads to the conclusion that 'membrane-effect' is not responsible for death of *E. coli*. The mode of action of lactoferrin B may be hypothesised as the following:

1. Attachment to bacterial surface.
2. Binding with the cytoplasmic membrane:
   * bacterial stress response?
   * induction of SOS-response?
   * inhibition of growth?
3. Intracellular action:
   * shut down of metabolism?
   * depolarisation?
   * bacterial action = MBC?

Preliminary results indicate that lactoferricin B affects the bacterial protein synthesis. At a concentration of 5 mg/ml suppression is observed, and 10 and 25 μg/ml further increase the effect. These *in vitro* findings do not necessarily reflect what may occur *in vivo* at these concentration levels. Through the bacterial response to defensin attack, bacteria may even be protected against the effect of other antibiotics, like aminoglycosides, which affect protein synthesis.

Lars-Göran Axelsson: Defensins and bacteria, a question of "live or let die?"

The human small intestine responds to bacterial challenge of the small bowel by secreting antimicrobial substances as in the mouse. Specific cells, Paneth cells, are strategically situated at the bottom of the small intestinal crypts and contain granula packed with bioactive substances. Paneth cell granules are degranulated upon stimulation and secrete e.g. lysozyme and phopholipase-2 into the lumen of the small intestinal crypts, together with the secretion of antibacterial defensins.

The importance of antimicrobial substances has been studied in patients having surgical by-pass treatment against morbid obesity. After Roux-en-y Gastric bypass (GBP), a standard surgical procedure for morbid obesity, food and oral-nasal-pharyngeal secretion pass directly into the small bowel without passing through the acid environment of the normal stomach. Postoperatively (3-6 weeks), complying patients underwent gastroscopy with jejunal biopsies using a sterile forceps. Postoperatively Gram-negative cocci, anaerobic bacteria and yeasts were found in a lower number than found in control individuals. The Paneth cell, histochemically, specific phloxine-tartrazine stain showed a striking reduction of stained granula in the postoperative biopsies, a result also obtained with the specific antibody to lysozyme. However, immunohistochemical staining of human defensin-5 showed an upregulation of the antibacterial peptide in granula in the entire lower crypt wall as well in the crypt lumen.

This indicates that the high exposure of the small intestine to environmental bacterial flora after GBS differentially regulates the secretions of antibacterial substances and thus controls bacterial colonisation and prevents deleterious bacterial overgrowth.

Mice having a conventional intestinal microbiota are known to produce at least 17 enteric antimicrobial peptides, i.e. defensins.

Germfree mice can be used to compare innate and classical immune responses to microbes. In the germfree mice, the basic activity of defensins can be studied and by mono-association with a single bacterial strain specific responses can be detected. HPLC fractionation of small intestinal crude extracts has shown that the sterile intestine

of germfree mice contains at least three antibacterial components. A mono-association with *Aeromonas hydrophila* (Bo-3N) produced two additional components. A pre-treatment with cortisone abolished the two first peaks with antibacterial activity, which implies a gene control by NFkB/IkB.

These results points to the role defensins can have in the new-born in controlling the establishment of a functional intestinal bacterial flora and later in protecting against unwanted colonisation of pathogens and deleterious microbes.

Conditions in humans where Paneth cell hyperplasia occurs, e.g. in the stomach, shows that there are genetic or mediator driven control of Paneth cells and its secretions. Lately there has been much interest in the pharmacological regulation or the administration of synthesised defensins to substitute for antibiotics to which microbes has acquired resistance against. Recently several methods to manufacture different antimicrobial peptides have been published.

In the near future there is the important and much promising possibility to induce and regulate or use defensins as new pharmacological entities in the control of infections caused by resistant or multi-resistant microbes.

Defensins might be important in the:

1. Regulation of the process of acquiring an functional healthy intestinal flora in the new-born:
   * are there qualitative differences between individuals,
   * if so, can the defensin production and secretion be up-, respectively down-regulated on demand in certain individuals under pathological conditions.
2. Maintaining a healthy intestinal flora under different environmental conditions:
   * maintaining the balance between commensals.
3. Protection against pathogens:
   * eradicate before critical infectious number of organisms is reached,
   * maintain commensal flora.
4. Treatment of disease:
   * maintain healthy flora vs. allergies,
   * maintain healthy flora vs. arthritis.
5. Treatment of cancer:
   * synthetic drugs.

Agnes Wold: Mucosal immunology.

Hygiene hypothesis by *Strachan* (1989): Microbial exposure reduces the risk of allergy development.

Children with elder siblings have fewer allergies. This could be due to a lower degree of contamination in the first child than in the siblings. Indeed, when "day care" is started early (<2 months) it seems to protect to allergy development. Also growing up on a "life-stock farm" reduces the risk for allergy.

Swedish infants develop IgA later than Pakistan children and children who develop allergy later on have a lower IgA in their saliva compared to infants who become not allergic.

When oral tolerance is induced in conventional mice (and in GF controls that do not become so easily tolerant following the same treatment) and their serum is injected into conventional nude (athymic) mice the latter become tolerant but not following injection of serum from the GF.

Strachan, D.P.: Hay fever, hygiene and household size. BMJ 289, 1259-1260 (1989).

Simon Murch: A link between mucosal regulatory lymphocytes and childhood food allergy.

A hypothesis linking parasites with development of allergy is presented. Low hygienic circumstances involving a high burden of pathogens/adjuvans disbalance the Th1/Th2 ratio. Viruses, bacteria and protozoa may stimulate DC1 cells and stimulate Th1 forming. Helminths and allergies on the other hand stimulate DC2 cells which stimulate Th2 formation.

Multiple Food Allergy:

* Breast-feeding has no preventive effect on allergy.
* Allergies are less severe in patients with enteropathies.
* May be found in subjects with "normal" bowel function (their villi however may be reduced)
* Transient IgA deficiency.
* IgG sub-clones deficiency like IgG2 and IgG4.
* Lymphocyte subset abnormality (low CD8, Low NK cells and CD19 cells).

Ovalbumin sensitisation can induce "allergic bowel dysmobility" in children.

Eotaxin attracts eosinophils, while IL-10 and TGF-β do the opposite as they induce development of suppressor cells.

"Para-cellular leakage" next to Peyer's patches attracts cells to infiltrate the lamina propria. In Gambian children this is seen at the age of one year and they may be dead by their second year. It seems that T-cells are involved but it is uncertain whether these are Suppresser cells. It is at the moment that "flora repair" will be of help to stop the process.

Stig Bengmark: Synbiotic treatment in clinical praxis.

Allergy is also important in human adults. People who grow fat and produce high amounts of volatile fatty acids, TNF and PAI-1. These patients may show Prostate hyperplasia, hypertension, diabetes, arteriosclerosis, hyperinsulinimia, hyperuraemia and obviously obesity. Their risk for development of cancer is increased.

Cows milk that comes from cows that are fed on hay (not fresh grass) may carry:

* trophic hormones,
* bovine growth factor (IGF-1), and
* xeno-estrogens.

According to Swidsinsky, intestinal mucus is normally free of bacteria. In patients with inflammatory bowel disease (IBD) on the other hand, the bacterial concentration in the mucus may be high. This may either be the cause or the result of IBD.

Elisabeth Norin: Phenotypic expressions in the small intestine.

In groups of 5 to 7 mice and rats the MACs were studied under several conditions:

* Fasting overnight.
* Vincristine injection.
* Similac diet.

In the rats, samples were taken at standard (same) places of the intestines. Significant differences were seen in the mitotic index of the crypt cells in the germfree animals. In their caecum a difference in mitotic index (MI) was seen between males and females. The MI was highest in rats following feeding of *Lactobacillus rhamnosis* GG and *Clostridium difficile* (in particular in toxin producing strains on the 3rd day).

In mice, similar results were obtained (young male animals had a higher mitotic index than females). Rats show higher mitotic indexes than mice.

Speculation: The effect of treatment on the MI is immunological, mediated by γδ T cells.

Barbara H. Iglewski: Quorum sensing in *Pseudomonas aeruginosa*.

Quorum sensing is bacterial communication co-ordinated activity of cells involved in the crosstalk. In this respect it is important to know whether bacteria of different species can "talk" with each other.

Cell-Cell signalling:

* Gram-positive bacteria: Post trans-stationally modified peptides are transported out of the cell to act on receptors on the cell membrane (outside the cell). Classic is two components systemic kinase.
* Gram-negative bacteria: Quorum sensing occurs by small molecular cyclates WSL.

AI-1 is an auto-inducer. These small molecules are diffusing (or pumped) out of the cell and may then diffuse back in to bind specific regulatory receptors. AI-2 are structures like antibodies are diffusing (or pumped) out to bind to specific receptors on the cell surface. This results in synthesis of two component kinases. Among AI-molecules much homology exists between different bacteria.

In mice, when the following bacteria are co-cultured in agar beads and put in the mouse lung, *Pseudomonas aeruginosa* produces 30-C^{12}-HSα which can activate has-R in *E. coli* containing HasR+LacB-Gfp.

The question rises whether bacteria can talk with host cells. Polarised lung tissue cells show their normal cillilar movement and other activities. When *P. aeruginosa* is added, the cells will produce AIs.

What type of bacterial behaviour is regulated by crosstalk (=quorum sensing)?

1. Virulence (toxins, exo-enzymes).
2. Invasion (swarming, chemotaxis, proteasis).
3. Antibiotic production (self-defence).
4. Siderophores (β-cepacia) to acquire iron.
5. Antibiotic sensitivity (efflux pumps) regulate siderophores which may act as two-edged sword.
6. Evade host defence (alter membrane proteins).
7. Plasmid transfer (transfers genetic info).

Pseudomonas gene chip has 5769 genes. *In vitro* 433 genes of which 259 genes upregulate and 179 down regulate. Efflux pump transport level in PAO1 effect of nutrients shows that three genes are involved at a very low level of quorum sensing.

Lessons for future experiments:

1. There is a need for three repeats per experimental variable.
2. High quality RNA is essential (free DNA and intact mRNA).
3. Gene 2 experiment in variable responses involving:
   a. growth stage,
   b. media,
   c. +/- O_2.
4. Limitations:
   a. can not see regulation short lived messenger RNA,
   b. can not see regulation if gene is poorly expressed,
   c. genes with multiple regulators pose challenges.
5. Transcript analysis is a good complement to other approaches (to mutagenesis, proteomics, gene transmission etc.

Alexander Parlesak: Developing an *in vitro* model on the investigation of the crosstalk among bacteria, enterocytes and leukocytes near the intestinal mucosa.

Model: It was clearly to be avoided to get a Graft versus Host effect between the monocyte layer underneath a filter, which separated them from the enterocytes. In this model crosstalk was

studied between bacteria and cells of host origin. The costs of testing one compound was high (€ 20,000.-).

Selective stimulation of Caco-2 occurred only in the presence of leukocytes on the basal side caused production of β-actin, IL-8, TNFα, IL-1β and TNF-γ.

Bacteria tested were human isolates:

1. *E. coli* K12 (two concentrations were tested).
2. *Lactobacillus johnsonii* (two concentrations were tested).
3. *Lactobacillus sakei* (two concentrations were tested).
4. LPS (endotoxin).
5. No treatment.

Both *E. coli* K12 and *Lactobacillus sakei* stimulated expression of IL-8, while all bacteria stimulated expression of β-actin.

The process follows three subsequent steps:

Step 1: leukocytes stimulate the bacteria,

Step 2: stimulate leukocytes stimulate Caco-2 cells (enterocytes),

Step 3: enterocytes affect bacteria.