Old Herborn University Seminar Monograph

14. INTESTINAL TRANSLOCATION

EDITORS:

PETER J. HEIDT
PAUL NIEUWENHUIS
VOLKER D. RUSCH
DIRK VAN DER WAAIJ

Old Herborn University Seminar Monograph 14

ISBN 3-923022-25-5
ISSN 1431-6579

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EDITORS:

Peter J. Heidt, Ph.D., B.M.
Department of Animal Science
Biomedical Primate Research Centre (BPRC)
Lange Kleiweg 139
2288 GJ - Rijswijk
The Netherlands

Paul Nieuwenhuis, Ph.D.
Department of Histology and Cell Biology
Section of Immunology
University of Groningen
A. Deusinglaan 1
9713 AV - Groningen
The Netherlands

Volker D. Rusch, Dr. rer. nat.
Institute for Microecology
Kornmarkt 34
D-35745 Herborn-Dill
Germany

Dirk van der Waaij, M.D., Ph.D.
Professor emeritus, University of Groningen
Hoge Hereweg 50
9756 TJ - Glimmen
The Netherlands

Verlag wissenschaftlicher
Schriften und Bücher
Am Kornmarkt 2
Postfach 1664
D-35745 Herborn-Dill
Germany
Telephone: +49 - 2772 - 921100
Telefax: +49 - 2772 - 921101

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Participating authors

J. Wesley Alexander, M.D., Sc.D., University of Cincinnati College of Medicine, Department of Surgery, 231 Bethesda Avenue, Cincinnati, OH 45267-0558, USA.

Stig Bengmark, M.D., Ph.D., Lund University, Ideon Research Park, Schelevägen 18, 22370 Lund, Sweden.

Rodney D. Berg, Ph.D., Department of Microbiology and Immunology, Louisiana State University Medical School, Louisiana State University Health Sciences Center-Shreveport, Shreveport, LA 71130, USA.

Ingeborg Bovee-Oudenhoven, Ph.D., NIZO Food Research, P.O. Box 20, 6710 BA – Ede, The Netherlands.

Mayra Veronica Herías, Ph.D., Department of the Science of Food of Animal Origin, Faculty of Veterinary Medicine, Utrecht University, P.O. Box 80175, 3508 TD - Utrecht, The Netherlands.

Mark A. Jepson, Ph.D., Cell Imaging Facility and the Department of Biochemistry, University of Bristol, Bristol BS8 1TD, United Kingdom.

Paul Nieuwenhuis M.D., Ph.D., Department of Cell Biology, Immunology Section, Faculty of Medical Sciences, University of Groningen, Building 3215, 11th floor, A. Deusinglaan 1, 9713 AV - Groningen, The Netherlands.

Reinhard Pabst, M.D., Ph.D., Department of Functional and Applied Anatomy, Medical School of Hannover, Carl-Neuberg-Straße 1, D-30625 Hannover, Germany.

Gerhard Volkheimer, M.D., Ph.D., Innsbrucker Straße 58, D-10825 Berlin, Germany.

Dirk van der Waaij, M.D., Ph.D., Hoge Hereweg 50, 9756 TJ - Glimmen, The Netherlands

Torkel Wadström, M.D., Ph.D., Department of Medical Microbiology, Dermatology and Infection, Lund University, Sölvegatan 23, S-223 62 Lund, Sweden.

INTESTINAL TRANSLOCATION: INTRODUCTION TO THE TOPIC

PAUL NIEUWENHUIS

Department of Cell Biology, Immunology Section
Faculty of Medical Sciences, University of Groningen,
Groningen, The Netherlands.

INTRODUCTION

At the end of the 13th Old Herborn University Seminar, which dealt with "the Role of Polyspecific Immunoglobulins in the Normal (physiological) Clearance of Micro-organisms", it was decided that the process of intestinal translocation per se (i.e. before any clearing mechanisms may become operative) should receive more attention at the next meeting. The present publication contains the proceedings of papers as presented at the 14th Old Herborn University Seminar devoted to the topic of intestinal translocation.

DEFINITION

Now what is "intestinal translocation"? In 1979, Berg and Garlington defined the term translocation as "the passage of viable bacteria from the gastro-intestinal tract to the mesenteric lymphnodes and possibly other organs"

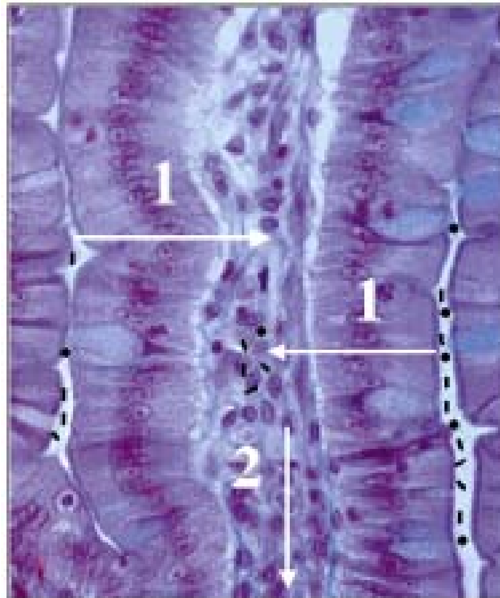

Figure 1: Photomicrograph of intestinal villus showing the two stages of bacterial translocation (arrows 1 and 2 respectively).

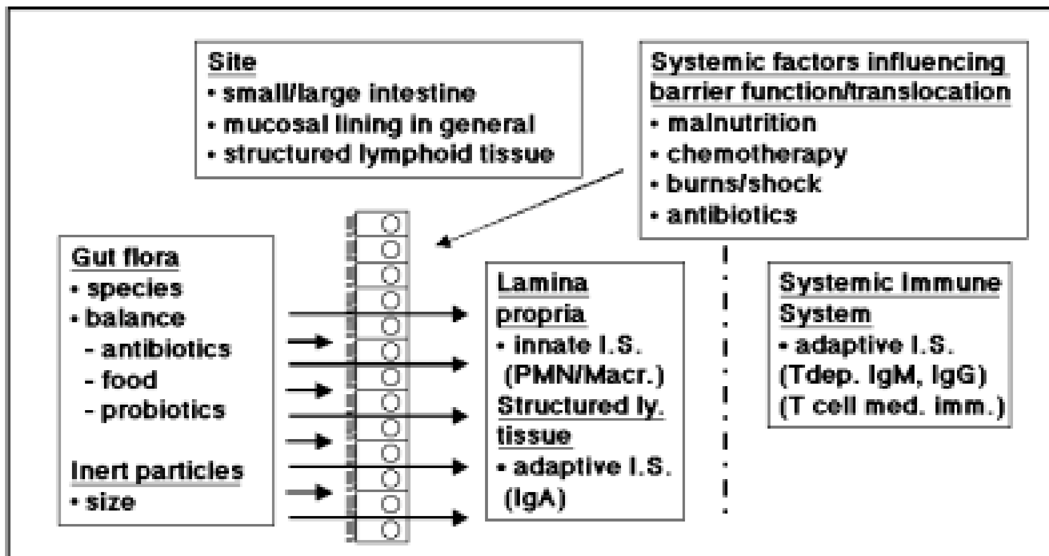

Figure 2: Factors involved in intestinal translocation as discussed in the text.
I.S.: Immune system; PMN: polymorphonuclear cells; Macr.: macrophages; T dep. IgM, IgG: T-cell dependent IgM, resp. IgG production; T-cell med. imm.: T-cell mediated immunity.

(*Berg* and *Garlington*, 1979). In this definition indigenous micro-organisms were explicitly included.

When analysing the process of translocation (Figure 1), several stages may be discriminated. Usually the mucosal lining (epithelial layer) is considered a physical barrier between the milieu extérieur and the milieu intérieur. Mutatis mutandis, the skin serves the same purpose. Crossing this barrier of epithelial cells (and as a result entering the milieu intérieur as such) could be defined as translocation in a more restricted sense (Figure 1, arrow numbered 1). Once having crossed this border (including the basement membrane) a translocating micro-organism is subject to the rules of homeostasis in the milieu intérieur which are quite different to those reigning at the site where it came from, i.e. the gut lumen. These rules now will determine whether the organism will live or die. However, with time during evolution, micro-organisms have developed several strategies to escape these rules, one being intracellular survival. If not eliminated after translocation into the lamina propria, the micro-organisms may travel on either by way of lymph or blood (Figure 1, arrow numbered 2). When the lymphatic route is chosen, the next step will be the mesenteric lymphnodes draining the gut wall (Figure 3). There again chances are that it will be eliminated. Some researchers feel that translocation to MLN is still within the boundary of physiology (as came up during the discussion), whereas others feel that reaching the MLN already is a sign of diminished resistance. However, if a micro-organism manages to get beyond the MLN, i.e. reach the blood stream through the efferent lymphatics draining into the thoracic duct, it is agreed that this is a sure sign of pathology, usually involving clinical manifestations of (serious) infection. From the blood stream in principle all organs of the body can be reached. Organs monitored for translocating micro-organisms usually include spleen, liver, kidney and brain.

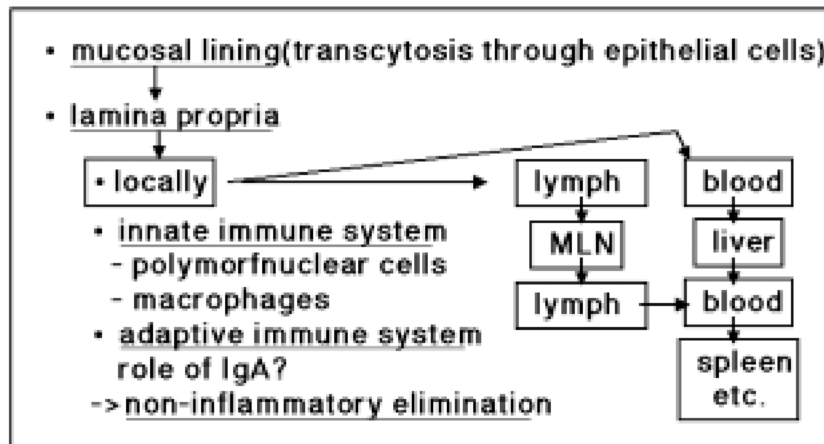

Figure 3: Events occurring after translocation across the mucosal lining in general. Arrows indicate possible routes of translocating micro-organisms.

WHAT "MATERIALS" DO TRANSLOCATE?

In principle any substance reaching the gut lumen by the oral route is eligible for translocation. This ranges from intact (macro) molecules to particles the size of tens of μ-meters. In this way, the definition as given above is extended to include all "particulate" matter crossing the epithelial border. These particles may be live or dead including inert particles like starch, carbon, latex etc. Dr. Volkheimer, and Dr. Hussain contributed papers on this issue.

Live micro-organisms like bacteria have predominantly been found to translocate. As to viruses and parasites, little information is available. In the following, intestinal translocation will be restricted to bacteria and inert particles (see also *Wells* et al., 1988).

FACTORS INVOLVED IN INTESTINAL TRANSLOCATION

Many factors are involved that determine whether a micro-organism will translocate or not and what happens once it has translocated (in a more restricted or in its broadest sense). Figure 2 shows an overview of these factors.

When considering factors involved, several compartments can be distinguished.

First (1) there is the *gut flora* itself. Aspects like species, their respective concentration, their adherence properties, whether they live under aerobic vs. anaerobic conditions and whether they are commensals or (opportunistic) pathogens, all play a role.

Under normal physiological conditions the composition of the gut flora is highly stable (dynamic equilibrium). This equilibrium, however, can be disturbed by the use of antibiotics both orally as well as systemically. Thus colonisation resistance may change resulting in overgrowth of resistant species thus enhancing chances of their translocation. Drs. Wadström, Herías, and Van der Waaij contributed papers related to this part.

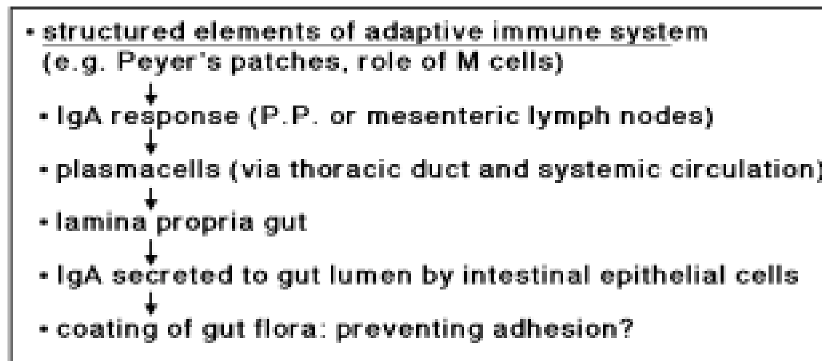

Figure 4: Events occurring after translocation at the level of structured lymphoid tissue. P.P.: Peyer's patches

In addition, the issue of manipulation of the gut flora in a positive way, i.e. to increase resistance to infection, was addressed. Special attention was given to the role of *Lactobacillus* species in this field. In a broader sense the possible beneficial role of prebiotics and probiotics were discussed. Related papers were presented by Drs. Bengmark and Bovée-Oudenhoven.

When preparing this seminar it seemed logical to pay special attention to the issue of translocation of inert particles in comparison with live micro-organisms as a different kind of particulate matter that may occur in the gut lumen. Is there an essential difference or are mechanisms involved virtually the same? (Dr. Volkheimer, Dr. Hussain; see above).

Second (2) there is the *intestinal barrier* itself. In a broad sense, elements of the innate immune system, like polymorphonuclear cells, macrophages and the adaptive immune system (especially IgA) as present in the lamina propria may be considered to be part of this barrier.

Issues like site of translocation i.e. ileum vs. jejunum vs. colon as well as mucosal lining in general vs. the specific M-cells associated with structured lymphoid tissue like Peyer's patches, were worthwhile considering.

Upon crossing the border both effector cells and molecules of the innate immune system (polymorphonuclear cells, macrophages, opsonising lectins, polyspecific IgM (?)) as well as effector cells and molecules of the adaptive immune system (T-cells, IgA) come into play (Figures 3 and 4). Assuming that under steady state conditions, translocation is a normal phenomenon, apparently translocated micro-organisms are eliminated in a non-inflammatory way. This process, however, still remains elusive. A major question here is if and to what extent locally secreted IgA is instrumental: opsonisation leading to non-inflammatory elimination, export of IgA coated bacteria through the epithelial lining back to the gut lumen? Or does IgA "only" play a role in the gut lumen by preventing adhesion? Papers in this area were contributed by Drs. Berg, Jepson, Pabst and -in the discussion- Bos.

Lastly (3) *systemic factors* may determine whether translocation may lead to pathology. In immunocompromised patients, (part of) the defence mechanisms as present in the lamina propria may fail. Malnutrition and chemotherapy may affect these mechanisms but may also affect the quality of the epithelial barrier. In critically ill patients e.g. due to extensive burn wounds, the ini-

tial hypovolaemic shock may lead to local ischaemia in the lamina propria consequently affecting the barrier function. As mentioned above, both oral and systemic administration of antibiotics may disturb the gut flora equilibrium thus facilitating translocation of now dominating species. To this area Drs. Alexander and Feltis contributed papers.

LITERATURE

Berg, R.D. and Garlington, A.W.: Translocation of certain indigenous bacteria from the gastrointestinal tract to the mesenteric lymph nodes and other organs in a gnotobiotic mouse model. Infect. Immunol. 23, 403-411 (1979).

Wells, C.L., Maddaus, M.A., and Simmons, R.L.: Proposed mechanisms for the translocation of intestinal bacteria. Review of Infectious Diseases 10, 958-979 (1988).

THE PHENOMENON OF PERSORPTION: PERSORPTION, DISSEMINATION, AND ELIMINATION OF MICROPARTICLES

GERHARD VOLKHEIMER

Innsbrucker Str. 58, D-10825 Berlin, Germany

SUMMARY

Solid microparticles, whose diameter lies far in the micrometer range (µm), such as pollen, spores, starch-granules, cellulose particles, silicates, crystals, diatoms, soot particles and other natural and industrial dusts are regularly incorporated in a noteworthy quantity in the alimentary tract. Their paracellular translocation through transitory leaks in the epithelial cell layer has been confirmed. Mechanical factors play an important role here: The solidity of the microparticles, the constantly hammering vascular pulsation communicated to the mucosa and the motility of the muscularis propria and muscularis mucosae are causal factors for the loosening of tight junctions and for the appearance of leaks in the epithelial cell layer. The microparticles are transported from the sub-epithelial region through lymph tracts via the thoracic duct but also through veins and disseminated with the blood stream. They are to be found in the peripheral blood already within a few minutes of peroral application.

There are numerous ways in which the microparticles can be eliminated from the blood stream. Their passage into the alveolar lumen, bile, urine, cerebrospinal fluid, peritoneal cavity, through the lactating mamma into the milk and also the transplacental transfer to the foetal blood circulation has been observed. Numerous ready-to-serve foods contain large quantities of solid microparticles capable of persorption.

INTRODUCTION

The enteral uptake of microparticles was observed for the first time in 1844 and subsequently confirmed on several occasions. However, it was not considered credible and little notice was taken of it. The term microparticle designates solid particles of a diameter less than 1/10th of a millimetre. Under the working title of “Persorption”, this phenomenon of the enteral translocation of solid microparticles and the question of where persorbed particles end was investigated in detail between 1959 and 1967 in the laboratories for experimental gastroenterology of the 1st Medical Clinic (Charité) of the Humboldt University, Berlin.

HISTORY

The enteral permeability for microparticles has been known for more than 150 years. *Herbst* gave a starch infusion to a female dog and three hours later found starch-granules in the chyle and in the blood. *Oesterlen* demonstrated some of the charcoal particles fed to chicken and rabbits in their blood: "Above all, I must indeed excuse myself for having thought at all of the possibility of solid, undissolved substances passing from the intestinal mucosa into the blood and of even carrying out experiments on this". *Eberhard* found charcoal particles fed to rabbits in the chyle and in the blood and also sulphur crystals fed to dogs in the chyle. *Donders* and his post-graduate student *Mensonides* found charcoal particles and starch-granules from the food fed to them in the mesenteric blood of the frog and rabbit. *Moleschott* had his assistant *Marfels* feed frogs with pigment particles and sheep erythrocytes, which they were then able to demonstrate in the blood. *Virchow* made the following comment on all this: "There has been much talk recently about the absorption of solid bodies. I cannot understand how this penetration of solid bodies can be called absorption or even resorption. In all these cases, it is a question of a mechanical perforation, a coarse form of permeability, a dissociation by the solid body. Should this be called absorption?". He subsequently considered

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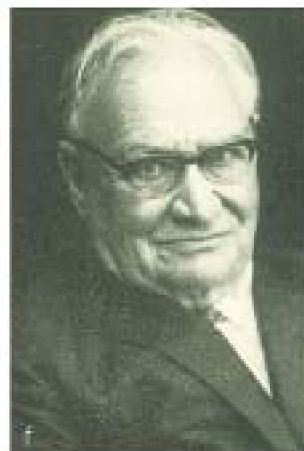
Figure 1:

a. E.F. Gustav HERBST (1803-1893), Göttingen, finds starch-granules in the chyle and blood three hours after administering starch flour to a dog (1844).
b. Franz Cornelis DONDERS (1818-1889), Utrecht, and his post-graduate student MENSONIDES find charcoal particles and starch-granules fed to frogs and rabbits in the mesenteric blood (1846, 1851)
c. Rudolf KOELLIKER (1817-1905) and Rudolf VIRCHOW (1821-1902) in Würzburg 1850. Koelliker's post-graduate student EBERHARD demonstrates charcoal particles fed to rabbits in the chyle and blood. He finds sulphur crystals fed to dogs in the chyle (1847, 1851). VIRCHOW believes that this is due to a mechanical perforation of epithelial layer (1852, 1854).
d. Jakob MOLESCHOTT (1822-1893), Heidelberg and his assistant MARFELS find pigments and sheep cells fed to frogs in the blood of the frogs (1854)
e. Rahel HIRSCH (1870-1953), Berlin finds occasional starch-granules after the ingestion of starch flour in the urine and blood of dogs and, for the first time, also in man (1906). When she reports these results in the "Society of the Charité Physicians", she is greeted with laughter.
f. Fritz VERZÁR (1886-1979), Budapest confirms the results of Rahel HIRSCH on mammals and man (1911). At the Institute for Experimental Gerontology in Basle, he subsequently concerns himself with the cell disintegration of the intestinal epithelium. In 1969, he discusses with VOLKHEIMER the influence of motor factors ("villous pump") on the persorption mechanism in the small intestine.
g. Theodor BRUGSCH (1878-1962), Berlin, Director of the 1st Medical University Clinic of the Charité, formerly the co-assistant of Rahel HIRSCH, recalls in 1956 her studies on the demonstration of starch-granules in urine.
h. Friedrich Horst SCHULZ (1915-1982), Berlin, the successor of Brugsch, generously encourages the studies of the persorption of microparticles taken up again by VOLKHEIMER in 1959 in the Laboratories for Experimental Gastroenterology at his clinic.



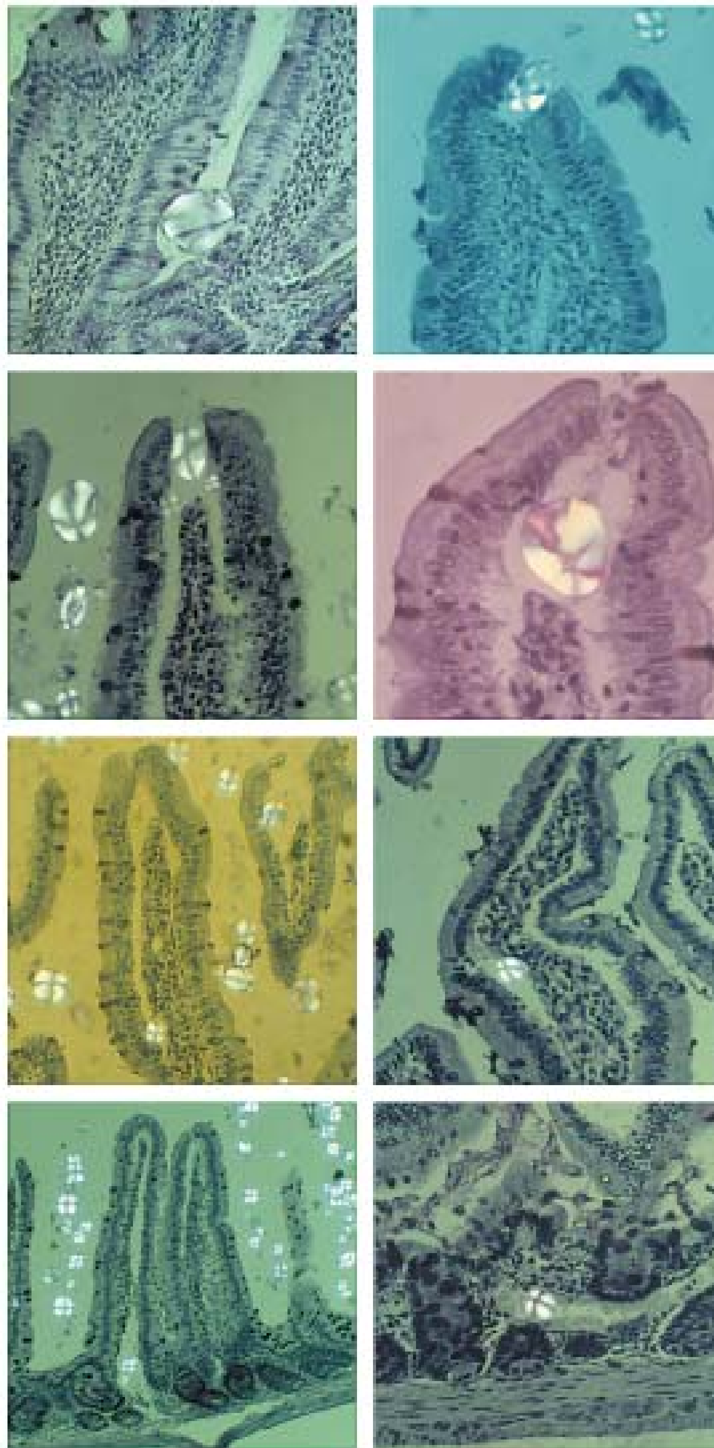


Figure 2: Reconstruction of the persorption process in rats after the ingestion of potato starch. Jejunum and colon. Starch-granules between enterocytes, in the subepithelial region and in the lumen of lymph vessels.

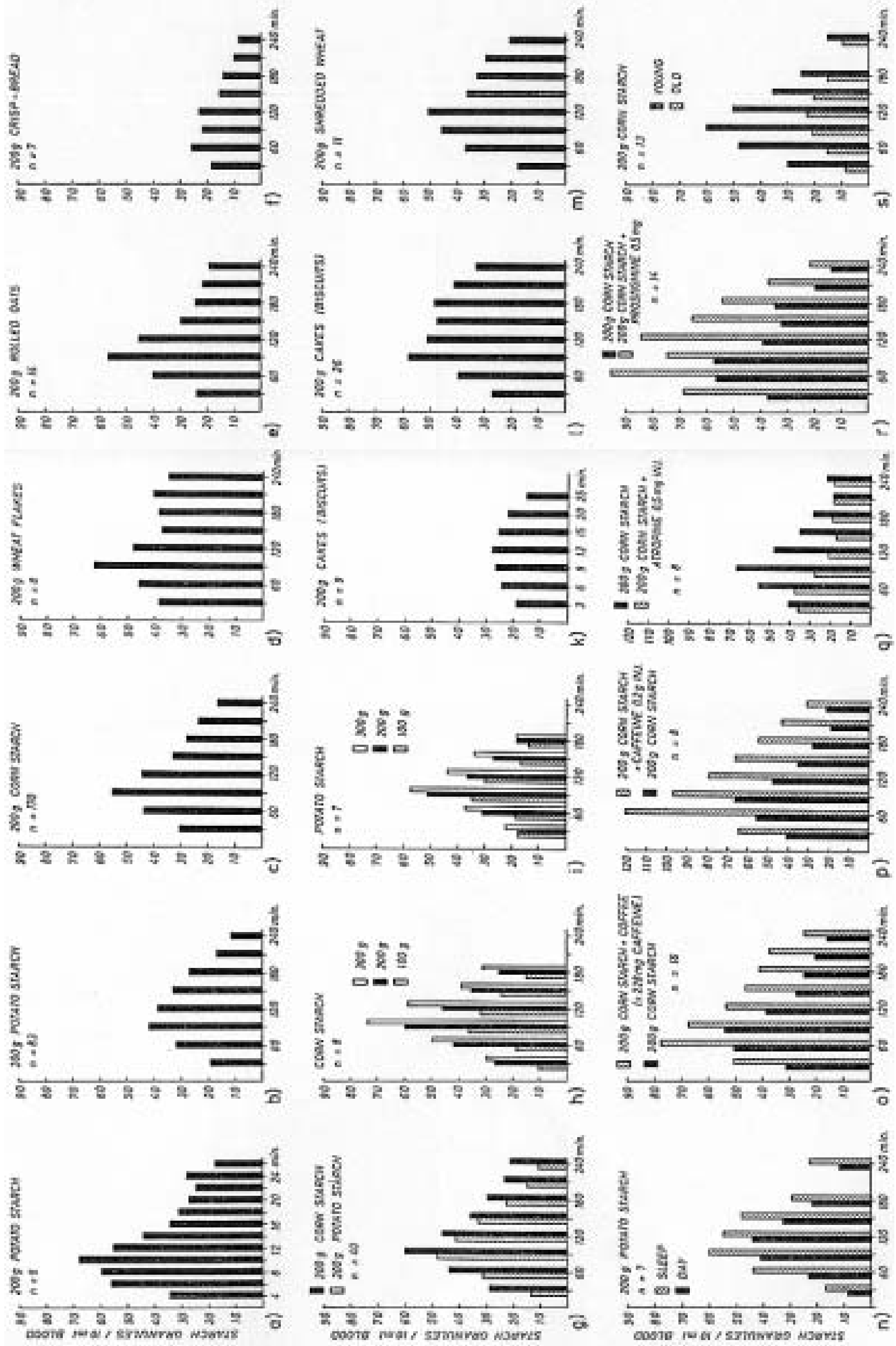
that "the passage of solid parts through the intestinal serosa and into the blood vessels, the so-called resorption of solid parts (*can*) be called at most a perforation of the soft parts".

In 1906, *Rachel Hirsch* administered starch to dogs and volunteers. She found starch-granules of the kind applied in each case in the blood and in the urine. She noted that "The identification of it would appear to be far less difficult than its acknowledgement." *Verzár* checked her results: "I must acknowledge that I was certainly prejudiced and indeed approached this question with the very greatest scepticism. Yes, I admit that I was totally convinced of the impossibility of this assertion. I thought of two possibilities. Either the granules observed were not starch-granules … or, however, …the work had not been carried out with absolute cleanliness and the starch-granules were an impurity, had got into the test-tubes and reactions as dust." After a very careful investigation in which he excluded any possibility of contamination, he considered it, however, "as proved in confirmation of the details given by *R. Hirsch* that starch-granules as such pass from the intestine into the blood stream and from there can be excreted via the kidneys in the urine" (Figure 1).

RESULTS

For the demonstration of the persorption process, starch-granules of a diameter of 5 to 110 µm are very suitable as model bodies and can be given with the food in generous quantities. In histological sections, they can be identified under the polarising microscope. After starch suspensions are fed to vertebrates, occasional starch-granules can be identified histologically between enterocytes. Others lie in the subepithelial region and many in the lymph vessels of mucosa, submucosa and mesenterium. This shows that the penetration mode for solid microparticles is the paracellular passage through the epithelial cell layer. The upper diameter limit for the persorption capacity was determined: In the chyle of mammals after they had been fed with particles of quartz, it was only very seldom that particles greater than 130 µm were found whereas particles of a diameter of up to 70 µm were frequently observed. Using the same method, microparticles such as pollen, spores, plant cells, diatomes, ground wood pulp, cellulose-particles, pulverised crab and lobster shells, lyophilised muscle fragments, PVC particles, iron powder, parasite eggs, hair fragments, asbestos fibres, soot and charcoal-particles, silicates and crystals can be found in the chyle (Figure 2).

This phenomenon was also quantitatively observed in self-experiments with a large team of colleagues and medical students. After native starch has been taken, starch-granules can be demonstrated in the venous blood already 100 seconds later. Their number displays a multi-peak characteristic with peaks at about 10, 100 and 210 minutes after the ingestion of particle suspensions. The persorption rate is dependent on the quantity of particles offered. The motility of the muscularis mucosae, drugs, circadian rhythm, age, caffeine and nicotine influence the absorption rate. Other microparticles of a comparable size such as cellulose particles, pollen and lycopodium spores are also found in the venous blood of volunteers after oral application (Figure 3).



On *elimination*, *degradation* and temporary *deposition*

Several possibilities for the elimination of persorbed microparticles from the circulation of the blood were observed: Microparticles of a size capable of causing embolism are arrested in small vessels. The transfer of embolising microparticles into the alveolar lumen can be histologically demonstrated at alveolar vessels. The elimination in the bile was quantitatively determined; it commences already within a few minutes of oral application. Comparable with this is the elimination in the urine after the embolisation of glomerular vessels. This can likewise be quantitatively determined: After the administration of 200 g starch to volunteers, about 100 starch-granules were excreted within 8 hours with the urine, most of this already in the first 4 hours. When caffeine is administered at the same time, the number of starch-granules found in the urine is almost three times higher whereas there is no significant change in the elimination rate under the influence of diuretics. The elimination through the lactating mamma and into the milk, cerebrospinal fluid and via the placenta into the foetal circulation was likewise quantitatively studied. Individual microparticles capable of embolisation are temporarily retained – even for a long time – and deposited in small blood vessels; in the pulmonary interstitium, they are enclosed and incorporated by multinuclear macrophages. Phagocytosis of persorbed starch-granules was observed in the spleen. A phagocytosis of fragments of starch-granules can be histologically demonstrated in the brain. In further series of tests, the fate of microparticle-induced embolisations in the brain was histologically investigated.

Figure 3: Quantitative studies of the rate of persorption of starch-granules given orally to volunteers (medical students at the Charité, Berlin). The number of granules per 10 ml of venous blood was determined at various time intervals.

a. Within a few minutes following consumption of 200 g of potato starch-granules are found in venous blood. A first maximum is reached after 10 minutes.
b. A second peak in appearance of starch-granules in blood is observed about 90 minutes after ingestion of potato starch.
c. Granules in venous blood upon consumption of 200 g of cornstarch.
d. The persorption rate of starch-granules is relatively high following intake of wheat flakes.
e. Persorption of particles upon consumption of rolled oats.
f. Persorption of particles upon consumption of crisp bread.
g. Administration of 200 g cornstarch and 200 g potato starch and persorption rates in comparison.
h,i. Consumption of various weights of starch and comparison of persorption rates.
k. Starch-granules in venous blood few minutes after ingestion of 200 g biscuit.
l. The persorption rate is relatively high following the consumption of 200 g of biscuit, although this amount contains much less granules than 200 g of pure starch.
m. Persorption of particles upon consumption of shredded wheat.
n. The persorption rate is higher during deep sleep than during day time.
o. Coffee increases the persorption rate significantly.
p,q,r. Simultaneous administration of caffeine or prostigmine increases the rate of persorption. Atropine decreases the persorption rate.
s. Comparison of the persorption rates in young and elderly persons.

DISCUSSION

Particles in the lower nanometer size range can be transcytotically passed through by enterocytes. Larger nanoparticles (up to 3000 nm) can be insorptively taken up by M-cells of the intestinal epithelium (*Sass* et al., 1990) and removed by macrophages.

But also very much larger, solid particles in the *micrometer* range regularly pass from the alimentary tract into the organism. Virchow's assumption of mechanical causes for the kneading of solid particles into and through the epithelial layer was confirmed. Doubts about a paracellular translocation of microparticles through transitory epithelial leaks have been excluded. Persorption of microparticles is possible where single-layer epithelium covers the mucosa of the alimentary tract, i.e., between the cardia and the anus. Apart from factors of cell disintegration, it is the solid microparticles adjoining the mucosa as an 'abutment' and the mechanical forces of vascular pulsation and motility acting on the particles from every direction that are responsible for the loosening of epithelial cell connections. In the small intestine, there is also the rhythmical change between the compression and suction of the villous "pump". When the intestinal motility is influenced pharmacologically, there is also a change in the persorption rate. What qualitative and quantitative part the constantly hammering vascular pulsation communicated to the mucosa has in the loosening of the epithelial cell connections, in the kneading in and by the epithelial cell layer and in their further transportation has not yet been adequately studied. After the paracellular penetration of the epithelial cell layer, the microparticles can be demonstrated in the subepithelial Grünhagen-Mingazzini area that may be optionally filled with variable quantities of tissue fluid from which they are rapidly removed. A participation of macrophages in the penetration and removal is not apparent. For the removal of the microparticles from the subepithelial region, use is made of lymph vessel veins; a size selection for the uptake in lymph or blood vessels is already apparent in this phase. The removal via lymph tracts can be traced histologically; it is also shown by the ample evidence of persorbed microparticles in the chyle of the thoracic duct.

An accumulation of persorbed microparticles in mesenteric lymph nodes was not apparent in the pig. In the mesenteric venous blood of dog intestinal segments filled with microparticles, significantly more particles are found than in arterial blood taken at the same time. This shows that some of the persorbed microparticles are removed by veins. Up to now, it has not been possible to make a more exact quantitative and qualitative determination of the proportions removed via the two routes.

The transfer to the peripheral blood stream always takes place as individual particles: microparticles transported via lymph tracts first of all pass through the thoracic duct to reach the pulmonary vascular system where they can embolise small vessels. They can also be seen there in the alveolar lumen after a short time. However, numerous microparticles, even larger ones, pass the pulmonary circulation with the blood stream. Essentially the same process is seen in the liver but the sinusoidal-cholangiolic translocation mechanisms of this copious elimination into the bile and its rapid onset have not yet been satisfactorily clarified in all its phases.

When the determination of the *persorption rate* is attempted, a surprising observation is that the first particles appear in the peripheral blood already

within a few minutes of ingestion and that a first peak value is reached after about 10-12 minutes. The reasons for the multi-peak curve of the number of particles in the venous blood after the peroral – and also rectal – application of microparticles have not yet been adequately clarified. The occurrence of persorption at the same time on the large areas of the alimentary tract and the rapid onset of the elimination from the blood circulation are to be considered here. In addition, there is the removal at varying rates from the mucosa via lymph tracts and mesenteric veins and other factors that quantitatively cannot be precisely determined with this rapid drift of microparticles into the circulatory blood. There is also the temporary embolisation in smaller blood vessels, distribution, degradation and phagocytosis.

Smaller microparticles, not capable of embolisation, circulate for a longer time in the blood stream than larger particles. Twelve hours after the ingestion of starch suspensions only a few starch-granules can still be demonstrated in the peripheral blood and after 24 hours they have almost completely disappeared. The persorption rate in dogs can be compared more or less with that in man whereas chicken and pigeons display a very much higher rate.

OUTLOOK

The persorption of microparticles is an effect that may be constantly observed in the passage of food through the organism. The embolisation of small vessels by persorbed particles is of interest from the viewpoint of micro-angiology. The long-term deposition of microparticles that are capable of embolisation and consist of potential allergens or contain contaminants is of immunological and toxicological importance. Environmental and industrial medicine is addressed since industrial and natural dusts passing via the nasopharynx to the alimentary tract are persorbed. A noteworthy observation is the passage via the placenta of persorbed microparticles into the foetal circulation. The phenomenon of the persorption of microparticles still requires numerous supplementary studies; the heuristic value has by no means been exhausted as yet.

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COLONISATION AND TRANSLOCATION OF BACTERIA IN THE INTESTINAL TRACT; GENERAL ASPECTS AND STUDIES IN A GNOTOBIOTIC RAT MODEL

MAYRA VERONICA HERÍAS

Department of the Science of Food of Animal Origin, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands.

SUMMARY

Bacterial attachment to surfaces or association to other bacterial species is an important subject for understanding the complex bacterial communities that populate the intestinal tract. Disruption of these ecologically stable communities can lead to harmful effects for the host, i.e. permitting the access of opportunistic or newly arrived pathogens to sterile areas of the body producing disease. Virulence factors present in commensal bacteria can be induced in stress situations and could favour translocation. Different colonisation experiments performed in gnotobiotic rats orally administered with pairs of *Escherichia coli* isogenic strains differing in a selected virulence factor suggest a role of P fimbriae and K5 capsule in intestinal colonisation. These *E. coli* traits however, did not favour translocation to mesenteric lymph nodes or other extra-intestinal organs only indirectly by increasing their bacterial numbers in the gut. Gnotobiologic studies are an excellent tool allowing a systemic approach for the study of bacterial traits in colonisation and translocation and should be encouraged for such purposes.

INTRODUCTION

Bacteria grow or attach to almost any surface. Within minutes of exposure of a solid object into sea or freshwater, the object becomes colonised by adherent organisms and the earliest to attach are bacteria (*Beachey*, 1981). Although marine microbiologists have known for long time that bacteria must stick to avoid being swept away, is not long that it has been recognised that adherence is an important ecological trait in the colonisation of specific sites in plants and animals (*Beachey*, 1981; *Zobell*, 1943). Adherence has been shown to be an important requisite in many bacterial infections (*Gibbons*, 1977; *Jones* et al., 1972; *Salyers* et al., 1994). In contrast, the role of adhesion in normal colonisation of the gut has not been proven, thus associations have been observed (*Edmiston* Jr. et al., 1982). An early study by *Hartley* et al. (1979) suggests that the predominant *E. coli* attach firmly to the mucosa. Thus, no mechanism of association was investigated.

BACTERIAL COLONISATION

Important definitions

Bacteria may associate with an inert surface, mucosal epithelium or mucus gel to be able to resist physical removal by washing or peristalsis without a known or specified interaction. Adhesion implies an interaction between a specified bacterial receptor (adhesin) and a corresponding ligand. Colonisation describes a bacterial population that is stable in size over time, implying that the bacteria multiply at least at the same rate as its physical elimination. Normal microbial flora, or more correctly microbiota, consists of a complex microbial community who colonise body surfaces such as the skin, upper airways, oral cavity and intestinal tract. The composition of this microbial population varies depending on nutrient and oxygen availability (*Tancrède*, 1992), where each species inhabits its specialised niche, which manifests as a selectivity in localisation and preferred substrates for metabolism (*Savage* et al., 1968).

BACTERIAL POPULATION IN THE GASTROINTESTINAL TRACT

The gastro-intestinal tract represents the major reservoir of bacteria in the human body. It is estimated that 400-500 different species reaching a total population level of 10^{14} inhabit the large intestine (*Luckey* et al., 1972; *Moore* et al., 1974). The bacterial populations and numbers vary along the gastro-intestinal tract, where the lowest levels are found in the stomach due to gastric acidity, and the highest concentrations are in the large intestine, where the contents are more static. It is important to mention the terms **transient** and **resident** bacteria, the former refer to bacteria that are isolated once, but who do not permanently colonise the intestine. Their presence is temporary resulting from food products or the environment. Residents, on the other hand, are bacteria that normally colonise or persist for long periods of time (*Savage*, 1999; *Sears* et al., 1949).

Ecological niches of the intestine

There are at least 5 microhabitats that can be inhabited by the bacterial population of the intestinal tract:

1. *The surface of epithelial cells.* They are identified by specific binding, often mediated by special organelles, such as fimbriae and afimbrial adhesins.
2. *Deep layer of the mucus gel of the crypts.* Micro-organisms colonising these sites are generally motile and spiral-shaped (*Borrelia*, *Treponema* and *Spirillum* spp.) (*Lee*, 1985). Active chemotactic directed motility towards the bottom of the crypts allows them to traverse the mucus gel and probably resist removal by the mucus flow (*Freter*, 1992).
3. *Mucus gel covering the epithelium.* Mucus is a viscous gel lubricating and protecting the epithelium. It is a mixture of mucin, water, electrolytes, sloughed epithelial cells, digested food components, exuded plasma and proteins. Some bacterial species have the capacity to degrade mucin molecules, notably, *Bacteroides*, *Bifidobacterium* and *Eubacterium* (*Salyers*, 1995). Released oligo- and monosaccharides may provide nutrients for other members of the microbial flora. Studies have shown that mucus is a good substrate for colonising bacteria and that bacteria associate with the mucus (*Cohen* et al., 1983, 1985; *Costerton* et al., 1983; *Guiot*, 1982).

4. *Intestinal lumen*. In the small intestine, where peristalsis is vigorous, bacteria may not persist in the intestinal lumen. In contrast, the colonic lumen contains large numbers of bacteria. It is however, not clear whether the lumenal bacteria are multiplying, or if they represent daughter cells of the actively replicating mucosa associated populations (*Freter*, 1992), since luminal contents are poor substrates for bacterial growth (*Wadolkowski* et al., 1988). In addition, it has been shown that adhesion of an enterotoxigenic *E. coli* strain to tissue culture cells gave a growth advantage compared to a non-adherent strain due to leakage of nutrients from the epithelial cells (*Zafiri* et al., 1987).

5. *Bacterial biofilms.* Indigenous bacteria form a thick multi layered population especially in areas rich with nutrients (*Freter*, 1981). Adhesion to existing micro-organisms rather than to any epithelial surface may be important in such instances. An example of this can be represented by members of the genus *Actinomyces* that adhere to streptococcal species in the oral cavity. Streptococci in turn, binds to the tooth surface (*Cisar* et al., 1979).

Successful association of bacteria to mucosal sites involves a large number of steps. The process, which has been studied mainly in pathogenic bacteria, may require or at least is facilitated by the presence of **virulence factors.** Virulence factors may simply have evolved to permit persistence in mucosal tissues and virulence may be coincidental. The major steps include:

1. Chemotactive attraction of bacteria to the surface of the mucosal gel, which can be facilitated by the production of motile organelles, such as flagella.
2. Penetration of the mucosal gel, as discussed above, may occur passively but can be enhanced by motility and chemotactive gradients.
3. Adhesion to receptors in mucus or the mucosa-associated layers of indigenous bacteria.
4. Adherence to epithelial surfaces, and finally
5. Multiplication of the mucosa associated bacteria (*Freter*, 1981).

The importance of adherence to mucosal cells in pathogenic bacteria may be summarised as follows:

1. Bacterial attachment protects the bacteria from being swept away (i.e. urinary flow in the urinary tract or by peristalsis in the small intestine).
2. Penetration can proceed after adherence to the tissues.
3. Toxic products can be secreted after cell contact or adhesion of the bacteria

We must not forget however, that pathogenic bacteria must overcome a number of local defences before they are able to attach to the epithelial cells.

COLONISATION RESISTANCE

It has been known for long time that normal microbiota limits the persistence of foreign or newcoming bacterial species. Early studies by *Sears* et al. (1951, 1955) show that ingested *E. coli* strains in human volunteers or dogs cannot displace existing resident ones. This restricting capacity of the indigenous microbiota has been known since long and identified by different names during the years, until a team of investigators conclusively established the term colonisation resistance (*van der Waaij* et al., 1971). It is defined as the resistance to colonisation of the alimentary canal by newly ingested micro-organisms (*van der Waaij*: History of recognition and measurement of colonization

resistance of the digestive tract as an introduction to selective gastro-intestinal decontamination. ISGNAS home page: http://www.isgnas.org/isgnas.htm).
The term is now being recognised by clinicians concerned with the negative effect of antimicrobial therapies on the commensal bacteria (*Donnelly*, 1993). The mechanisms controlling colonisation resistance are not completely understood but probably include: competition for substrate, competition for attachment sites, production of bacteriocins which directly kill or inhibit other bacteria, and production of short chain fatty acids. In addition, indirect effects could include stimulation of intestinal motility and mucosal immunity of the host (as revised in *Herías*, 1998).

BACTERIAL TRANSLOCATION

It is defined as the process by which bacteria cross the intestinal barrier and reach the bloodstream or other extra-intestinal sites such as liver or kidneys (*Berg*, 1983). The passage of endotoxin has also been discussed by *van Leeuwen* et al. (1994). The study of translocation has become increasingly important because is being considered as an initial step in the pathogenesis of sepsis, meningitis or other serious conditions that could eventually lead to multiple organ failure and death (*van Leeuwen* et al., 1994). Various studies agree in that the gut bacteria are a principal source of postoperative sepsis, bacteraemia and meningitis in debilitated patients and in neonates (*Lambert-Zechovsky* et al., 1992; *O'Boyle* et al., 1998; *Sarff* et al., 1975; *Tancrède* et al., 1985). Pathogens like *Salmonella*, *Shigella* and *Listeria* as well as members of the normal microbiota, including *E. coli*, *Klebsiella*, *Proteus*, enterococci, staphylococci and lactobacilli have been shown to have the ability to translocate, while obligate anaerobes (with some exceptions like *Bacteroides fragilis* and *Clostridium perfringens*) do not usually translocate (*Berg* et al., 1979; *O'Boyle* et al., 1998; *Steffen* et al., 1988; *Tancrède,* 1992).

Translocation is also considered as an important process for immune priming (*van Leeuwen* et al., 1994; *Wells* et al., 1988). It has been shown that bacteria that are able to persist in the Peyer's patches stimulate a better immune response than those non-persistent (*Hohmann* et al., 1979). The site for bacterial translocation is still a debating issue, but many studies agree that Peyer's patches, specifically through the M-cells seems one of the most likely sites (*Jones* et al., 1995; *Owen* et al., 1986; *Pappo* et al., 1989, *Wells* et al., 1988). Invasion through epithelial cells or passage through tight junctions are also possible, but have been documented mainly for pathogenic bacteria (*Perdomo* et al., 1994; *Rüssmann* et al., 1996; *Savage*, 1972).

With the data mentioned above, I do believe that translocation can be divided or occurs in two different circumstances:
1. During the process of antigen recognition, as a physiological condition and consequent priming of the immune system. This can be supported by studies of (*Shroff* et al., 1995).
2. During catabolic stress, starvation, turgor pressure, altered temperature, antibiotics and osmolality changes in the host that induce strong signals in bacteria which must then struggle to cope and adapt to the harsh environmental changes. As stated by *Alverdy* et. al.: "Harming the host is not the microbe's intent; its goal is to prevail. Injury to the

host by a microbe struggling to survive is the inadvertent consequence of a threatening environment" (*Alverdy* et al., 1994).

IMPORTANT VIRULENCE FACTORS IN *E. coli* (Figure 1).

Fimbriae mediate attachment to host structures. The word originates from the latin meaning "threads" or "fibres" and was introduced by Duguid in 1955 (*Duguid* et al., 1955). In general, they consist almost entirely of protein, are around 7 nm wide x 0.5-2 μm long rigid helical polymers and are found on Gram-negative bacilli (e.g. enterobacteria), Gram-negative cocci (*Neisseriae*) and in some Gram-positive bacteria such as *Corynebacterium* spp. (*Johnson*, 1991, *Ørskov* et al., 1983). **Type 1 fimbriae** are present in about 80% of the wild-type *E. coli*, and are also found in many other species of the family *Enterobacteriaceae* (*Klemm* et al., 1994). They bind to mannose-containing carbohydrate moieties of various human tissues. The exact role of this fimbriae has not been elucidated, but it has been suggested to be involved in cystitis (*Johnson*, 1991). **P fimbriae** bind to oligosaccharides containing an internal or terminal Galα1-4Galα moiety. It is the most important virulence factor for *E. coli* causing urinary tract infection (*Johnson*, 1991). **S fimbriae** mediate binding to sugar moieties comprising sialic acid in α2-3 or α2-6 linkage to lactose in glycoproteins. They bind to many host structures including laminin and brain microvascular endothelial cells (*Hacker* et al., 1994). It is a main factor associated with neonatal meningitis, being present in around 30% of the isolates (*Korhonen* et al., 1985).

Phase variation is defined as a reversible on-off switch in the expression of a property. The phenotypic expression of fimbriae is affected by the bacteria's surrounding environment (temperature, osmolarity, solid or liquid growth media, etc).

Capsules coat the bacterial cell interfering with O-antigen detection and protecting from host immune defence mechanisms. They consist of linear polymers or repeating carbohydrate sub-units that can also contain a prominent amino acid or lipid component (*Jann* et al., 1990; *Johnson*, 1991). There are around 70-80 known capsular (K) antigens in *E. coli.* **K1** and **K5**

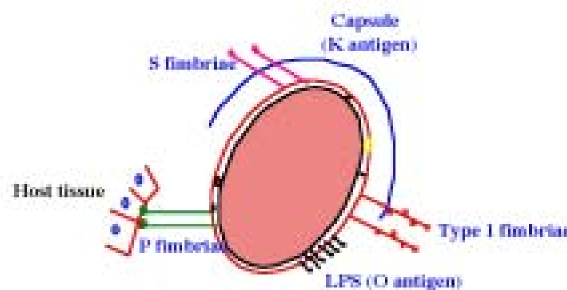

Figure 1: Virulence factors that were studied in *E. coli*. The explanation for each is provided in the text.

Table 1: Identification and characteristics of the bacterial strains used. Each pair includes a different colonisation group and the difference between each one is highlighted in bold letters (Modified from *Herías*, 1998).

E. coli isogenic strains	Serotype	Reference
506 transformants		
506 MS (**Type 1 Fim**+)	O19,22:K1:H-	(*Hagberg* et al., 1983)
506 MR (**Pfim**+)	O19,22:K1:H-	(*Hagberg* et al., 1983)
GR-12 mutants		
742 (**Type 1 fim**+)	O75:K5:H-	(*Svanborg-Edén* et al., 1982)
824 (**Pfim**+)	O75:K5:H-	(*Svanborg-Edén* et al., 1982)
972 (Type 1 +Pfim+)	O-**K5**:H-	(*Svanborg Edén* et al., 1987)
998 (Type 1 +Pfim+)	O-**K-**:H-	(*Svanborg Edén* et al., 1987)
973 (Type 1 +Pfim+)	O75:**K5**:H-	(*Svanborg Edén* et al., 1987)
997 (Type 1 +Pfim+)	O75:**K-**H-	(*Svanborg Edén* et al., 1987)
3034 S-fimbriated mutants		
3034 Sm (**Sfim**+)	O18:K1:H7	(*Pouttu* et al., 1999)
3034-8 (**Sfim-**)	O18:K1:H7	(*Pouttu* et al., 1999)

capsules have been implicated in the majority of extraintestinal infections, because both cross-react with human tissue structures, sialic acid for K1 (*McGuire* et al., 1964) and a precursor of heparin for K5 (*Vann* et al., 1981), which permit them avoid immune recognition.

O antigen is the serologic name given to the lipopolysaccharide (LPS) covering the outer membrane of Gram-negative bacteria. LPS is formed by the O side chains, a core region and lipid A. The antigenic specificity of the O antigen is determined by the composition and linkage of the sugars that form the O side polysaccharide chains (*Hammond* et al., 1984). There are about 164 O antigens typable for *E. coli*, and only a few relative number seem to account for the majority of pathogenic species (*Schiffer* et al., 1976, *Ørskov* et al., 1985).

DO BACTERIAL TRAITS ASSOCIATED WITH VIRULENCE ENHANCE COLONISATION AND/OR TRANSLOCATION IN *E. COLI*? STUDIES IN A GNOTOBIOTIC RAT MODEL.

Pathogenicity can be enhanced by virulence factors, but probably they can also enhance persistence in the intestine as a normal colonisation process or could favour translocation. To study this hypothesis, we colonised germfree rats with *E. coli* strains differing in some recognised virulence factors (*Herías*, et al., 1995; 1997, *Herías* et al., 2001). The approach included **isogenic strains** that are bacterial species that have the same parental origin but differ in the chosen characteristic (*i.e.* capsule or fimbriae). The bacteria were orally administered and allowed to colonise for around 13-15 days.

Importance of type 1 and P fimbriae

Two types of isogenic strains were used to test the ability of type 1 and/or P fimbriae to colonise the intestine of germfree rats (Table 1). The 506 family derived from a human faecal isolate that expressed neither P nor type 1 fimbriae. The strains were transformed with a plasmid conferring either type 1 fimbriae and chloramphenicol resistance (506 MS) or P fimbriae and tetracycline resistance (506 MR) (*Hagberg* et al., 1983). After the colonisation period of two weeks, we observed that the 506 strains were not suitable to test the role of P or type 1 fimbriae for *in vivo* colonisation, because the plasmids enabling the fimbrial expression were lost, and thus, no adhesin advantage could be tested (*Herías* et al., 1995).

The second study included the GR-12 mutants (serotype O75:K5:H-), derived from a pyelonephritic isolate which originally expressed both type 1 and P fimbriae and therefore capable of phase variation. The derivatives used, 742 (expressing type 1 but not P fimbriae) and 824 (expressing P but not type 1 fimbriae), were obtained by chemical mutagenesis (*Svanborg-Edén* et al., 1982). With these strains (see Table 1), both capable of phase variation, it was shown that strain 824 colonised at much higher levels than 742, its type 1-fimbriated counterpart. The difference was highly significant ($p<0.001$), suggesting the advantage of P fimbriae over type 1 fimbriae for persistence in the intestine (*Herías* et al., 1995).

Importance of K5 capsule

The O75:K5:H- family was further manipulated and four mutants were generated differing in the expression of the K5 capsule and the O75 LPS (*Svanborg-Edén* et al., 1987). For the colonisation experiments, we used two different combinations of the strains (see Table 1). In the first colonisation, both strains lacked the O75 antigen, but differed in the expression of the K5 capsule. After colonisation for 11-12 days, the strain expressing K5 (strain 972) reached about 3.8 log higher levels ($p<0.001$) than the K5 negative mutant (strain 998) (*Herías* et al., 1997).

In the second colonisation, the two strains used expressed the O75 antigen, but differed in the K5 expression, where 973 was K5+ and 997 was K5- (See Table 1). After the colonisation period of two weeks, the strain expressing K5 capsule was also established at higher level compared with the K5 negative (1.3 log higher, $p<0.01$). The results were also confirmed by serology (*Herías* et al., 1997).

Importance of S fimbriae

For the study of S fimbriae, we colonised germfree rats of different age groups (adult, infant and neonatal) with two isogenic *E. coli* O18:K1:H7, strain 3034 Sm (which expressed S fimbriae, Sfim+) and 3034-8 (which lacked a functional gene for S fimbriae, Sfim-) (Table 1, (*Pouttu* et al., 1999). *E. coli* reached similar population levels in the colon of all three age groups of rats. The population levels in the small intestine were irregular in all groups, but both neonatal and infant rats had higher levels of *E. coli* than the adult rats (mean 6 vs. 3^{10} log). The Sfim+ and the Sfim- mutants colonised at similar levels in the colon and the small intestine. Thus, no importance of S fimbriae for colonisation was obtained (*Herías* et al., submitted for publication).

Translocation

The dependence of translocation on intestinal bacterial population numbers was also noted in our study, as has been reported before (*Steffen* et al., 1983). Translocation to mesenteric lymph nodes was rarely seen when *E. coli*

colonisation levels were below 1 x 10^8 CFU/g of intestinal contents (see Figure 2). The O75:K5:H- did not usually translocate in high numbers, even if reaching very high colonisation levels. Conversely, the 506 family generally did not reach high levels in the intestinal contents, but translocated in high numbers in the cases when high enough levels were reached in the intestine (*Herías*, 1998).

When comparing isogenic strains, neither K5 capsule, nor P or S fimbriae seemed to influence translocation, other than indirectly by affecting intestinal colonisation levels. In this respect, although all isogenic strains studied had similar translocation capacity, those expressing K5 capsule or P fimbriae by increasing the numbers in the intestine, may indirectly increase translocation (*Herías*, 1998).

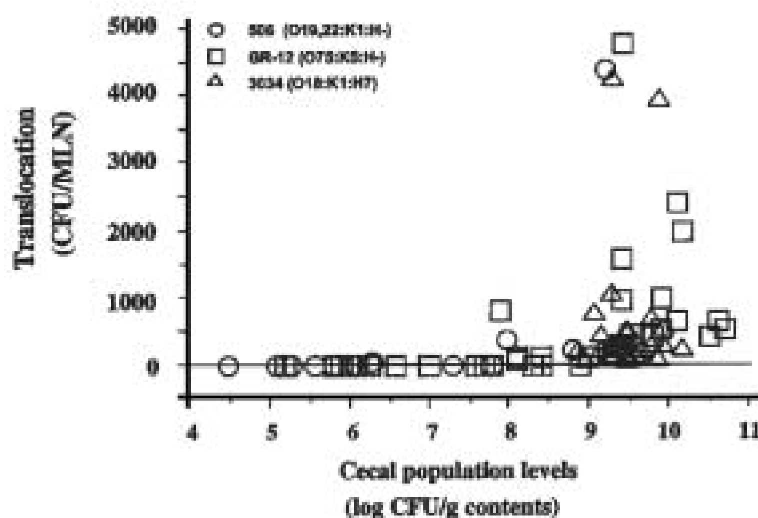

Figure 2: Relationship between bacterial levels in the cecal contents and translocation to mesenteric lymph nodes (MLN). Each symbol represents one rat and one bacterial strain. The different symbols represent the three families of *E. coli* mentioned above. CFU= colony forming units.

CONCLUDING REMARKS

Colonisation of commensal bacteria in the intestine is a complicated process, which includes a serial succession of bacterial species. The bulk of bacteria, which permanently reside in the intestine, include beneficial strains but also potential pathogens. Both bacterial populations live in a balanced ecosystem that when disturbed (by *i.e.* antibiotics or disease) could lead to detrimental consequences to the host.

Bacterial translocation occurs as a normal process for priming the immune system, but it also happens as a consequence of a microbial imbalance. Studies in germfree rats colonised with *E. coli* show that some virulence factors (P fimbriae and K5 capsule) could help for the colonisation process favouring persistence in the intestine. These traits however, did not favour translocation in this model. It will be interesting to determine if factors allowing translocation during a physiological process (if any) are the same allowing potential pathogenic bacteria to invade in stressful or debilitated conditions of the host.

ACKNOWLEDGEMENTS

The gnotobiologic studies were performed as part of my Ph.D. studies in collaboration with Dr. Agnes Wold and Professor Lars Å Hanson at the Department of Clinical Immunology, Göteborg University, and Professor Tore Midtvedt and coworkers at the Department of Microbial Ecology, Karolinksa Institute, Stockholm, Sweden, who provided the germfree animals and its facilities. The work was supported by the Swedish Medical Research Council.

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MECHANISMS PROMOTING BACTERIAL TRANSLOCATION FROM THE GASTROINTESTINAL TRACT

RODNEY D. BERG

Department of Microbiology and Immunology,
Louisiana State University Medical School,
Louisiana State University Health Sciences Center-Shreveport,
Shreveport, USA.

SUMMARY

Bacterial translocation is defined as the passage of viable bacteria from the gastro-intestinal tract to extra-intestinal sites, such as the mesenteric lymph node complex (MLN), liver, spleen, kidney, and bloodstream. Three primary mechanisms promote bacterial translocation from the GI tract: (a) intestinal bacterial overgrowth, (b) immunodeficiencies, and (c) increased intestinal permeability. These mechanisms can act in concert to promote synergistically translocation and the systemic spread of the translocating bacteria to cause lethal sepsis. In animal models of translocation with a normal intestinal epithelium, indigenous bacteria translocate by an intracellular route through the epithelial cells lining the intestines and travel via the lymph to the MLN. In animal models exhibiting increased intestinal permeability or physical damage to the mucosal epithelium, indigenous bacteria translocate intercellularly between epithelial cells to directly access the lymph and blood. The indigenous bacteria found to translocate most readily in animal models also are the bacterial types most commonly causing septicaemia in hospitalised patients. Indigenous GI bacteria also have been cultured directly from the MLN of patients with haemorrhagic shock, bowel cancer, bowel obstruction, Crohn's disease, ulcerative colitis, inflammatory bowel disease, or trauma. Thus, evidence is accumulating that translocation from the GI tract is an important early step in the pathogenesis of opportunistic infections caused by the indigenous GI microflora. Furthermore, bacterial translocation is strongly suspected in the pathogenesis of human septicaemia, acute respiratory death syndrome (ARDS), and multiple organ failure syndrome (MODS). The study of translocation of indigenous bacteria from the GI tract is becoming even more relevant with the dramatic rise in numbers of hospitalised patients with compromised immune systems and/or increased intestinal permeability, such as the elderly, and those with cancer, diabetes, transplants, invasive devices, trauma, or AIDS.

INTRODUCTION

We define bacterial translocation as the passage of viable indigenous bacteria from the gastro-intestinal (GI) tract to extra-intestinal sites, such as the mes-

enteric lymph node complex (MLN), liver, spleen, kidney, and bloodstream (*Berg* and *Garlington*, 1979). Translocation is an appropriate term since it simply describes the relocation of bacteria from one site (intestinal) to another (extra-intestinal) without implying the mechanisms. In the healthy adult host, indigenous bacteria are "spontaneously" translocating across the intestinal epithelial barrier at a low rate (*Berg*, 1980; 1981a). The translocating bacteria are killed, however, by innate host immune defences during transit through the intestinal lamina propria, the draining lymph, and in reticulo-endothelial organs, such as the MLN. Thus, only rarely are viable indigenous GI bacteria cultured from the MLN or other extra-intestinal organs of normal adult rodents with an intact intestinal barrier and a competent immune system (*Berg* 1980).

Indigenous bacteria translocate primarily through the intestinal epithelial cells (intracellular route) rather than between the epithelial cells (intercellular route) in animals exhibiting a normal intestinal epithelium (*Berg*, 1981a; 1985). Thus, the epithelial cells lining the intestinal tract can be considered "non-professional phagocytes" readily taking in any bacterium that comes in close contact. The translocating bacteria pass through the individual epithelial cells and are transported via the lymphatics to the MLN and then spread to other extra-intestinal sites, such as the liver, spleen, peritoneal cavity, and even bloodstream.

Indigenous bacteria translocate intercellularly between intestinal epithelial cells when there is increased intestinal permeability or physical damage to the intestinal mucosa. For example, indigenous bacteria translocate intercellularly or through ulcerations left by denuded epithelial cells in rodents subjected to haemorrhagic shock or endotoxic shock (*Deitch* et al., 1988). In this case, the translocating indigenous bacteria may travel directly to the blood bypassing the MLN.

In 1980, we identified three primary mechanisms promoting bacterial translocation from the GI tract: (a) intestinal bacterial overgrowth, (b) immunodeficiencies, and (c) increased intestinal permeability (*Berg*, 1980). Since that time, no other mechanisms promoting bacterial translocation have been added to this list. Thus, one or more of these three primary promotion mechanisms is operating in all the animal models of translocation examined to date.

INTESTINAL BACTERIAL OVERGROWTH

Intestinal bacterial overgrowth is the most effective of the three primary translocation promoting mechanisms in initiating bacterial translocation from the GI tract to the MLN (*Berg*, 1998). That is, a greater percentage of MLN cultures are positive in animal models exhibiting only intestinal bacterial overgrowth than in animal models exhibiting only immunodeficiency or only increased intestinal permeability. Intestinal bacterial overgrowth promotes bacterial translocation in the animal models listed in Table 1.

Bacterial translocation is directly promoted by an increase in GI population levels. That is, there is a "cause and effect" relationship between the GI population level of a particular bacterial species and its translocation from the GI tract. For example, *E. coli* attains caecal populations of 10^{9-10}/g in *E. coli*-monoassociated gnotobiotic mice (ex-germ-free), and 100% of the MLN are positive for translocating *E. coli* (*Berg* and *Owens*, 1979). If these *E. coli*-monoassociated mice are then inoculated with

Table 1: Promotion of bacterial translocation by intestinal bacterial overgrowth.

- Oral antibiotics
- Associated gnotobiotic (ex-germfree) mice
- Endotoxic shock
- Zymosan shock
- Starvation
- Protein malnutrition
- Parenteral (i.v.) alimentation
- Enteral (oral) alimentation
- Bowel obstruction
- Bile duct ligation
- Streptozotocin-induced diabetes

an indigenous microflora (i.e. the whole caecal contents from conventional mice), the *E. coli* caecal population decreases 1000-fold and *E. coli* no longer translocates to the MLN. If the obligate anaerobes of the indigenous microflora antagonistic to *E. coli* are removed by antibiotic treatment, *E. coli* again increases in intestinal population levels and readily translocates from the GI tract to the MLN.

This relationship is even more dramatically demonstrated by comparing caecal populations of three different species of *Enterobacteriaceae* in tri-associated gnotobiotic mice with their translocation rates to the MLN. Germfree mice were tri-associated with indigenous *E. coli*, *Klebsiella pneumoniae*, and *Proteus mirabilis* and the proportion of each of the three bacterial species in the caecum compared to the proportion of each of the three bacterial species translocating to the MLN (*Steffen* and *Berg*, 1983). Thus, *P. mirabilis* comprised 10% of the caecal bacteria and 14% of the bacteria translocating to the MLN, *K. pneumoniae* comprised 66% of the caecal bacteria and 54% of the MLN bacteria, and *E. coli* comprised 25% of the caecal bacteria and 32% of the MLN bacteria. Thus, the proportion of each of the three bacterial species in the caecum is statistically similar to the proportion of each of the three bacterial species in the MLN, demonstrating again a direct relationship between intestinal bacterial overgrowth and bacterial translocation.

In the intestinal bacterial overgrowth model, the translocating indigenous bacteria do not establish an "infection" in the MLN. That is, the indigenous bacteria are continuously translocating from the GI tract to the MLN rather than multiplying within the MLN. For example, the numbers of *indigenous E. coli* translocating to the MLN of *E. coli*-mono-associated gnotobiotic mice is similar after either 2 days of mono-association or 100 days of mono-association (*Berg* and *Owens*, 1979). *E. coli* maintains constant level of $10^{9\text{-}10}$/g caecum throughout the entire 100-day test period. If the *E. coli*-mono-associated gnotobiotes are treated orally with a non-absorbable antibiotic to remove *E. coli* from the GI tract, MLN cultures become negative within 24 hours for translocating *E. coli*. These results suggest translocating *E. coli* do not establish in the MLN but rather continuously seed the MLN from the GI tract.

The intestinal bacterial overgrowth model has several advantages over other translocation models. As already mentioned, intestinal bacterial overgrowth is more efficient in promoting initial bacte-

rial translocation from the GI tract than either immunodeficiency or increased intestinal permeability. Most importantly, promotion of bacterial translocation in the intestinal overgrowth model is due directly to bacterial overgrowth and not confounded by other variables, such as increased intestinal permeability. Another advantage of the intestinal overgrowth model is that bacterial challenge is by the "natural" oral route rather than by "artificial" intravenous or intraperitoneal injection as is often the case in bacterial pathogenesis studies. Bacterial translocation to the MLN and other organs also is extremely sensitive. Theoretically, a single translocating bacterium will produce a positive MLN culture. In fact, culture of the MLN for viable translocating bacteria has proved more sensitive in detecting bacterial translocation than tests employing fluorescein-labelled or even radiolabelled bacteria (*Alexander* et al., 1990).

The various species of indigenous bacteria do not all translocate at the same rate. The Gram-negative, facultatively anaerobic *Enterobacteriaceae*, such *E. coli*, *K. pneumoniae*, and *P. mirabilis*, translocate most readily from the GI tract (*Steffen* et al., 1988). The Gram-positive, oxygen-tolerant bacteria, such as *Staphylococcus epidermidis*, translocate at an intermediate rate. Surprisingly, the strict anaerobes, such as *Bacteroides*, *Clostridium*, and *Fusobacterium*, translocate at the lowest rate even though they normally maintain very high GI population levels (10^{10-11}/g caecum). There is some evidence that oxygen sensitivity may be a limiting factor in the translocation of strictly anaerobic bacteria (*Berg* and *Itoh*, 1986).

Intestinal bacterial overgrowth is not just a laboratory phenomenon, but occurs readily in a variety of patients including those receiving antibiotic therapy. Patients treated with antibiotics often exhibit intestinal overgrowth by antibiotic-resistant indigenous bacteria or are colonised by more pathogenic, antibiotic-resistant, exogenous bacteria. This clinical situation is readily produced in rodent models given oral antibiotics (*Berg*, 1981b). Parenteral alimentation, enteral alimentation, protein malnutrition, starvation, bowel obstruction, diabetes, and endotoxic shock also induce intestinal bacterial overgrowth and subsequently promote translocation (*Berg*, 1983b; 1985; 1992a; 1992b). Interestingly, the bacterial types that translocate the most readily from the GI tract following intestinal bacterial overgrowth, namely the *Enterobacteriaceae*, also are the bacterial types that most commonly cause septicaemia in hospitalised patients, with *E. coli* being the most prominent (*Donnenberg* et al., 1994).

IMMUNODEFICIENCY

Translocating bacteria are cultured from the MLN and other extra-intestinal sites only if they survive transit through the intestinal lamina propria and are not killed by immune mechanisms in the lymph or other extra-intestinal sites. If the translocating bacteria are killed in route, cultures of the MLN and other organs will be negative and bacterial translocation will not have taken place even though the bacteria crossed the intestinal epithelial barrier. Host immune defences are therefore integral components in the dynamics of bacterial translocation.

Table 2 lists animal models in which deficiencies in host immune defences allow bacterial translocation. The injection of mice with immunosuppressive agents, such as cyclophosphamide,

Table 2: Promotion of bacterial translocation by immunodeficiency

- Injection of immunosuppressive agents
- Genetically athymic (*nu/nu*) mice
- Beige/nude (*bg/bg;nu/nu*) mice
- Thymectomised (*nu*/+) mice
- T-cell-depleted (anti-CD4/anti-CD8) mice
- Lymphoma
- Leukaemia
- Streptozotocin-induced diabetes
- Thermal injury
- Endotoxic shock
- Haemorrhagic shock

prednisolone, methotrexate, 5-fluorouracil, or cytosine arabinoside, readily promotes translocation of indigenous bacteria from the GI tract to the MLN, spleen, liver, and kidneys (*Berg*, 1983a). Bacterial translocation due to immunodeficiency also occurs in animal models of diabetes (*Berg*, 1985), leukaemia (*Penn* et al., 1986), endotoxaemia (*Deitch* and *Berg*, 1987), thermal injury (*Maejima* et al., 1984), and haemorrhagic shock (*Baker* et al., 1987). Likely all components of the host immune system, such as systemic immunity (serum immunoglobulins), mucosal immunity (secretory IgA), and cell-mediated immunity (T-cells and macrophages) are involved in immune defence against bacterial translocation. Very little research has been conducted to date, however, to delineate the relative roles of these immune compartments or to identify the immuno-effector mechanisms responsible for killing the translocating bacteria.

Serum immunoglobulins act as opsonins to increase the effectiveness of phagocytosis and killing of bacteria by macrophages and polymorphonuculear leukocytes. Surprisingly, i.v. or i.p. injection with anti-*E. coli* antibodies do not decrease the numbers of *E. coli* translocating from the GI tract to the MLN (*Gautreaux* et al., 1990). Anti-*E. coli* antibodies, however, reduce the spread of the translocating E. coli from the MLN to the spleen, liver, or kidney. Thus, serum antibodies appear to be more effective in reducing the spread of translocating bacteria that have already penetrated the intestinal barrier than inhibiting the initial translocation of bacteria across the intestinal mucosa to the MLN. Nonetheless, systemic immunity has not been studied sufficiently to draw any firm conclusions concerning its relative role in translocation defence.

It seems likely that specific anti-bacterial secretory IgA on intestinal mucosal surfaces would be a major factor in the defence against translocation. Indigenous bacteria must come in close contact with the intestinal epithelial cells prior to their translocation across the intestinal mucosa and secretory IgA is known to inhibit bacterial adherence to mucosal surfaces. For example, specific anti-bacterial secretory IgA inhibits association with the intestinal epithelium of certain pathogenic bacteria, such as *Vibrio cholerae* and *Salmonella typhimurium* (*Winner* et al., 1991; *Michetti* et al., 1992). However, secretory IgA has not been demonstrated to decrease association of indigenous bacteria with the intestinal epithelium nor is there any information concerning the importance of secretory IgA in defence against indigenous bacterial translocation.

Thymectomy of neonatal mice pro-

motes the translocation of indigenous bacteria from the GI tract to 46% of MLN, spleen, liver, and kidneys compared with only 5% positive organs in control sham-thymectomised mice (*Owens* and *Berg*, 1982). Athymic (nu/nu) mice exhibit "spontaneous" translocation of aerobic, facultative, and obligately anaerobic bacteria to 50% of the MLN, spleen, liver, and kidneys compared with 5% positive organs in control euthymic (nu/+) mice (*Owens* and *Berg*, 1980). Since IgA development is T-cell-dependent, athymic mice lack intestinal secretory IgA. Neonatal thymuses grafted from heterozygous donor (nu/+) mice to homozygous (nu/nu) recipients decrease translocation in the recipients from 58% to 8% of these organs. These results suggest T-cell-mediated immunity contributes to host defence against translocation and is especially effective in preventing the spread of translocating bacteria from the MLN to other extra-intestinal sites.

The importance of T-cells in host defence against translocation is confirmed in mice depleted of T-cells to promote bacterial translocation and then adoptively transferred with T-cells to inhibit translocation. Thymectomised mice depleted of CD4+ and/or CD8+ T-cells via injections of specific anti-T-cell monoclonal antibodies exhibit increased bacterial translocation (*Gautreaux* et al., 1993). As demonstrated by flow cytometry, the T-cell depletion regimen depletes 100% of the CD4+ and CD8+ T-cells in the spleen, MLN, intestinal lamina propria, and intestinal epithelium. CD4+ and/or CD8+ T-cells harvested from donor mice and adoptively transferred to the T-cell-depleted mice inhibit bacterial translocation (*Gautreaux* et al., 1995). The adoptive T-cells (Thy-1.1+) migrated to the sites of interest, namely the MLN and intestinal lamina propria, in the Thy-1.2+ recipients.

The fact that either CD4+ or CD8+ adoptively-transferred T-cells reduces bacterial translocation suggests an effector function common to both subsets of T-cells. Direct cytotoxicity by T-cells as a defence against bacterial translocation is unlikely because both CD4+ and CD8+ T-cells kill only MHC-restricted targets. Phagocytic cells are likely the ultimate immune effector cells in the defence against bacterial translocation. Consequently, it is possible that the translocating bacteria are engulfed by MHC I and II-expressing macrophages. In this case, both CD4+ and CD8+ T-cell subsets would provide protective function. CD4+ and CD8+ T-cells also secrete gamma interferon and granulocyte/macrophage colony-stimulating factor, both of which activate phagocytic cells.

Translocating bacteria are always cultured from the MLN prior to their appearance in organs, such as the liver, spleen, kidney, or bloodstream (*Berg*, 1992c; 1995). Thus, resident macrophages in the MLN are strategically located along the translocation route from the GI tract. It is not surprising, therefore, that non-specific immunostimulation of macrophages by vaccination with formalin-killed *Propionibacterium acnes* (formerly classified as *Corynebacterium parvum*) inhibits translocation of indigenous bacteria to the MLN (*Fuller* and *Berg*, 1985).

P. acnes vaccination induces splenomegaly, a lymphoreticular response commonly reported to indicate macrophage activation. Furthermore, plastic-adherent spleen or MLN cells (predominantly macrophages) from *P. acnes*-vaccinated mice adoptively transferred to non-vaccinated recipients inhibit bacterial translocation whereas non-adherent control cells (predominantly lymphocytes) do not. (*Gautreaux* et al., 1990). These results suggest macrophages are important effector cells

Table 3: Promotion of bacterial translocation by increased intestinal permeability

• Ricinoleic acid (castor oil)
• Endotoxic shock
• Zymosan (yeast polysaccharide) shock
• Thermal injury
• Haemorrhagic shock

in the host defence against bacterial translocation. Non-specific activation of macrophages and polymorphonuclear leukocytes to more efficiently engulf and kill a variety of bacterial types would be a particularly effective defensive measure since it cannot be predicted with certainty which of the 400-500 indigenous bacterial species in the GI tract will translocate under particular clinical conditions.

Unexpectedly, *P. acnes* vaccination does not reduce *E. coli* translocation in gnotobiotic (ex-germfree) mice mono-associated with *E. coli* (*Fuller* and *Berg*, 1985). This is despite the fact that the gnotobiotic mice exhibit marked splenomegaly indicating immunologic stimulation. The only difference between the germfree and conventional mice is the presence of an indigenous microflora in conventional mice. Consequently, the indigenous microflora appears to "prime" the immune system of conventional mice so that subsequent *P. acnes* vaccination is effective in inhibiting *E. coli* translocation.

To test this hypothesis, adult germfree mice were colonised for 8 weeks with the whole caecal microflora from conventional mice prior to *P. acnes* vaccination (*Berg* and *Itoh*, 1986). The mice then were decontaminated with oral antibiotics to remove the indigenous microflora, mono-associated with *E. coli*, vaccinated with killed *P. acnes*, and tested for inhibition of *E. coli* translocation. Surprisingly, *P. acnes* vaccination did not reduce *E. coli* translocation to the MLN in these adult gnotobiotes exposed to the entire indigenous GI microflora for 8 weeks. Further studies then revealed that the germfree mice must be colonised with the indigenous microflora within 1 week after birth in order for *P. acnes* vaccination at 8 weeks of age to inhibit *E. coli* translocation (*Berg* and *Itoh*, 1986). These results are another dramatic demonstration of the profound influence of the indigenous GI microflora on the immunologic development of the host (*Berg*, 1983b).

Activated macrophages and/or polymorphonuclear leukocytes appear to promote rather than inhibit bacterial translocation in mice with inflamed abdominal abscesses (*Wells* et al., 1987). It is suspected that polymorphonuclear leukocytes engulf the indigenous GI bacteria and carry them to the abdominal abscess. In this case, macrophages and polymorphonuclear leukocytes promote rather than defend against bacterial translocation.

Bacterial translocation from the GI tract to the MLN and liver is neither decreased nor increased in op/op mice genetically deficient in CSF-1-dependent macrophage populations (*Feltis* et al., 1994). Only a few op/op mice were tested, however, so it is difficult to draw firm conclusions from these experiments. Nonetheless, it seems likely that macrophages are important effector cells in the defence against translocation. More study is required to determine the exact role of macrophages in translocation defence and to determine if macrophages promote translocation under certain circumstances.

INCREASED INTESTINAL PERMEABILITY.

The intact intestinal mucosa provides a physical barrier preventing bacteria colonising the GI tract from translocating to extra-intestinal sites. Translocation of indigenous GI bacteria readily occurs, however, when the intestinal barrier is compromised as in the animal models presented in Table 3. Chemical agents can damage the mucosal barrier and promote bacterial translocation. For example, ricinoleic acid (12-hydroxy-9-octadecenoic acid), the pharmacologically active constituent of castor oil, when given to mice once intragastrically severely damages the intestinal mucosa and readily promotes the translocation of indigenous GI bacteria (*Morehouse* et al., 1986). Massive exfoliation of columnar epithelial cells in the proximal small intestine occurs within 2 hrs after the ricinoleic acid administration. Both facultatively anaerobic and strictly anaerobic bacteria translocate to the MLN, spleen, and liver in the ricinoleic acid-treated mice. The incidence of bacterial translocation is greatest at 4 days following the ricinoleic acid administration and ceases completely by 7 days when the damaged mucosal epithelium has regenerated.

Shock with the accompanying ischaemia/reperfusion injury to the intestinal mucosa also readily promotes bacterial translocation from the GI tract. For example, mice injected once i.p. with *E. coli* O26:B6 endotoxin exhibit ischaemia/reperfusion injury and concomitant translocation of indigenous bacteria (*Deitch* and *Berg*, 1987). To determine which components of the endotoxin structure are involved in promoting bacterial translocation, mice were injected with endotoxin from six R-mutant strains of *Salmonella* all differing in their endotoxin composition (i.e. Ra, Rb, Rc, Rd, Re, or lipid A) (*Deitch* et al., 1989). Injection of intact *Salmonella* endotoxin (wild type), the Ra endotoxin fragment, or the Rb endotoxin fragment increased bacterial translocation to the MLN. Injection of the smaller endotoxin fragments, Rc, Rd, Re, or lipid A alone, did not promote bacterial translocation. Apparently, endotoxin must contain the terminal three sugars of the core polysaccharide in order to induce sufficient mucosal damage to promote translocation.

Both endotoxin and lipid A produce their toxic manifestations by stimulating host cells, especially macrophages, to release mediator substances that then act as second messengers to disrupt various homeostatic systems. For example, oxygen-free radicals generated by xanthine oxidase are released during ischaemia/reperfusion (*Parks* et al., 1982). Endotoxin-challenged mice exhibit intestinal oedema and separation of the epithelium from the lamina propria. Injection of the translocation-promoting Ra endotoxin fragment increases ileal xanthine oxidase and xanthine dehydrogenase activities, whereas injection of the non-promoting Rc or Re endotoxin fragments does not (*Deitch* et al., 1989b). Consequently, intestinal damage and subsequent bacterial translocation are reduced when animals are pretreated with allopurinol (xanthine oxidase inhibitor), dimethylsulphoxide (hydroxyl scavenger), or deferoxamine (iron chelator) (*Deitch* et al., 1988; 1989). Intestinal mucosal damage and bacterial translocation also are inhibited in animals fed for 2 weeks prior to endotoxin challenge a tungsten-supplemented molybdenum-free diet to deplete intestinal xanthine oxidase (*Deitch* et al., 1989). Thus, xanthine-generated hydroxyl radicals are responsible, in part at least, for the intestinal mucosal damage and increased bacterial translocation following endotoxic shock.

Haemorrhagic shock and the shock of thermal injury also induce intestinal ischaemia/reperfusion injury and promote bacterial translocation (*Baker* et al., 1987; 1988; *Maejima* et al., 1984). For example, rats submitted to haemorrhagic shock for 90 minutes exhibit ileal mucosa necrosis and increased bacterial translocation. The mucosal damage occurring in haemorrhagic shock and thermal injury also is due to oxidants generated by xanthine oxidase (*Deitch* et al., 1988; 1989). As with endotoxic shock, pre-treatment with allopurinol or maintenance on a tungsten-supplemented molybdenum-free diet ameliorates the damage due to xanthine oxidase and lessens bacterial translocation (*Deitch* et al., 1988; 1989).

Large populations of Gram-negative indigenous bacteria are normally present in the GI tract and endotoxin is continuously released during their cell growth and, particularly, during cell death and lysis. Fortunately, endotoxin is not readily absorbed from the GI tract. In certain clinical situations where intestinal permeability is increased, however, there is increased bacterial translocation and endotoxin can be detected in the lymph and portal and systemic circulations. In these severely ill patients, bacteria and endotoxin readily cross the mucosal barrier to gain access to extra-intestinal tissues and the bloodstream. The translocated endotoxin activates plasma protein cascades, resident macrophages, and circulating neutrophils to release monokines and proteins that, in turn, further increase gut mucosal permeability. Thus, a cycle is initiated with increased translocation of both bacteria and endotoxin and increased damage to the intestinal barrier. In fact, it is hypothesised that failure of the intestinal barrier in conjunction with hepatic dysfunction promotes or potentiates multiple organ failure syndrome, a newly recognised syndrome leading to death in a variety of patients (*Carrico* et al., 1986). Thus, bacterial translocation from the GI tract may be an early step in the pathogenesis of acute respiratory death syndrome (ARDS) and multiple organ failure syndrome.

CONCLUSIONS

In complex animal models of translocation, such as endotoxic shock, haemorrhagic shock, thermal injury, and streptozotocin-induced diabetes, multiple mechanisms operate to promote bacterial translocation (*Berg*, 1996; 1998). In fact, in some models these promotion mechanisms act synergistically. This is easily demonstrated in mice given the combination of an oral antibiotic (e.g. penicillin) plus an immunosuppressive agent (e.g. prednisolone) (*Berg* et al., 1988). Oral penicillin disrupts the GI ecology allowing intestinal bacterial overgrowth by indigenous *Enterobacteriaceae* resistant to penicillin and thereby promotes *Enterobacteriaceae* translocation. The translocating Enterobacteriaceae usually do not spread beyond the MLN to other extra-intestinal sites in these antibiotic-treated mice. Prednisolone given alone also promotes *Enterobacteriaceae* translocation to the MLN and, furthermore, allows spread of the translocating bacteria from the MLN to other organs, such as the spleen, liver, and kidney. The combination of the antibiotic plus the immunosuppressive agent is even more effective and synergistically promotes *Enterobacteriaceae* translocation. Thus, mice given the combination of penicillin plus prednisolone die within 2 weeks of lethal sepsis caused by the translocating indigenous bacteria.

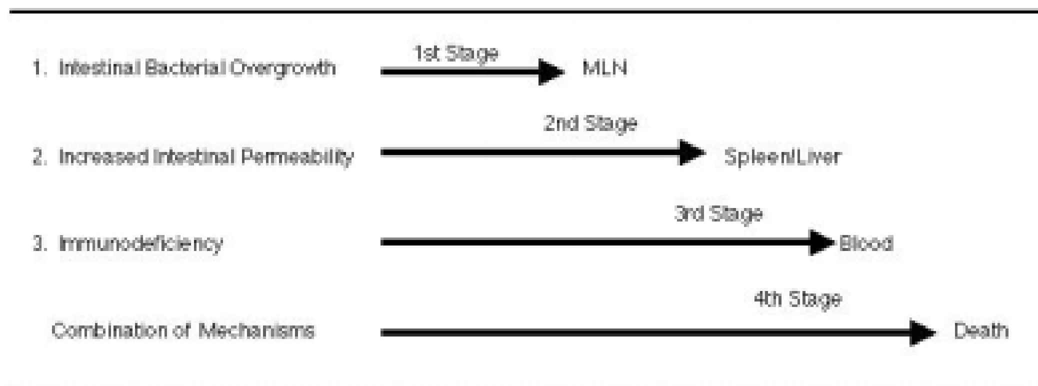

Figure 1: Stages in pathogenesis of bacterial translocation.

Other translocation promotion mechanisms also can act synergistically. For example, protein malnutrition alone produces histologic atrophy of the mucosa of the small bowel and caecum but the mucosal barrier remains intact and bacterial translocation does not occur (*Deitch* et al., 1987). The combination of protein malnutrition plus one endotoxin injection, however, produces intestinal ulcerations with concomitant bacterial translocation (*Ma* et al., 1989). Similarly, a 30% total body surface area burn plus one IP injection with endotoxin causes intestinal mucosal damage and synergistically promotes bacterial translocation (*Deitch* and *Berg*, 1987).

The pathogenesis of indigenous bacterial translocation from the GI tract occurs in several stages (Figure 1). Indigenous bacteria are translocating continuously from the GI tract in low numbers in the healthy adult animal. However, the translocating bacteria are killed in route or in situ in the MLN by host immune defences and indigenous bacteria are not usually cultured from the MLN or other extra-intestinal organs. This low level of "spontaneous" translocation by indigenous bacteria to the MLN may actually be beneficial to the host by stimulating the host immune system to respond more rapidly and more effectively to other more pathogenic exogenous micro-organisms.

In the first stage of translocation pathogenesis, bacteria translocate to the MLN but the host immune defences are able to confine the bacteria to the MLN and they do not spread to other sites. The second stage of translocation occurs when the host immune system is compromised and the translocating bacteria are allowed to spread from the MLN to organs, such as the liver, spleen, and kidney. Depending upon the degree of immunodeficiency and the virulence characteristics of the translocating bacteria, the host may still be able to confine the translocating bacteria to these organs. If host defences cannot control the translocating bacteria, they will spread systemically to the peritoneal cavity and bloodstream producing the third stage of translocation pathogenesis. Again, the host may recover depending on the degree of immunosuppression, the extent of intestinal mucosal damage, and the virulence properties of the translocating bacteria. The fourth and final stage occurs when the host succumbs to septic shock or multiple organ failure caused by the translocating bacteria. The fourth stage usually only occurs with a combination of promotion mechanisms, such as intestinal

bacterial overgrowth plus immunosuppression or intestinal bacterial overgrowth plus shock.

Indigenous bacteria also translocate from the GI tract in humans. Surveillance cultures of faecal samples from patients with leukaemia or other immunosuppressive disorders demonstrate an association between the bacterial biotype/serotype present at the highest population level in a patient's faeces and the bacterial biotype/serotype most likely to cause septicaemia in that patient (*Trancrede* and *Andremont*, 1985). Indigenous GI bacteria are detected in the blood or MLN of patients exhibiting haemorrhagic shock (*Rush* et al., 1988; *Moore* et al., 1991; 1992). Indigenous GI bacteria also have been cultured directly from the MLN of patients with bowel cancer (*Vincent* et al., 1988), bowel obstruction (*Sedman* et al., 1994;), trauma (*Brathwaite* et al., 1993; *Reed* et al., 1994), and from patients exhibiting increased intestinal permeability, such as those with Crohn's disease, ulcerative colitis, or inflammatory bowel disease (*Ambrose* et al., 1984; *Peitzman* et al., 1991; *Sedman* et al., 1994). A volunteer who ingested large quantities of *Candida albicans* exhibited *C. albicans* in large numbers in his urine and blood (*Krause* et al., 1969).

Thus, evidence is accumulating that translocation from the GI tract is likely an important early step in the pathogenesis of opportunistic infections caused by the indigenous GI microflora. Bacterial translocation is strongly suspected in the pathogenesis of human septicaemia, acute respiratory death syndrome (ARDS), and multiple organ failure syndrome. The translocation of indigenous bacteria from the GI tract is becoming even more relevant with the dramatic rise in numbers of hospitalised patients with compromised immune systems and increased intestinal permeability, such as the elderly, and those with cancer, diabetes, transplants, invasive devices, trauma, or AIDS.

In animal models of bacterial translocation, the bacterial species most likely to translocate from the GI tract, namely *Pseudomonas aeruginosa*, *Enterococcus faecalis*, and especially the Gram-negative enteric bacilli (i.e. *Enterobacteriaceae*) are also the most common causes of septicaemia in hospitalised patients. Selective antibiotic decontamination may eventually prove beneficial in certain types of patients to remove the indigenous *Enterobacteriaceae* but leave intact the indigenous obligate anaerobes to exert colonisation resistance against the indigenous *Enterobacteriaceae* and even more pathogenic exogenous bacteria. Oral and even systemic antibiotics, however, must be employed with caution, since intestinal bacterial overgrowth is an efficient mechanism promoting bacterial translocation. Maintaining a stable ecological balance in the GI tract therefore is a major defence mechanism preventing intestinal bacterial overgrowth and subsequent translocation. In this regard, the use of probiotics, such as *Lactobacillus acidophilus* or *Saccharomyces boulardii*, may have merit as an alternative to antibiotic therapy in maintaining an ecological balance. However, more information is required concerning the complex ecological interrelationships between the host and its indigenous GI microflora in order to design therapeutic measures effective against bacterial translocation but disruptive to the ecological GI balance.

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GLYCOSAMINOGLYCAN AND SIALIC ACID BINDING MICROBIAL PROTEINS IN GUT TISSUE ADHESION AND INVASION

TORKEL WADSTRÖM and ÅSA LJUNGH

Department of Medical Microbiology, Dermatology and Infection, Lund University, Lund, Sweden

SUMMARY

Glycosaminoglycans (GAGs), heparin, heparan sulphate (HS) and other sulphated molecules and hyaluronic acid, form part of the extracellular matrix (ECM), mediate cell-ECM adhesion, cell migration and growth, and bind growth factors and growth factor-binding proteins. Bacterial pathogens, like *Helicobacter pylori, Staphylococcus aureus* and *Streptococcus pyogenes,* and parasites such as *Trypanosoma cruzi* and *Leishmania* were shown to express cell surface proteins binding specific HS molecules on macrophages, triggering cell uptake and adhesion to fibronectin and other molecules involved in the phagocytic process. So, in addition to acting as a mechanism of tissue adhesion GAG binding may interfere with phagocytosis. It is tempting to speculate that GAG binding may play an important role in intracellular survival in macrophages. Several microbial cell surface proteins interact with highly negatively charged sialic acid-containing glycoconjugates, e.g. fimbriae of *Escherichia coli* and *Plasmodium falciparum*, recognising glycophorin on erythrocytes. *Yersiniae* cells can utilise HS binding for gut translocation, and *Listeria monocytogenes* cell entry is mediated by HS binding. Heparin was shown to mediate the erythrocyte invasion by *P falciparum* merozoites. *H pylori* invades through tight junctions which may be enhanced by expression of plasminogen binding. Heparin binding may interfere with vitronectin binding and complement activation. GAG binding proteins of *Borrelia* sp. are vaccine candidates for prevention and treatment of infections. Likewise, with *H pylori* a similar anti-adhesion approach is promising. Heparin binding microbes may interfere with the effect normally exerted by heparin binding growth factors, like wound healing and tissue integration. Heparin was shown to inhibit the mucosal inflammation and enhance tissue healing in mice infected by *H pylori.* Likewise, in patients with ulcerative colitis, heparin was shown to enhance the healing process. Before anti-adhesion treatment directed against GAG- and sialic acid binding proteins is developed effects on the normal intestinal microbial flora have to be elucidated.

INTRODUCTION

Glycosaminoglycans (GAGs), i.e., heparan sulphate (HS), dermatan sulphate (DS), chondroitin sulphate (CS), keratin sulphate, heparin and other sul-

Table 1: Some examples of glycosaminoglycan microbial binding,

Organism	Mol.wt (kDa)	GAG recognised
Bordetella pertussis (FHA)		
Borrelia burgdorferi	20 and 22	decorin
Helicobacter pylori	55-60	heparin, HS, DS
Listeria monocytogenes (ActA)		heparin, HS
Mycobacterium tuberculosis	28	heparin
Mycoplasma pneumoniae		DS
Neisseria gonorrhoeae (Opa)		heparin
Staphylococcus aureus	60	heparin, HS
Streptococcus pyogenes		
Yersinia enterocolitica (LcrG)		heparin, DS
Plasmodium falciparum		CSA
Toxoplasma gondii	-	heparin, HS, CSA, CSC
Herpes simplex glycoprotein C, B		HS
Cytomegalovirus	30, 34	HS
Candida albicans		GAG

phated molecules, and unsulphated hyaluronic acid form a major part of the extracellular matrix (ECM). Cell surface GAG molecules mediate many cellular processes: cell-ECM adhesion, cell migration and growth, and bind growth factors and growth factor-binding proteins (*Wadström* and *Ljungh*, 1999; *Lane* and *Lindahl*, 1989). Many pathogenic bacteria, viruses, fungi, and parasites use cell surface glycolipids, glycoproteins and GAGs as receptor molecules for cell and ECM attachment, as well as for eukaryotic cell invasion and intercellular migration processes. These interactions usually involve specific surface proteins of micro-organisms, named adhesins (*Rostand* and *Esko*, 1997). Proteins, interacting with specific carbohydrate structures of glycoconjugates, are defined as microbial surface lectins (*Wadström* and *Trust*, 1984).

Microbial binding to glycolipids and glycoproteins by specific surface lectins, often with haemagglutinating (HA) activity, has been extensively studied. In contrast, binding to cell surface ECM proteins and GAGs has been studied less (Table 1) (*Ljungh* and *Wadström*, 1995; *Wadström* and *Ljungh*, 1999; *Ljungh* et al., 1996; *Conrad*, 1998). More recently, bacterial pathogens such as *Helicobacter pylori* (*Ascencio* et al, 1993a; *Ascencio* et al, 1995; *Utt* and *Wadström*, 1997), *Staphylococcus aureus* (*Liang* et al., 1992), and *Streptococcus pyogenes* (group A streptococci) (*Bergey* and *Stinson*, 1988), and parasites like *Trypanosoma cruzi* (*Prioli* et al., 1987) and *Leishmania* (*Love* et al., 1993) were shown to express cell surface proteins that bind specific HS molecules on macrophages which triggers cell uptake and adhesion to specific ECM molecules involved in phagocytic processes, such as fibronectin (*Chmiela* et al., 1995a; *Duensing* et al., 1999). *Mycobacterium tuberculosis* binds HS by specific surface molecules, and binding to this GAG molecule on macrophages and other cells may trigger cellular uptake (*Menozzi* et al., 1996).

Table 2: Intracellular pathogens using heparan sulphate-like molecules in lectinophagocytosis

Organism	Reference
*Borrelia burgdorferi**	*Guo* et al. (1995)
Listeria monocytogenes	*Alvarez-Dominguez* et al. (1997)
Mycobacterium tuberculosis	*Menozzi* et al. (1996)
Neisseria gonorrhoeae	*Chen* et al. (1995)
*Helicobacter pylori**	*Chmiela* et al. (1995)
Yersinia enterocolitica	*Boyd* et al. (1998)
Leishmania donovani	*Love* et al. (1993)
Plasmodium falciparum	*Chen* et al. (1997)
Toxoplasma gondii	*Franklin* et al. (1996)
Trypanosoma cruzi	*Ortega-Barria* and *Pereira* (1991)

*Possibly not intracellular.

Interestingly, *H. pylori* and *Neisseria gonorrhoeae* (*Chen* et al., 1995) bind heparin and HS, like some pathogens commonly defined as extracellular microbes, i.e. *Borrelia spp.* and *Treponema pallidum* (Table 2) (*Guo* et al., 1995; *Alderete* and *Baseman*, 1989). These pathogens may adapt to multiply in the ECM of various body tissues without triggering uptake by macrophages and other phagocytes. Initial cell binding is usually followed by binding to other cell surface glycoconjugates, such as cell surface glycolipids, like with cytomegalovirus (CMV) and dengue virus (*Compton* et al., 1993; *Chen* et al., 1997). Prions may also use HS or other similar cell surface and ECM molecules, such as laminin, in cellular interactions in the brain and other body tissues.

GLYCOSAMINOGLYCAN CHEMISTRY

Cell membrane proteoglycans have single transmembrane-spanning domains in a type I orientation, with syndecan-1 as the prototype. Each of these molecules has conserved attachment sites for 3 to 5 GAG chains (*Linhardt* et al., 1992; *Lane* and *Lindahl*, 1989). The other major family of proteoglycans is named glypicans, which appear to contain only HS chains. Interestingly, expression of these proteoglycans occurs in a tissue-specific manner. It is noteworthy that heparin is cleaved from a core protein in mast cells. Free heparin chains form complexes with basic proteases and peptidases which are packed in secretory granules. Upon degranulation, these are released and dissociate. In contrast, HS is secreted intact from cells (*Kusche* et al., 1991).

Proteoglycans exhibit an enormous structural heterogeneity caused by great variations in glycosylation patterns, variations in glycan chain length of GAG chains, and variation in the extent and pattern of sulphation (*Lane* and *Lindahl*, 1989; *Heremans* et al., 1989). HS chains usually contain 0.8-1.4 sulphate groups/disaccharide per unit, while heparin, synthesised in intracellular granulae of mast cells, contain ≥2.4 sulphate groups/unit. Various modifications occur to a greater extent in heparin than in HS (e.g., >80% of glycosamine residues are N-sulphated compared to <60% of HS).

GAG BINDING IN MICROBIAL ADHESION TO MUCOSAL SURFACES

Intramolecular variations in the GAG chain, chain length and degree of sulphatation define how these molecules interact with specific ECM proteins - fibronectin, collagen type I, laminin, vitronectin, and various microbes (*Bober-Barkalov* and *Schwarzbauer*, 1991; *Casu*, 1994; *Conrad*, 1998; *Hayashi* et al., 1980; *Keller*, 1994; *Murphy-Ullrich* et al., 1993; *Zou* et al., 1992). Heparin-dependent growth factors, the acidic and basic growth factors (aFGF, bFGF) and platelet-derived growth factor (PDGF) bind to distinct pentasaccharide units with specifically positioned 3-0 sulphated glucosamine residues, shown also to bind to antithrombin III (*Baird* and *Klagsbrun*, 1991; *Dowd* et al., 1999; *Hileman* et al., 1998; *Kinsella* et al., 1998; *Kost* et al., 1992; *Ljungh* and *Wadström*, 1995; *Schlessinger* et al., 1995). The HS-binding peptide from *Plasmodium falciparum* is also a CS-binding protein like the N-terminal region of an actin-binding protein, ActA, of *Listeria monocytogenes* (*Pouvelle* et al., 1997; *Alvarez-Dominguez* et al., 1997).

Several microbial cell-surface proteins interact with highly negatively charged sialic acid-containing glycoconjugates (S fimbriae, K88, K99, CFAI and CFA II surface lectins of *Escherichia coli*) (*Ascencio* et al., 1993b; *Lindahl* et al., 1988; *Sun* et al., 2000; *Virkola* et al., 1993; *Wadström*, 1993; *Wadström* and *Trust*, 1984). Similarly, *P. falciparum* recognises sialic acid and glycophorins on erythrocytes (*Templeton et al*, 1998). Heparin and some other GAG molecules also express a negatively charged surface *in vivo*

The precise mechanism by which heparin blocks cell adhesion of microbes has been difficult to elucidate because of lack of commercially available purified heparins and heparin-derived fragments, now in clinical use in drugs like Fragmin® (KABI-Pharmacia, Stockholm, Sweden). Such fragments of heparin and other GAG chains, as well as those from various natural sulphated carbohydrate polymers like fucoidan and some carrageenans, often called 'heparinoids', are used as adhesion inhibitors to define the specificity of sulphate interactions in the binding of viruses, bacteria and parasites to specific tissue culture cells and ECM derived from cells grown on various biosurfaces (*Duensing* et al., 1999; *Hoffman*, 1993; *Kinsella* et al., 1998; *Pascu* et al., 1995; *Yahi* et al., 1994). Bacteria may also produce heparin and HS lyases with certain specificities (*Nader* et al., 1999).

Moreover, a number of specific glycosidases, such as heparinase, heparitinases and chondroitin sulphatases were used as 'receptor-destroying enzymes' in an analogy to the use of sialidases (neuraminidases) to destroy sialoglycoconjugate cell receptors for influenza virus and *T. cruzi* (*Prioli* et al., 1987).

Examination of the binding properties of ^{125}I-HS with sialoglycoconjugates, and HA assays with fetuin, glycophorins and hyaluronic acid in combination with a great number of 'heparinoids' as potential HA inhibitors showed that heparin-inhibitable ^{125}I-HS binding occurs with all strains of *H. pylori* tested (so called class I, compared to class II strains, or *cagA* pathogenicity 'island' positive) (*Utt* and *Wadström*, 1997). Likewise, expression of sialic acid haemagglutinin and other sialic acid-binding surface lectins (SALs; present in ~50% of strains of mainly class I strains; *T. Wadström* et al., unpublished results) did not influ-

ence binding of heparin. Interestingly, *Bordetella pertussis* expresses a filamentous haemagglutinin (FHA) that binds heparin and other GAG molecules by its C-terminal, whereas the pertussis toxin binds specific sialoglycoconjugates (*Geuijen* et al., 1998; *Menozzi* et al., 1994; *van ´t Wout* et al., 1992). Other *Helicobacter* spp. isolated from animals, such as *H. felis* and *H. mustelae,* commonly express GAG-binding surface molecules but not SALs (*T. Wadström* et al., unpublished results).

Further analyses of the molecular properties of the first *H. pylori* heparin binding protein or HEBP (57 kDa) (*Utt* and *Wadström*, 1997) will reveal if this protein belongs to a new class of *microbial* GAGBPs (see below). Interestingly, HEBP of *M. tuberculosis* and *M. bovis* is a haemagglutinin that agglutinates rabbit erythrocytes (*Menozzi* et al., 1996).

With *Mycoplasma pneumoniae,* DS has been shown to inhibit adhesion to pulmonary epithelium (*Krivan* et al., 1989). DS and other GAG molecules bind primarily to heparin-binding ECM molecules like fibronectin and, in so doing, cover or mask potential binding ligands in ECM, e.g. for *Candida albicans* (*Klotz* and *Smith*, 1992; *Hileman* et al., 1998). DS, which is more negatively charged than heparin, did not show such an effect indicating that specific interactions occur between heparin or HS and sub-endothelial ECM.

For the recently described GAGBPs of *M. tuberculosis* and *L. monocytogenes* (*Menozzi* et al., 1996; *Alvarez-Dominguez* et al., 1997), similar studies on expression of these proteins *in vivo* are lacking. GAG molecules have been shown to block *Chlamydia* infection *in vitro,* but this infection was not blocked in a mouse model of infection. Specific biovariants (of the trachoma-*Lymphogranuloma venerum* (LGV) group) also express neuraminidase-sensitive eukaryotic cell receptors. However, as heparin inhibits cell binding of all *Chlamydiae*, expression of GAG binding is likely to be common in the genus, including *Chlamydia pneumoniae* (*Stephens*, 1994).

POSSIBLE ROLE OF GAG BINDING IN INTRACELLULAR SURVIVAL

Survival and multiplication of intracellular micro-organisms in macrophages and other eukaryotic cells is vital in the pathogenesis of infections caused by such microbes. It has been suggested that GAG-binding surface molecules confer the resistance to phagocytosis seen with *L. monocytogenes, H. pylori* and *N. gonorrhoeae* (Table 2) (*Chen* et al., 1995; *Chmiela* et al., 1995a,b; *Chmiela* et al., 1996). It is tempting to speculate that GAG binding may play an important role in intracellular survival in specific cells, such as macrophages, known to express mannose-sialoglycoconjugates and HA on the surface.

However, more *in vitro* studies in serum-containing and serum-free systems with macrophages are necessary to define a possible interplay of GAG molecules with individual pathogens. Such studies will form a basis for deciding if GAG molecules such as heparin and various ‘heparinoids’ can be used in combination with other glycoconjugates to block uptake into macrophages, other professional phagocytes and other target cells in pathogenesis, including the human gastric epithelium for studies of *H. pylori.*

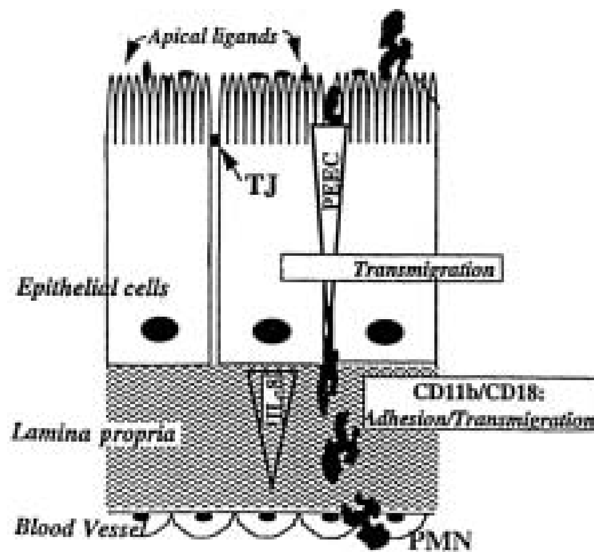

Figure 1: Model of gastro-intestinal epithelium. *Salmonella* sp. invades epithelial cells directly or through tight junctions (TJ) whereas *H. pylori* invades probably only via TJ.

GAG BINDING IN INVASION AND TRANSLOCATION

Yersiniae cells can utilise HS exposed on eukaryotic cells for gut translocation (*Boyd* et al., 1998; *Cornelis* and *Wolf-Watz*, 1997; *Pusztai* and *Bardocz*, 1995), and *L. monocytogenes* cell entry is mediated by HS binding (*Menozzi* et al., 1996). GAG-binding is responsible for the gliding motility by *Toxoplasma gondii,* and GAG-deficient mutant host cells could not be invaded by this parasite (*Carruthers* et al., 2000). Likewise, heparin binding is involved in erythrocyte invasion by *P. falciparum* merozoites (*Kulane* et al., 1992). HS binding by Type 1 glycoprotein B and C of *Herpes simplex* was shown to be involved in attachment, cell-to-cell spread and invasion of eukaryotic cells but in different ways (*Laquerre* et al., 1998). Whether or not gastro-intestinal (GI) pathogens, like *Salmonella* and *H. pylori* invade epithelial cells by GAG binding ability has not been elucidated. *H. pylori* invades through tight junctions (Figure 1). Possibly, expression of plasminogen binding may enhance gut translocation and tissue invasion, as described for several other invasive pathogens (*Pantzar* et al., 1998; *Ljungh*, 2000; *Lähtenmääki* et al., 2000). Moreover, heparin binding may interfere with complement and vitronectin binding, and modulate microbe-phagocyte interactions as well as binding to other cells, as shown for *C. albicans* and staphylococci (*Calderone* et al., 1988; *Duensing* et al., 1999; *Ljungh* and *Wadström*, 1995; *Lundberg* et al., 1997). Epithelial cells undergo rapid apoptosis and loss of contact with the underlying matrix. As several ECM proteins have heparin-binding domains, it is possible that expression of heparin binding by microbes may enhance apoptosis by interfering with the matrix-epithelium contact (*Duensing* et al., 1999; *Wadström* and *Ljungh*, 1999).

At least three growth factors require heparin for activation. These are aFGF, bFGF and PDGF (*Baird* and *Klags-*

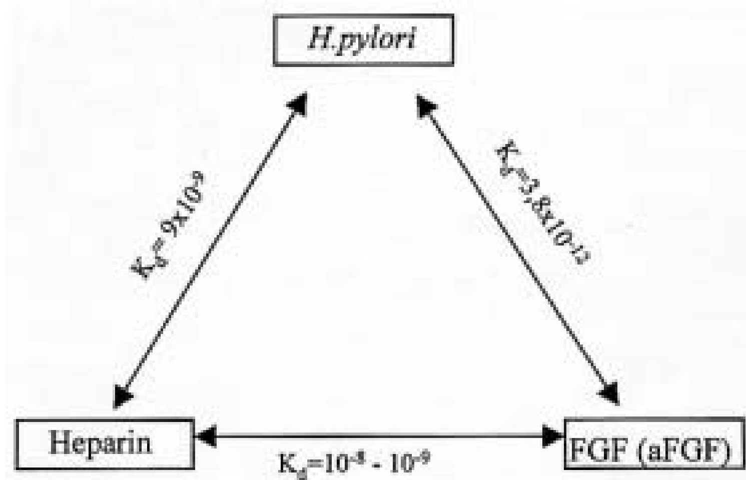

Figure 2: Interactions between *H. pylori*, heparin and heparan sulphate, and heparin-dependent growth factors.

brun, 1991). *H. pylori* binds bFGF and PDGF specifically which may indicate that binding of heparin or growth factors can interfere with wound healing (Figure 2) (*Ascencio* et al., 1995). In addition, it has been suggested that such heparin depending growth factor interactions target *Pseudomonas aeruginosa* exotoxin to susceptible cells (*Mesri* et al., 1993).

Surface domains may express proteins rich in charged and hydrophobic domains ('hydrophobins') of importance for the pathogen to evade a strong immune response. Thus, studies on *Borrelia sp.*, *H. pylori* and other GAG-binding pathogens seem to be of great importance because GAGBPs, as mucosal epithelium adhesins, are vaccine antigen candidates and for targeting molecules (beside other surface lectins and ECM-binding proteins) for an anti-adhesion approach to prevent and treat infections by a 'non-antibiotic' strategy (*Breithaupt*, 1999; *Feng* et al., 1998; *Hanson* et al., 1998; *Ruiz-Bustos* et al., 2000). Preliminary studies involving the use of modified heparin molecules and specific sialoglycoconjugates to block *H. pylori* GAGBPs and SALs in a BALB/c mouse and a primate model have been promising (see below).

Standard commercial heparin and low molecular weight or LMW heparin (Fragmin) were shown to disrupt rosette formation between *P. falciparum* and both infected and non-infected red blood cells from patients with cerebral malaria. Other investigators have confirmed this proposal and shown that chondrotin-4-sulphate is the natural receptor for *Plasmodium* spp. in the placenta, and is involved in transmission of the parasite from the mother to the foetus (*Gysin* et al., 1999; *Reeder* et al., 2000).

Altogether, these findings suggest that modified LMW heparin and other GAG molecules, or heparinoids, are indeed candidates for future development of antibacterial, antiviral and antiparasitic drugs.

MICROBIAL AND PLANT LECTINS IN THE GASTRO-INTESTINAL TRACT

We know from pioneer studies by *Pusztai* and *Bardocz* (1995) that feeding plant lectins to young animals such as rats and guinea pigs affect the maturation process of the GI mucosa. More recently, it has been debated that gene-modified plants with high lectin contents may enhance these effects and maybe predispose to overproliferation of the gut mucosa.

Pioneer studies in gnotobiology a few decades ago showed that maturation of the gut mucosa and lymphoid GALT system is much delayed in germfree (GF) compared to conventional rats, and is more associated with specific species of the indigenous gut microflora such as *Enterococcus faecalis.* Despite these observations we have still a very poor knowledge of how microbial lectins with various carbohydrate specificities and plant lectins (fucose, galactose and mannose specific lectins) are involved in such growth stimulation of mucosal epithelium and cells associated with the GALT lymphoid system of the gut (*Pusztai* and *Bardocz*, 1995; *Gabius* and *Gabius*, 1993).

However, the situation is different for some bacterial lectins, such as SALs and heparin or GAG binding proteins. These latter proteins are not often referred to as lectins for the simple fact that the carbohydrate binding studies often involved several units unlike plant lectins mostly recognising one or two units (*Gabius* and *Gabius*, 1993). Moreover, modification of sulphate groups can drastically affect binding of heparin, HS and other GAG molecules such as CS.

We know that in the evolution of pathogenic microbes of the GI mucosa, SALs are common lectins among enteropathogenic *E. coli* and *H. pylori,* and less well characterised microbes such as *Campylobacter jejuni* and other campylobacter species (*Wadström* and *Trust*, 1984; *Gabius* and *Gabius*, 1993).

GAG-binding proteins are common among various tissue invading pathogens like staphyloccoci, streptococci, *L. monocytogenes* and various parasites (Table 1). For some of these organisms such as gonococci and *M. pneumoniae* GAGBP seem crucial to trigger later stages of glycolipid cell receptor binding and uptake in gut mucosa cells as well as professional phagocytes (*Krivan* et al., 1989).

On the contrary, our knowledge on putative GAGBPs and SALs of members of the indigenous microbial microflora on various mucosal surfaces is nearly non-existing. Such studies are needed since tissue trauma of the gut mucosa exposes ECM and GAG molecules. Based on studies of GAGBPs of *H. pylori* binding to GAGs on cell surfaces and in the ECM in experimental studies, we propose that other GAG binding pathogens and maybe also members of the indigenous microflora can colonise tissue wounds further down the GI tract. Studies on various new Helicobacters colonising the GI mucosa of rodents, dogs and cats as well as primates are now underway. Recent reports on how such organisms may cause chronic colitis as well as hepatitis (“gut-liver link”) as defined in the pathogenesis of primary sclerosing cholangitis and chronic ulcerative colitis (UC) will encourage further studies (*Kirsner*, 2000; *Franklin* et al., 1996). Furthermore, early observations of ECM binding *E. coli* colonising lesions in patients with UC (*Ljungh* and *Wadström*, 1988; *Ljungh* et al., 1988; *Ljungh*, 1992) suggest that studies on GAG binding properties of other GI

Table 3: History of anti-peptic ulcer drugs*

1907	Deklug	Theory of mucosal protection by gastric mucus
1931	Fogelson	Clinical application of gastric mucin
1932	Babkin and Komarov	Discovery of antipeptic effect of gastric mucus
1954	Levey and Sheinfeld	Antiulcerogenic effect of chondroitin sulphate
1959	Anderson	Antiulcerogenic effect of carageenan
1967	Cayer	Clinical application of amylopectin sulphate
	Hino Nao	Clinical application of aluminum sucrose sulphate (sucralphate)
1968	Ishimori	Clinical application of sorbitol sulphate, dextran sulphate, amylopectin sulphate and aluminum dextran sulphate

* Modified from A. Ishimori (*Hollander* and *Tytgat*, 1995)

inhabitants like *Bacteroides* and lactic acid bacteria (LAB) now are needed. We have earlier characterised binding of collagen by *Lactobacillus reuteri,* and ECM binding by various anaerobic species (*Aleljung* et al., 1994; *Szöke* et al., 1996). Interestingly, *Bacteroides* species were recently shown to produce glycosaminoglycan-degrading enzymes (*Ahn* et al., 1998).

PEPTIC ULCERS DISEASE (PUD) OF THE STOMACH AS A MODEL FOR ULCERATIVE COLITIS AND OTHER CHRONIC GUT-BARRIER DESTROYING DISEASES

Already studies in the 1960's in Japan showed that sulphated natural carbohydrates often called 'heparinoids' enhance the wound healing process in chronic PUD (Table 3) (*Hollander* and *Tytgat*, 1995). These studies stimulated the development of sucralphate by the Chugai Company as a drug to enhance PUD healing and as a mucosal protecting agent. Later studies have proposed that this drug accumulates in mucosal lesions, and protects various heparin dependant growth factors against rapid breakdown by tissue and microbial proteases such as ECM associated metalloproteases, also proposed to play a key role in the pathogenesis of UC and maybe of Mb Crohn (*Keusch* et al., 1880; *Kirsner*, 2000; *Parks* and *Mecham*, 1999). Interestingly, recent studies in mouse models for *H. pylori* gastritis showed that heparin inhibits mucosal inflammation and enhances tissue healing (*Wang* et al., 2001). Moreover, studies in patients with UC have shown that various forms of heparin such as low molecular weight heparin enhance the healing process of the colonic mucosa (*Gaffney* et al., 1995; *Korzenik* et al., 1998; *Törkvist* et al., 1999). We should then remember that early studies on sucralphate in colonic enemas showed a mucosal healing effect in patients with UC (*Hollander* and *Tytgat*, 1995). These observations will stimulate more studies on the effect on heparin and other GAG-models in inflammations and ulcer healing in various parts of the GI tract.

HEPARIN GROWTH FACTORS AND CYTOKINES

A first study on how *H. pylori* GAG-binding surface factors can attenuate growth suggests that this pathogen may deplete growth factors from mucosal lesions, and thus delay the natural healing process in type B gastritis and PUD (*Ascencio* et al., 1995). Recently, hepatocyte growth factor and other heparin dependant growth factors (aFGF and bFGF) were shown to be upregulated in mucosal lesions in rats (*T. Watanabe*, personal communication), and oral treatment trials with synthetic stable acid growth factors enhanced the healing process in a proton pump based therapy in PUD, and might also enhance the healing in treatment with antibiotics (*I. Kondo*, personal communication).

However, recent findings that heparin also affects tissue and ECM binding of various growth factors such as interleukin 6 (IL-6) and IL-8 suggest that heparin or other GAGs may wash out a surplus of cytokines in mucosal lesions (*Dobosz* et al., 1996). On the contrary, other sulphated carbohydrates such as DS and carrageenan under experimental conditions enhance the mucosal inflammation in rats. Thus, more research is needed to study how various 'heparinoids' and GAG binding molecules trigger mucosal inflammation in some situations, and other molecules suppress tissue inflammation mediated, probably by heparin-binding growth factors and mucosa associated cytokines (*Korszenik* et al., 1998; *Kuschert* et al., 1999). Most likely such compounds are candidates for a novel therapy of various inflammatory and infectious processes in the GI tract. However, possible side effects on the indigenous flora have to be studied to rule out that these compounds cause side effects as broad spectrum antibiotics to destroy the normal GI indigenous microflora (*Breithaupt*, 1999).

During chronic tissue inflammation processes such as transplant rejection, there is a selective loss of HS. Soluble GAG molecules can reduce the inflammation in experimental animal models and a low dose of heparin inhibits delayed type hypersensitivity reactions, adjuvant arthritis, allergen induced eosinophilic infiltration maybe interfere with both heparin binding cytokines growth factors as well as L and P selectins (*Kuschert* et al., 1999). Moreover, heparin may inhibit mast cell granulation and complement activation, which may also decrease the inflammation process.

Thus, GAG binding microbes involved in chronic infections such as *H. pylori* and *M. tuberculosis* may modulate and affect tissue inflammation in a complex fashion by modulating GAG/heparin binding surface proteins and indirectly also affect GAG binding tissue molecules such as heparin binding growth factors and cytokines. This may indirectly affect multiple chemoattractants in control of leukocyte homing into inflammation target tissues such as the gut mucosa (*Naef* et al., 1996; *Kuschert* et al., 1999).

It has been postulated that heparin and DS bind to similar tissue substrates but have opposite effects on the same pathway (*Korzenik* et al., 1998). Experimental models for chronic colitis in rats and mice have shown that DS can induce a chronic colitis in conventional but not in Germ-free animal models (*T. Midtvedt*, personal communication). It seems likely that DS as well as other sulphated glycoconjugate molecules such as carrageenans, can modulate the binding of growth factors to ECM like bFGF-β1 and PDGF. Heparin and

other GAG molecules act as antagonists for DS in these experimental models and probably also in patients with inflammatory bowel disease (IBD) (*Korzenik* et al., 1998). Heparin has also been shown to decrease the cell concentration of the IL-6 cytokine in tissue inflammation in UC and have a beneficial role on the microcirculation and to inhibit nitric oxide synthase (*Dobosz* et al., 1996). Thus, heparin and GAGs are interesting candidates for therapy of UC and other forms of IBD. If mast cells play a key role in the first step of this inflammation process like for the *H. pylori* induced type B gastritis should be further studied.

Early studies on enterotoxigenic *E. coli* (ETEC) infecting young pigs, calves and lambs revealed that galactose and sialic acid-specific lectin associated with fimbriae and fibrillar surface structures determine colonisation of the mucus layer and the intestinal epithelium (*Lindahl* et al., 1988; *Sun* et al., 2000). Interestingly, studies of ETEC causing diarrhoea in young children and adult travellers revealed that ETEC fimbriae-associated HA's or lectins, such as CFAI and CFAII (CFA, colonisation factor antigen) which recognise terminal sialic acid in α-2,3, α-2,6, and also α-2,8 linkages (*Wadström* and *Trust*, 1984). More recent studies showed that *H. pylori* SAL also recognise α-2,3 linkage (*Hirmo* et al., 1996). An experimental therapy based on polysialyllactose was shown to inhibit the gastritis of monkeys experimentally infected by *H. pylori* (*Mysore* et al., 1999). Promising studies have recently shown that sialic acid rich glycoconjugates from bovine milk inhibit *H. pylori* induced gastritis in a mouse model (*Wang* et al., 2001). However, since *H. pylori* also produces Lewis B blood group fucose specific lectin and GAGBP, an optimal therapy has to be designed (*Guruge* et al., 1998; *Hileman* et al., 1998).

FUTURE PERSPECTIVES

With the rapidly increasing knowledge on microbial infections in the last two decades it is most encouraging to enhance development of an anti-adhesin therapy to combat *H. pylori* and other GI infections. A non-antibiotic approach seems attractive to eradicate or suppress *H. pylori* infection to avoid an overuse of antibiotics and antibiotic resistance development in *H. pylori* and in the indigenous oral and GI flora (*Breithaupt*, 1999). That HS and DS inhibit *H. pylori* infection in the gastric mucosa but have opposite effects on infections in the large intestine may indicate different modulating effect of members of the normal intestinal microflora (*Utt* and *Wadström*, 1997). Hence, future studies have to include an analysis on possible effects of these glycoconjugates on the normal GI microflora. Since a number of glycosialidases are produced by the complex GI microflora, studies on break-down of sialoglycoconjugates, GAGs and heparin-like molecules should be studied especially among strict GI anaerobes, such as various species of *Bacteroides* and *Eubacteria*, and among LAB and *Bifidobacteria* (*Ahn* et al., 1998; *Nader* et al., 1999).

ACKNOWLEDGEMENTS

The experimental part of this study was supported by grants from the Swedish Medical Research Council (16X-04723), the Swedish Board for Agricultural Research (497/98) and grants from the Medical Faculty and University Hospital in Lund.

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DIETARY MODULATION OF THE RESISTANCE TO INTESTINAL INFECTIONS

INGEBORG BOVEE-OUDENHOVEN and ROELOF VAN DER MEER

Department of Nutrition, Quality, and Safety,
Wageningen Centre for Food Sciences/NIZO Food Research,
Ede, The Netherlands

SUMMARY

Gastrointestinal infections are still a major health problem. These infections may be modulated by dietary components. Diet determines the composition of (gastro-)intestinal contents, which in turn affects gastro-intestinal survival of food-borne pathogens, the composition of the autochthonous microflora, and functioning of the mucosal barrier. These non-immunological parameters are essential for host defence, especially during the first encounter with a pathogen. Although numerous *in vitro* studies showed beneficial effects of dietary components on microflora- or infection-related issues, sound scientific evidence to confirm their functionality in animal and human health is surprisingly scarce.

We have studied the protective effects of dietary calcium against salmonella infection. It was already known that calcium precipitates irritating bile acids and fatty acids in the intestinal lumen and diminishes epitheliolysis. We speculated that reduced epitheliolysis might strengthen the barrier function of the gut mucosa and improve the resistance to intestinal infections. In addition, intestinal Gram-positive bacteria like lactobacilli might also benefit from the reduced luminal cytotoxicity, as these lactic acid bacteria are very sensitive to bile acids and fatty acids *in vitro*. Several infection studies were performed with rats consuming purified diets differing only in calcium content. After adaptation to these diets, the rats were orally infected with *Salmonella enteritidis*. Indeed, calcium stimulated the intestinal lactobacilli. More importantly, calcium supplementation decreased colonisation and translocation of salmonella, as judged by the reduced faecal shedding of this pathogen in time and the diminished infection-induced urinary NO_x excretion, respectively.

In literature, prebiotics are claimed to improve resistance to intestinal infections, though evidence is merely lacking. Therefore, we studied the effect of non-digestible carbohydrates on the course of a salmonella infection. Lactulose and oligofructose decreased colonisation of *S. enteritidis* when compared with resistant starch, wheat fibre, and cellulose. However, lactulose and oligofructose concomitantly increased translocation of this pathogen, an effect probably due to impairment of the gut mucosal barrier.

So, diet does modulate the resistance to intestinal infections, but more research is needed to prove the high expectations regarding functional foods and to elucidate the mechanisms involved.

INTRODUCTION

Gastrointestinal infections are still a major health problem. The world-wide incidence of acute infectious diarrhoeal disease is estimated to be 3-5 billion cases per year, resulting in 3-5 million deaths each year (*Gianella*, 1993). It is certainly not a problem of developing countries only. Even in industrialised societies the yearly incidence of food-borne intestinal infections is about 10% of the population. Bacterial pathogens (35%) are the leading cause of food-borne disease outbreaks of known aetiology, followed by toxins, viruses, and parasitic organisms other than bacteria and viruses. In the Western world, the bacterial pathogen most frequently isolated from patients suffering from acute gastro-enteritis is *Campylobacter* spp. (50%), followed by salmonella spp. (25%) (*Altekruse* and *Swerdlow*, 1996; *Lacey*, 1993). Moreover, infections caused by these two pathogens have emerged importantly in the last two decades (*Altekruse* and *Swerdlow*, 1996).

While most intestinal infections result in a self-limiting gastro-enteritis, severe complications like gut-derived septicaemia regularly occur in immunocompromised people, patients suffering from inflammatory bowel diseases, and patients in intensive care units on (par)enteral nutrition regimen (*Brooks* et al., 1993; *Deitch*, 1994). Treatment of many food-borne intestinal infections is often discouraging, since it hardly ameliorates the severity of illness and often prolongs asymptomatic carriage (*Tenover* and *Hughes*, 1996). Another concern is the growing resistance of bacterial pathogens, including Salmonella species to clinically important antibiotics (*Lee* et al., 1994; *Osterholm*, 2000). The high frequency of antimicrobial resistance today is probably a consequence of the widespread use or the inappropriate therapy of infections in both humans and animals (*Osterholm*, 2000; *Fey* et al., 2000). This stresses the importance of prevention and to search for alternative approaches to cope with the problem of emerging infections. One attractive approach is to improve host resistance by modulation of the diet. Scientific interest in dietary modulation of host resistance to intestinal infections is just emerging. Notwithstanding the results of numerous *in vitro* studies, strictly controlled infection studies showing the importance of the diet (e.g. supplemented with pre- or probiotics) to inhibit or ameliorate intestinal infections *in vivo* are scarce. Without the pretension of being complete, the following review highlights and discusses relevant scientific literature on dietary modulation of the primary (non-immunological) resistance to intestinal infections and combines it with results obtained from infection studies performed by our lab.

NON-IMMUNOLOGICAL HOST DEFENCES OF THE GASTRO-INTESTINAL TRACT

Of course, the most effective way of protecting the human body from pathogenic bacteria is to prevent contact with the bacterium in the first place. As that is a utopia in daily practice, we have to rely on our defences in the gastro-intestinal tract to get rid of swallowed disease-causing bacteria. Especially during the first encounter with a pathogen, the non-immunological defences are very important for host resistance to intestinal infections. The low pH of gastric

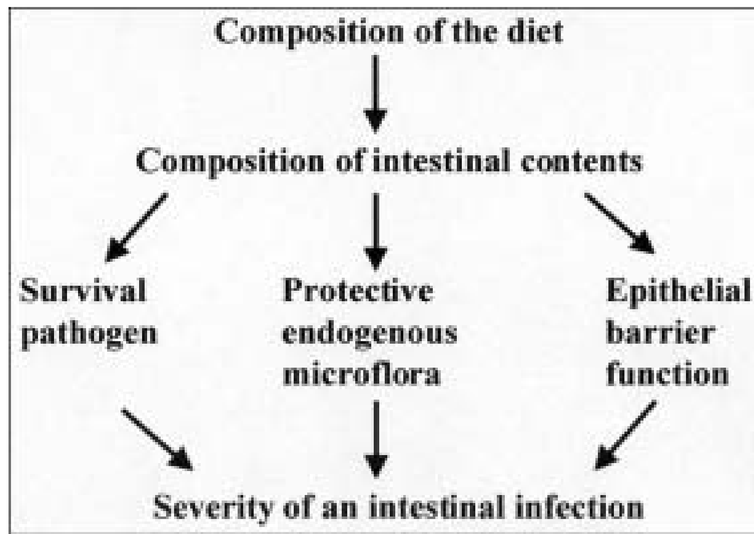

Figure 1: Dietary modulation of non-immunological host defences of the intestinal tract, which determines the severity of an intestinal infection.

juice has strong sterilising properties, because many food pathogens are acid-sensitive (*Gorden* and *Small*, 1993). Hypochlorhydria, in otherwise healthy elderly or in users of H_2 receptor antagonists and proton pump inhibitors, leads to increased intragastric bacterial counts and susceptibility to gastro-intestinal infections (*Duncan* and *Edberg*, 1995; *Gianella* et al., 1973), including salmonella (*Neal* et al., 1994). Buffering of gastric content and physical protection of bacteria by food, in combination with the rate of gastric emptying, are additional factors influencing the amount of pathogens surviving the gastric barrier (*Gianella* et al., 1973; *Sarker* and *Gyr*, 1992). The small intestine is protected to infective bacteria by a thick mucus layer covering the epithelium. Mucus not only acts as a lubricant to protect the delicate epithelial cells from gastric acid, bile acids and physical damage, but is also a trap for microbes to prevent their attachment to the mucosa. The mucus layer obtains its viscosity from mucins. The sugar chains of these glycoproteins mimic epithelial receptors for bacteria and act like a physical sieve trapping microbes and bringing them in close contact with secreted antibodies (sIgA), lactoferrin, lysozyme, and lactoperoxidase (*Duncan* and *Edberg*, 1995). The small intestine has a relatively high motility, which prevents adhesion to the mucosal epithelium and subsequently bacterial overgrowth (*Sarker* and *Gyr*, 1992). Duodenal secretions, such as bile and pancreatic enzymes, have potent bactericidal activity (*Rubinstein* et al., 1985; *Williams* et al., 1975). As a result of the combination of gastric acid, bile salts, and rapid flow of contents, the small intestine is relatively sparsely populated by bacteria. Though not very numerous, the endogenous microflora colonising the ileal mucosa might still be important in host defence, considering that many food-borne pathogens mainly elaborate their noxious effects in this region of the gastro-intestinal tract (*Salyers* and *Whitt*, 1994). In contrast to the small intestine, the most important defence mechanism of the colon is the presence of its luxuriant microflora (*Sarker* and

Gyr, 1992). Probably due to the slow transit of contents, the bacterial density is so great that nearly half of the volume of human colon contents is accounted for by bacteria (*Stephen* and *Cummings*, 1980). Potential invaders have to compete with this extensive established bacterial population for nutrients and adhesion sites on the epithelium. The production of antibacterial substances by the colonic microflora, such as lactic acid, short-chain fatty acids, and bacteriocins, may also inhibit growth of pathogenic bacteria (*Salyers* and *Whitt*, 1994). Animal (*Wells* et al., 1987) and human (*Bartlett*, 1992; *McFarland*, 1998) studies have shown that use of antibiotics, affecting the endogenous microflora, increases the susceptibility to contract intestinal infections. All the above mentioned non-immunological defence mechanisms cooperate with the gut-associated lymphoid tissue (GALT) in eliminating microbial pathogens (*Hazaoui* and *Pringault*, 1998).

DIETARY MODULATION OF THE RESISTANCE TO INFECTION

Diet directly affects the non-immunological host defences of the gastro-intestinal tract (Figure 1). It is obvious that the composition of the diet, especially the amount of non-absorbed nutrients, determines the composition of gastro-intestinal contents. The latter affects the survival of pathogenic bacteria in the gastro-intestinal tract. In addition, intestinal contents may influence the composition and the activity of the protective endogenous microflora and subsequently their antagonistic activity towards pathogens. Besides an effect on bacteria, luminal contents also affect functioning of the intestinal epithelium and its barrier function against invading microbes. Diet also directly affects host defence. For instance, if the habitual diet is deficient in one or more essential nutrients, normal functioning of all cells, including these belonging to the immune system, is impaired (*Kelley* and *Bendich*, 1996). Currently, there is a keen scientific and commercial interest in foods containing specific ingredients that modulate the intestinal microflora and subsequently the resistance to infection. The majority of these ingredients can be divided in two classes, the so-called probiotics and prebiotics. Besides these pro- and prebiotics, other dietary components (like minerals) may influence the course of an intestinal infection as well. Considering that several reviews about probiotics and intestinal health were published recently (e.g. *Erickson* and *Hubbard*, 2000; *Rolfe*, 2000), the discussion below focuses on the effects of non-digestible carbohydrates and minerals (particularly calcium) on host defence against intestinal infections.

NON-DIGESTIBLE CARBOHYDRATES

Most of the predominant species of the intestinal microflora require a fermentable carbohydrate for growth. It is generally assumed that the carbon and energy needed to maintain bacterial mass are derived from host secretions or from dietary carbohydrates (or other components) that escape digestion in the small intestine. Mucins, extensively glycosylated proteins secreted by goblet cells, might be excellent endogenous bacterial growth substrates (*Salyers* and

Leedle, 1983). Dietary fibres (vegetable polysaccharides) and oligosaccharides are more or less fermented in the intestinal tract, depending on their sugar composition, type of glycosidic linkages, and degree of branching. Dietary fibres are essential for maintenance of intestinal mucosal integrity, considering that oral administration of fibre-free elemental liquid diets induces spontaneous translocation of the gut microflora (*Sedman* et al., 1994). The protective effect of fibres on the mucosal barrier function might be independent of their fermentability and mediated by stimulation of the release of trophic gut hormones, preventing mucosal atrophy (*Jenkins* and *Thompson*, 1994). By definition, prebiotics are fermented by the endogenous microflora in contrast to the non- or low-fermentable dietary fibres (e.g. cellulose). Selective stimulation of the growth or the activity of a limited number of resident bacteria (preferably lactobacilli and bifidobacteria) and improvement of the host health are often mentioned criteria a prebiotic should meet. Polysaccharides like inulin, pectin, and resistant starch, and oligosaccharides based on fructose and galactose are potential prebiotics (*Roberfroid*, 1993).

A characteristic all the above mentioned potential prebiotics share is that they are fermented by the intestinal microflora in the lower gut to lactic acid and short-chain (C_2-C_6) fatty acids, resulting in a decreased pH of luminal contents (*Roberfroid*, 1993). Consumption of prebiotics may change the intestinal microflora. For instance, dietary inulin and oligofructose increase human faecal bifidobacteria (*Gibson*, 1999). Whether the *in vivo* growth advantage of bifidobacteria is due to selective utilisation of these non-digestible sugars by these genera or due to their relative resistance to the organic acids formed is not established yet. The latter explanation seems more likely, considering that other genera (for instance *Bacteroides*) can also degrade oligofructose at least *in vitro* (*Roberfroid*, 1993). Indeed, a recent study from our lab showed that oligofructose has a general growth-stimulating effect because significantly higher numbers of lactobacilli as well as *Enterobacteriaceae* were detected in faeces of rats fed these rapidly fermentable oligosaccharides (*Bovee-Oudenhoven* et al., submitted for publication).

It is frequently assumed that stimulation of intestinal lactobacilli or bifidobacteria (e.g. *Gibson*, 1999) is beneficial to the host and will result in an improved resistance to intestinal infections. This is mostly based on results of *in vitro* experiments showing that these carbohydrate-fermenting bacteria produce bactericidal organic acids and possibly other inhibitory compounds (hydrogen peroxide and bacteriocins) which suppress growth of pathogens like salmonella, *Escherichia coli* (*Gorden* and *Small*, 1993), and *Clostridium difficile* (*May* et al., 1994). However, stimulation of intestinal lactic acid bacteria or inducing a change in gut flora composition as such, is not directly a functional effect or a direct health advantage. In our opinion, evidence to establish the protective effect of prebiotics against intestinal infections can only be obtained from strictly controlled infection studies *in vivo*. Surprisingly, studies showing the efficacy of fermentable dietary fibres to reduce the severity of intestinal infections are rare. Lactulose therapy has been investigated in the management of salmonella and Shigella infections in humans. This non-digestible disaccharide increased faecal clearance of these pathogens in a significant proportion of the patients (*Hanssen* et al., 1981; *Liao* et al., 1994). Notwithstanding the beneficial effect of dietary fibre on intestinal

physiology, restraint should be applied in increasing the intake of non-digestible and rapidly fermentable oligo- or polysaccharides. High concentrations of short-chain fatty acids may damage the intestinal epithelium, resulting in an increased permeability and epithelial cell proliferation (*Argenzio* and *Meuten*, 1991; *Rémésy* et al., 1993; *Wasan* and *Goodlad*, 1996). In addition, a recent infection study of our lab actually showed that lactulose and oligofructose stimulated killing of *Salmonella enteritidis* in the intestinal lumen but concomitantly severely reduced the resistance of rats to translocation of this invasive pathogen, which was likely due to significant impairment of the mucosal barrier (*Bovee-Oudenhoven* et al., submitted for publication). The amount of lactulose and oligofructose added to the diets was realistic for human intake of highly fermentable dietary fibres (*Alles* et al., 1999). The mechanism of this unexpected detrimental effect of these oligosaccharides is subject of current research. Another observation of this infection study was that dietary oligofructose, but especially lactulose, greatly increased the number of faecal lactobacilli (> 100 times) when compared with supplemental cellulose. So, in contrast to often made claims, stimulation of intestinal lactobacilli is absolutely no guarantee that the resistance to intestinal infections will be improved.

DIETARY MINERALS

Very little is known about the effects of dietary minerals on the course of an infection. In addition to our studies on the protective effect of dietary calcium against Gram-negative bacterial infections, only data about the role of iron and zinc are reported in literature. Individuals with iron-overload, whether induced by excess dietary iron intake or due to diseases like β-thalassaemia major and sickle cell disease, are more susceptible to infection (*Walter* et al., 1997). Iron is essential for bacterial growth and may increase oxidative damage to host membranes or DNA during inflammation, as it is a catalyst in the production of hydroxyl radicals (*Shenkin*, 1995). The observed drop in plasma iron levels during infectious diseases, the so-called anaemia of infection, is a protective response of the body to combat the infection and to limit oxidative damage (*Beisel*, 1977). On the other hand, there is also evidence that severe iron deficiency is associated with an increased incidence of infections (*Walter* et al., 1997). Iron deficiency compromises host resistance by suppressing the cellular immune response (*Omara* and *Blakley*, 1994).

Zinc is a cofactor of about 120 mammalian enzymes. Zinc deficiency has a pronounced effect on nucleic acid metabolism, thus influencing protein synthesis and cell growth. Furthermore, an inadequate zinc intake is associated with an impaired immune function (*Mocchegiani* et al., 2000). In zinc deficiency, the organism is more susceptible to toxin-producing bacteria or enteroviral pathogens that activate guanylate and adenylate cyclases, which stimulate chloride secretion into the intestinal lumen. The resulting diarrhoea and diminished absorption of nutrients exacerbate an already compromised mineral status (*Wapnir*, 2000). Zinc supplementation can reduce the incidence and prevalence of acute infectious diarrhoea (*Black*, 1998). Besides restoration of immune cell functioning, normalisation of intestinal epithelial cell proliferation and strengthening of the mucosal barrier might be responsible

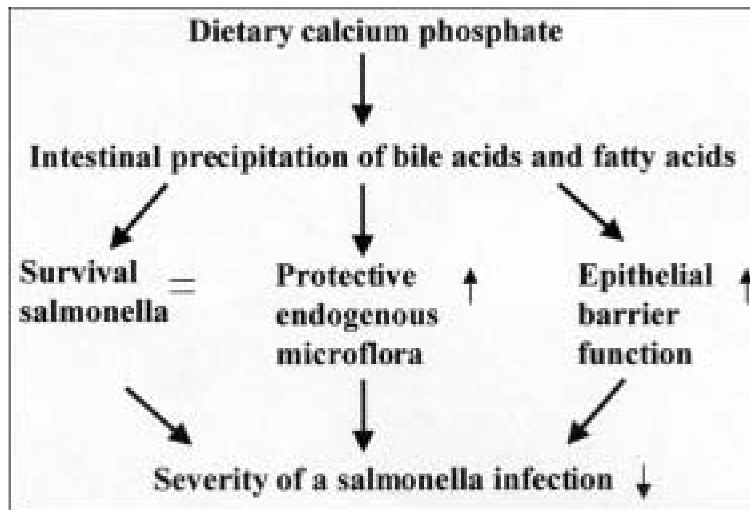

Figure 2: Mechanism by which dietary calcium phosphate may decrease the severity of an intestinal Salmonella infection.

for the observed beneficial effects of zinc supplementation (*Alam* et al., 1994; *Black*, 1998).

Until recently, the effects of calcium on the resistance to intestinal infections were unknown. At the same time, evidence accumulated showing the protective effects of dietary calcium on the development of colorectal cancer (*Baron* et al., 1999; *van der Meer* et al., 1997). A substantial amount of dietary calcium reaches the lower gut because the intestinal absorption of calcium is limited. After passing the stomach calcium forms an insoluble complex with dietary phosphate, which strongly adsorbs and precipitates intestinal surfactants, like bile acids and fatty acids. Bile acids and fatty acids are damaging to the intestinal epithelium and stimulate epithelial cell proliferation, which may increase the risk of colon cancer (*Govers* et al., 1996; *van der Meer* et al., 1997). We speculated that these cytoprotective effects of dietary calcium on the intestinal epithelium are not only relevant tot colon carcinogenesis but also have major implications for the resistance to intestinal infections. Diminishing epitheliolysis, by decreasing the cytotoxicity of gut contents, might strengthen the mucosal barrier. Conversely, increased epithelial cell damage provoked by dietary lectins or chemical irritants leads to disruption of the physical barrier function of the intestinal mucosa and gut-derived septicaemia, as shown by others (*Gardiner* et al., 1993; *Shoda* et al., 1995). By precipitating and thus inactivating intestinal bile acids, calcium may also indirectly affect the intestinal microflora. Bile is a classical supplement used in the preparation of several selective microbiological culture media to suppress contaminating flora. This indicates that bacterial species differ in their sensitivity to bile acids. Additionally, the restraining effect of bile in general on the intestinal microflora can be deduced from *in vivo* studies on obstructive jaundice. A reduced or absent bile flow results in intestinal bacterial overgrowth and an increased risk for endotoxaemia and septic complications. Oral administration of bile acids can normalise intestinal bacterial numbers and alleviate some of the symptoms in such cases (*Erbil* et al., 1999).

DIETARY CALCIUM AND RESISTANCE TO SALMONELLA INFECTION

We performed several animal studies to address whether dietary calcium indeed protects against intestinal infections. The proposed mechanism responsible for this protective effect is shown in Figure 2. *Salmonella enteritidis* was chosen as infective agent, because non-typhoidal salmonellosis is one of the most common, food-borne, bacterial infections in Europe and the United States (*Rampling*, 1993). Equally important, the infective dose and the development and pathology of salmonellosis in humans and rodents is largely similar and well described (*Salyers* and *Whitt*, 1994). Because *S. enteritidis* has invasive properties, dietary modulation of the resistance to colonisation as well as to translocation can be studied. Before performing dietary intervention studies, we first developed a new method to quantify intestinal bacterial translocation because classical organ cultures suffer from some major drawbacks. Bacteriological determination of pathogens in tissue samples (e.g. mesenteric lymph nodes) is invasive, only applicable as a one-point measurement, and rather insensitive considering that more than 99% of the bacteria is rapidly killed by the immune system upon translocation (*Gianotti* et al., 1993). Daily nitric oxide-derived urinary NO_x (sum of nitrite and nitrate) excretion appeared to be a sensitive marker to quantify intestinal bacterial translocation. Total translocation-induced urinary NO_x excretion highly correlated with weight of the mesenteric lymph nodes and its viable bacterial content (*Oudenhoven* et al., 1994). Moreover, determination of urinary NO_x might also be applicable to monitor bacterial sepsis in humans (*Krafte-Jacobs* et al., 1997).

After validation of this new translocation marker, strictly controlled infection experiments were performed to study whether dietary calcium protects against salmonella infection. Specific pathogen-free rats (n=8) were fed purified 'humanised' diets differing only in calcium phosphate content (20 and 180 µmol/g). After adaptation to the diets, they received a single oral infection with *S. enteritidis* (10^8-10^9 CFU) to mimic a food-borne intestinal infection. Calcium supplementation reduced the soluble bile acid and fatty acid concentration in ileal lavages and in faecal water, resulting in concomitantly decreased luminal cytotoxicity, as measured with a bioassay. This coincided with significantly higher numbers of lactobacilli in the intestinal tract: in the intestinal lumen as well as adhering to the intestinal mucosa (*Bovee-Oudenhoven* et al., 1999). Probably due to the absence of an extra outer-membrane, the Gram-positive lactobacilli appeared to be very sensitive to the bactericidal activity of bile acids and fatty acids, as shown in additional *in vitro* experiments. In contrast, the viability of a Gram-negative pathogen, like *S. enteritidis* was totally unaffected by physiologically relevant concentrations of these surfactants (*Bovee-Oudenhoven* et al., 1999). Compared with the low-calcium control group, the calcium-supplemented group shedded 10-1000 times less salmonella in their faeces indicating a significantly decreased intestinal colonisation of this pathogen (Figure 3). Even more important, the translocation of *S. enteritidis* was also significantly inhibited, considering the reduced urinary NO_x excretion in time (Figure 3). As shown in another study, the decreased infection-induced urinary NO_x excretion in

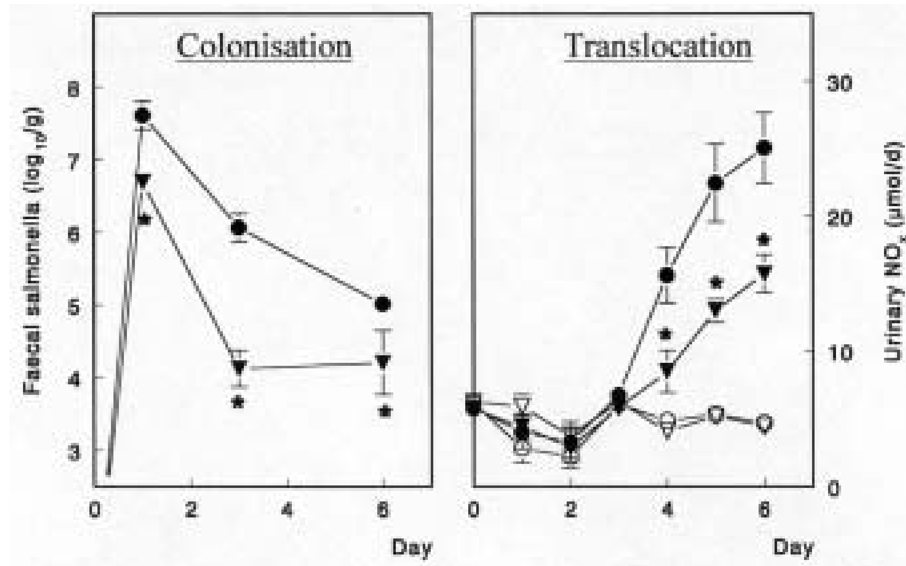

Figure 3: Effect of dietary calcium phosphate (CaP_i) on the colonisation and translocation of *Salmonella enteritidis* after oral infection of the rats (n=8) on day 0. The colonisation of salmonella was determined by measuring the faecal excretion of this pathogen in time and the translocation to extra-intestinal organs was quantified by analysis of urinary NO_x excretion. Symbols: ✿ infected control group, ✿ infected CaP_i-supplemented group, and the corresponding open symbols represent the non-infected groups. No salmonella was detected in faeces of non-infected animals. The asterisk indicates a significant difference from the control group ($p<0.05$).
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the calcium-supplemented groups correlated highly with the significantly less viable numbers of salmonella in ileal Peyer's patches and spleen (*Bovee-Oudenhoven* et al., 1997). The protective effect of calcium against salmonella infection was not only observed during administration of high amounts of calcium as usual in standard rodent diets (*Reeves* et al., 1993), but also during supplementation of 60 µmol calcium per g diet, which is more realistic for the human calcium intake in Western societies (*Becker* and *Kumpulainen*, 1991). Whether these beneficial effects of dietary calcium can also be extrapolated to humans is subject of current research of our lab.

CONCLUSION

Due to emerging problems of antibiotic resistance the need for alternatives to prevent and treat intestinal infections is growing. Though sound scientific evidence is still not very abundant, several *in vivo* experiments now show that the resistance to intestinal infections can be improved by changing the composition of the diet. Combined biochemical and microbiological *in vitro* experiments are very useful to elucidate the mechanisms responsible for protection. However, animal and finally human infection studies are essential to verify whether dietary components (including probiotics) indeed strengthen host resistance and protect against infectious disease.

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QUALITATIVE AND QUANTITATIVE DIFFERENCES BETWEEN SPECIES IN THE GUT IMMUNE SYSTEM: CONTROVERSIAL AND UNSOLVED ASPECTS

REINHARD PABST and HERMANN-JOSEF ROTHKÖTTER

Department of Functional and Applied Anatomy,
Medical School of Hannover, Hannover, Germany

SUMMARY

Many parameters of the gut immune system, such as the development of tolerance or protective immune responses, can only be studied in detail in experimental animals. Species differences have to be considered when such results are extrapolated to humans with their mixed genetic background, non-standardised eating conditions and variable exposure to microbial stimulation. In this review some of the major species differences in the gut immune system are discussed: the development, structure and function of Peyer´s patches show enormous differences, and the M-cells differ in number and reagents which label them. Recently discovered lymphoid structures in the gut wall, i.e. cryptopatches and lymphocyte-filled villi, have so far only been described in mice and humans & rats, respectively. Why are there huge species differences in the number of $\gamma\delta^+$ T-cell receptor intraepithelial lymphocytes? What is the function of the different antigen presenting cells in the gut wall, e.g. dendritic cells? The functional relevance of the unique cellular composition of the gut wall in early childhood for tolerance development or vaccination protocols has to be studied in more detail with respect to the role of cytokines, chemokines and the enteric nervous system. Despite the great advances in recent studies on the gut immune system there still seem to be many unsolved questions for future research in laboratory animals and even more in humans.

INTRODUCTION

The gut immune system has two diametrical functions: on the one hand oral tolerance to enormous numbers of nutritional and microbial antigens must be induced early in life (*Weiner*, 1997) and on the other hand specific immune reactions against bacteria, viruses and parasites must protect the body in future contact with these microbial aggressors. The basic concepts of these functions are more complex than previously thought (*McGhee* et al., 1992) and are of major relevance for the development of oral vaccines (*Shalaby*, 1995). Mice with all their well-characterised genetic parameters, transgenic and knockout techniques and wealth of reagents are most often used in characterising the basic mechanisms of tolerance induction and protective immune reactions. However, the species differ greatly, not only in eating habits (omnivora, carnivora and ruminants) but also in the size of the different sections of the gastrointes

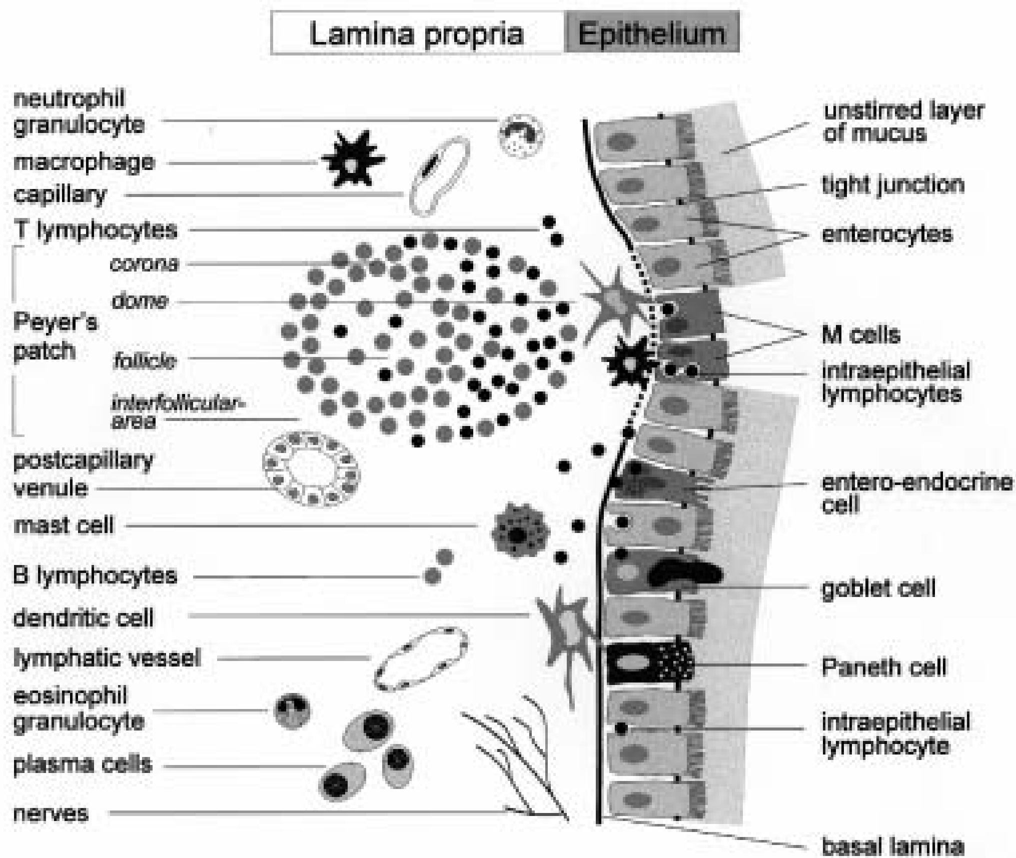

Figure 1: Schematic drawing of the composition of cells of the innate and specific immune system (modified after *Pabst* and *Rothkötter*, 1998).

tinal tract, as well as the amount and composition of bacteria in the gut lumen.

The gut wall consists of different compartments, each with a specific composition of cells of the immune system. The afferent branch of the mucosa-associated immune system is found in isolated follicles and Peyer´s patches, in which antigen is taken up by M-cells and immune reactions initiated. The efferent branch consists of effector T-lymphocytes in the epithelium and the lamina propria and effector cells of the B-lymphocyte family, the plasma cells (Figure 1).

Many aspects of immune reactions in the alimentary canal can only be studied by serial biopsies, local application of antigens and other experimental procedures, which can hardly be performed in humans. Here, we will give examples of species differences of the gut immune system at different ages which stresses the importance of always mentioning the species in studies on the intestinal immune system and the need for caution in extrapolating data from animals to humans.

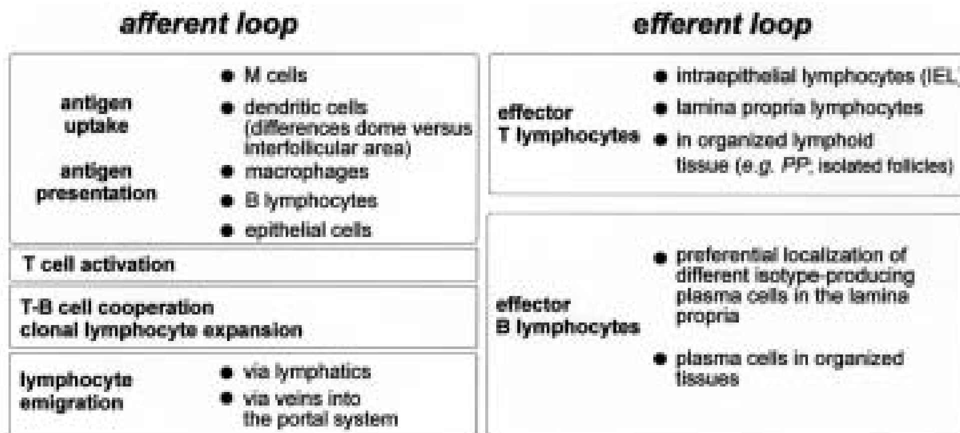

Figure 2: Comparison of the afferent and the efferent branch of the gut immune system

PEYER´S PATCHES DIFFER IN NUMBER, SIZE, AND FUNCTION BETWEEN SPECIES

Peyer´s patches (PP) consist of four well defined compartments: the follicles in several aspects resemble germinal centres in lymph nodes, tonsils or the spleen; the corona or mantle zone surrounds a follicle and consists of more mature B-lymphocytes; the interfollicular area contains mainly T-lymphocytes and can easily be identified by the high endothelial venules in the compartment; and finally the dome area with a mixture of T- and B-lymphocytes, dendritic cells and macrophages is found directly beneath the basal lamina. The most interesting cells in the epithelium of the dome are specialised epithelial cells, the M-cells (Figure 2).

In rats and mice PP develop after birth while PP are already found in the human foetus. In sheep a continuous PP in the ileum, which is up to 2 m in length, is fully mature in the foetus and shows an extremely high rate of lymphocyte production. This ovine ileal PP plays a critical role in B-lymphocytopoiesis: the animal´s B-cell repertoire is expanded by somatic hypermutations (for review see *Griebel* and *Hein*, 1996; *Reynolds* et al., 1999). In this respect the sheep's PP in the jejunum are totally different and more like human PP. *Butler* et al. (1996) proposed a concept of grouping species according to the development of the repertoire of B-lymphocytes: in primates and rodents a so far unknown organ is responsible for an antigen-independent development of the B-lymphocyte repertoire. In chicken, rabbits, ruminants and pigs, a B-cell repertoire develops in the PP in the terminal ileum. A further interesting aspect is the regression of the ileal PP in sheep from about 18 months and a similar disappearance in adult pigs, while the PP in the jejunum alter neither their size nor structure during ageing. Furthermore, the lymphocyte subset composition differs between the continuous ileal PP and the discrete jejunal PP (for review see *Pabst* and *Rothkötter*, 1999). A comparison of the development, lymphocyte subset composition and function between different species has been summarised by *Griebel* and *Hein* (1996).

All too often researchers focus on PP as entry sites for antigens and the isolated follicles in the intestinal lamina

propria are forgotten. In humans a total of 30,000 isolated follicles have been counted. Comparable data on isolated follicles in different species are urgently needed under defined microbial situations to calculate the total area of lympho-epithelium covering PP and isolated follicles. It has to be stressed that there is great variability of antigen uptake between the different dome areas of one PP, between individual PP and between animals bred and kept under comparable conditions (*Rothkötter*, *Pabst*, unpublished). It would be of great interest to synchronise the activity of M-cells along the small intestines before giving an oral vaccine.

MAJOR DIFFERENCES IN NUMBERS AND MARKERS OF M-CELLS

The relative number of M-cells in proportion to enterocytes reaches 50% in rabbits which is in contrast to about 10% in rats and mice and about 3.5% in humans (for review see *Gebert*, 1997). The uptake of many different bacteria, viruses or particles has been described (for review see *Gebert* et al., 1996). M-cells can hardly be identified by light microscopy and no generally accepted monoclonal antibodies exist for these cells. In addition to electron microscopy some markers have been described which can be used by light microscopy but without knowing why these markers differ between species: in rabbits M-cells are vimentin positive, in pigs cytokeratin 18 positive and in mice cytokeratin 8 identifies M-cells (reviewed by *Gebert*, 1997).

Meanwhile a long list of soluble or particulate tracers, viruses and bacteria has been described as being effectively transported by M-cells through the gut wall (for review see *Gebert* et al., 1996). The vast majority of these have only been taken up by M-cells in one species. It remains to be shown whether one can extrapolate from these specific species to all other mammals including humans.

LYMPHOID STRUCTURES RECENTLY FOUND IN THE GUT WALL

It was very surprising that only recently organised lymphoid structures other than PP and isolated follicles were discovered, since for generations anatomists, immunologists and pathologists have cut the gut wall and missed these structures. For mice, *Kanamori* et al., (1996) described the so-called "cryptopatches". These consist of about 1000 lymphoid cells around epithelial crypts in the small intestine and colon. The most interesting aspect is their subset composition: mostly negative for markers of B, CD3, CD4, CD8 but dependent on IL-7 and c-kit$^+$ (*Saito* et al., 1998). They are also found in nude and SCID mice and these structures obviously play a central role in the development of the gut intraepithelial lymphocytes (*Oida* et al., 2000). It has recently been suggested that cryptopatch-like structures might have been conserved in the animal kingdom as a local source of intestinal T-cells during the evolution of vertebrates (*Nanno* et al., 1999).

A further lymphoid structure of the gut wall in humans only recently de-

scribed and characterised are the "lymphocyte-filled villi" (LFV) (*Moghaddami* et al., 1997). These structures in the human small intestine contain MHC-class II$^+$ dendritic cells, many memory T-lymphocytes, few B-cells and no evidence of immature lymphocytes expressing c-kit or CD1a. Thus, the cellular composition is totally different from that of cryptopatches in mice. In rats LFV have also been described: In new-born rats the lymphoid cells expressed neither surface immunoglobulin nor CD3, but a different more mature lymphocyte composition has been described for 4-5 week old rats (*Mayrhofer* et al., 1999). It has been speculated that in species with a long life span as in humans, LFV might have different functions in different phases of life. In a recent short article *Kanamori* et al. (2000) discussed the similarities and differences between cryptopatches and LFV and indicated the field of future research on these burning issues.

The obvious questions for future research programs are: are there cryptopatch-like structures in species other than mice? Are LFV unique to rats and humans? What are their size, number, and cellular composition at different ages in humans? Which role do these structures play in the gut immune system?

WHY ARE THERE SUCH DIFFERENT NUMBERS OF INTRA-EPITHELIAL LYMPHOCYTES EXPRESSING THE γδ T-CELL RECEPTOR?

The proportion of $\gamma\delta^+$ IEL has been described as about 10% in humans, approx. 50% for bovine gut and a huge variation in mice (20 - 80%). Age, antigen-exposure and the presence of the thymus all influence the number of $\gamma\delta^+$ IEL (for review see *Mowat* and *Viney*, 1997). *Lefrançois* and *Puddington* (1999) have recently summarised the great species differences with respect to number, subset composition and functional role of the IEL. It remains to be studied which immune functions of the gut wall might be influenced by the presence or absence of certain subsets of IEL. It can be predicted that chemokines will play a central role in lymphocyte migration to the different compartments of the gut wall (e.g. *Sallusto* et al., 1999; *Mackay*, 1999). In a recent study nearly all lymphocytes in the lamina propria and the epithelium of the human gut expressed the CXCR3 and CCR5 receptors, which are associated with TH1-lymphocytes. The pattern of chemokine receptors seems to be typical for the gut wall and during intestinal inflammation an upregulation is observed (*Agace* et al., 2000).

The exact function of γδ IEL was long under debate. In respect to the interaction of gut epithelial cells and IEL some recent findings are of interest. *Boismenu* (2000) summarised *in vitro* and *in vivo* data, demonstrating an important role of IEL in stimulating gut epithelial cells to divide and thus repair epithelial damage via keratinocyte growth factor (KGF). These protective effects of γδ IEL by inducing re-epithelialisation in a mouse model of colitis were conclusively shown by in situ hybridisation and PCR of KGF in γδ IEL. Thus, there is growing evidence that not only epithelial cells regulate IEL function by secreting cytokines or processing and presenting antigen to IEL (*Hershberg* and *Mayer*, 2000) but also γδ IEL stimulate epithelial cell growth by recognising self-antigens expressed by damaged epithelial cells.

ANTIGEN PRESENTATION IN THE GUT WALL

The number and function of antigen-presenting cells in the gut wall differs depending on the markers and species studied. Much more is known about dendritic cells, their antigen uptake and transport to regional lymph nodes in the respiratory tract (summarised by *Stumbles* et al., 1999). The number of dendritic cells in the epithelium of the trachea increased several hundred folds within two days after exposure to viruses, live or killed bacteria and to a lesser degree after chronic exposure to dust. These dendritic cells then migrated to the draining bronchial lymph nodes. There are fewer data on dendritic cells in the lamina propria of the gut and Peyer´s patches (*Liu* and *McPherson*, 1995; *Kelsall* and *Strober*, 1996; *Maric* et al., 1996). *Ruedl* and *Hubele* (1997) described the maturation of dendritic cells by cytokines of PP in the rat. A surprising finding was that in pigs endothelial cells of vessels in the lamina propria were strongly MHC-class II^+ (*Bailey* et al., 1996). It can be questioned why these cells express MHC-class II and what functional relevance it might have in this species. The antigen presentation in respect to mucosal pathogens has been summarised by *Reyes* et al. (1997).

IgA-PRODUCING PLASMA CELLS IN THE GUT WALL HAVE DIFFERENT ORIGINS

Undoubtedly IgA is the prevailing antibody class produced by plasma cells in the gut lamina propria. In the human small intestine about 80% and in the colon 90% of all plasma cells are IgA^+ (IgA_2 approx. 66% in contrast to only 7% IgA_2 in the nasal mucosa) (for review see *Brandtzaeg* and *Farstad*, 1999). For years the concept was accepted that the IgA-producing cells all originate from B-cells stimulated in the gut wall, emigrate via lymphatics and return preferentially to the gut lamina to mature to IgA-producing plasma cells. Meanwhile, there are growing numbers of studies mainly in mice, differentiating the IgA precursor B-lymphocytes into B1- and B2-cells. The $CD5^+$ B1-cells are found in the peritoneal cavity and produce a more unspecific IgA in the gut wall (*Su* and *Tarakhovsky*, 2000), while the B2-cells are the precursors of the classical IgA-producing plasma cells (*Kroese* et al., 1994). In a very recent study *Hiroi* and *Kiyono* (2000) summarised their data showing that IL-15 secreted by enterocytes (as shown for mice, rats and humans) induces the differentiation of B1-lymphocytes and only B1-cells have the IL-15 receptor. The differentiation of B2-cells in contrast is dependent on IL-5.

POSTNATAL DEVELOPMENT OF THE GUT IMMUNE SYSTEM

There are major species differences in the early postnatal development of the gut barrier. In species with a multilayered placenta, such as pigs and sheep, the animals are born without antibodies, and these have to be absorbed postnatally from the colostrum. The phenomenon of the so-called gut closure is still ill-defined for many species. Cytokines are critical for the development of PP as shown in knockout mice, e.g. $TNF^{-/-}$ and $lymphotoxin^{-/-}$ animals show obvi-

ous defects (for review see *Mowat* and *Viney*, 1997). Once more details about these regulatory factors are known, it might be possible to stimulate the development of PP with active M-cells before an oral vaccination. In the human foetal gut 2 IEL with the αβ receptor per 100 enterocytes were found. IEL with the γδ receptor are hardly seen before birth but later about 10% of the IEL express this marker (*Cerf-Bensussan* and *Guy-Grand*, 1991). It is still unclear how much the entry of lymphocytes and how much local proliferation contribute to the expansion of IEL after birth and after antigen exposure. In mice and pigs IEL incorporate the DNA analogon bromodeoxyuridine (*Penney* et al., 1995; *Rothkötter* et al., 1999) which seems not to be the case in humans. Further experiments have to clarify the role of age, nutritional and microbial factors which regulate the number of IEL in the many different species.

In all developmental aspects the balance between entry and exit, local proliferation and apoptosis have always to be considered when explaining an increase or decrease of cell numbers in a compartment (*Pabst* and *Rothkötter*, 1999). Further details on the role of adhesion molecules and lymphocyte traffic to the gut can be found in the reviews by *Pabst* and *Westermann* (1997) and *Dunkley* et al. (1995).

In conclusion, the many species differences in the number, structure and function of immune cells in the gut and the effect of nutritional and microbial antigens on the barrier function have to be studied in much more detail. Extrapolation of data from species living under very artificial SPF conditions in animal breeding facilities should only be extrapolated to the situation in humans with great caution.

ACKNOWLEDGEMENTS

The authors own studies were supported by the Deutsche Forschungsgemeinschaft (SFB 280, C1). The preparation of the figures by Marita Peter and the correction of the English by Sheila Fryk are gratefully acknowledged.

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M-CELLS AND THEIR ROLE IN INFECTION: A REVIEW

MARK A. JEPSON[1] and M. ANN CLARK[2]

[1]Cell Imaging Facility and the Department of Biochemistry,
University of Bristol, Bristol, UK.
[2]Department of Physiological Sciences, University of Newcastle upon Tyne,
Newcastle upon Tyne.

SUMMARY

M-cells are located in the epithelia overlying mucosal tissues where they function as the antigen sampling cells of the mucosal immune system. Paradoxically, M-cells are also exploited by a range of pathogens as a route of host invasion. Despite their pivotal roles in immunity and infection, the mechanisms responsible for micro-organism interaction with M-cells and the origin and developmental pathway of these specialised cells has, until recently, remained obscure. Now, the availability of improved methods for identifying M-cells coupled with a variety of modern research tools is permitting more detailed analysis of M-cell origin and function. The majority of evidence now suggests M-cells primarily arise from undifferentiated crypt cells under stimulation from the local lymphoid environment. However, under appropriate antigenic stimulation, enterocytes may also contribute to the M-cell population. The mechanisms by which pathogens target M-cells are now also partially defined and it has become clear that some of the microbial infection mechanisms previously defined *in vitro* similarly mediate M-cell infection. However, it also appears that M-cell invasion may proceed via additional routes not previously identified by *in vitro* studies. Furthermore, it is clear that M-cells, which constitute only a minute proportion of the gastro-intestinal epithelium, are not the sole site of microbial translocation from the gut. These findings indicate that many micro-organisms possess multiple alternate routes of intestinal epithelial invasion; reflecting the critical importance of this initial step in microbial infection. In this paper we review the current state of knowledge about the origin of M-cells and their role in pathogen invasion.

INTRODUCTION

Membranous epithelial (M-) cells transport antigens across mucosal epithelia to the underlying lymphoid tissues where protective immune responses are initiated (*Gebert* et al., 1996; *Neutra* et al., 1996; *Gebert*, 1997). Paradoxically, some micro-organisms exploit M-cells as a route of host invasion. The bulk of our knowledge of M-cells has been derived from studies of those present in the follicle-associated epithelium (FAE) overlying isolated and aggregated lymphoid follicles in the intestinal tract. The proportion of M-cells in the FAE

varies from 10% in humans and mice to 50% in rabbits and 100% in the terminal ileum of pigs and calves. The existence of cells analogous to M-cells has also been reported in the epithelia of a variety of other mucosal tissues in a diverse range of species. However, it should also be borne in mind that the presence of functional equivalents of M-cells at some of these sites has been disputed (for a critical review of the evidence for and against M-cell analogues at extra-intestinal sites see *Gebert* and *Pabst*, 1999).

In writing this review, it is not our intention to provide a thorough description of the morphology and function of M-cells or a comprehensive list of the pathogens suggested to utilise the M-cell route of infection; these topics have been covered extensively in other reviews (*Gebert* et al., 1996; *Neutra* et al., 1996; *Siebers* and *Finlay*, 1996; *Gebert*, 1997; *Owen*, 1998a; *Sansonetti* and *Phalipon*, 1999). Instead, we will focus primarily on recently published data that has advanced knowledge of M-cell function and development, and, most importantly, their role in infection. In considering these topics, we shall specifically concentrate on intestinal M-cells and their role in bacterial infection.

M-CELL CHARACTERISTICS AND THEIR IDENTIFICATION

The most important characteristic of M-cells is of course their ability to transport material across epithelial barriers (*Gebert* et al., 1996; *Jepson* et al., 1996; *Neutra* et al., 1996; *Gebert*, 1997). In addition to possessing an increased endocytic capacity for a broad range of materials, ultrastructural analysis has suggested that M-cells may have a reduced degradative capacity. In comparison to neighbouring enterocytes, intestinal M-cells generally have less organised brush borders which may promote interaction of material with the apical membrane. M-cells also have a characteristic basolateral invagination that harbours leukocytes. This provides a short-cut for transcytosis of antigens and permits their rapid delivery to lymphoid tissue where a mucosal immune response may be induced. These well-established morphological features of M-cells not only have implications to their function as antigen-sampling cells but also facilitate their identification by transmission electron microscopy (TEM). However, since TEM studies tend to focus on small numbers of cells they are not always the most appropriate means of examining a cell type such as M-cells, which constitute a relatively minor cell population. Furthermore, the interaction of bacteria and inert particles varies considerably both between and within individual Peyer's patches and regions of the FAE, necessitating the examination of numerous M-cells (*Clark* et al., 1994a, 1998a; *Frey* et al., 1996). The unusual brush border morphology of M-cells also facilitates their identification by scanning electron microscopy (SEM) which readily allows examination of almost the entire FAE and thus a greater proportion of the M-cell population. However, SEM only allows a view of the epithelial surface so is limited to studies of the binding of material to M-cells and any subsequent alterations in surface morphology. It yields little information about the uptake of material by M-cells and its subsequent fate. For these reasons, considerable effort has been put into developing methods of identifying M-cells at the light microscopical level. The most widely used cytochemical methods for M-cell identification rely on their relative lack of alkaline phosphatase (e.g.

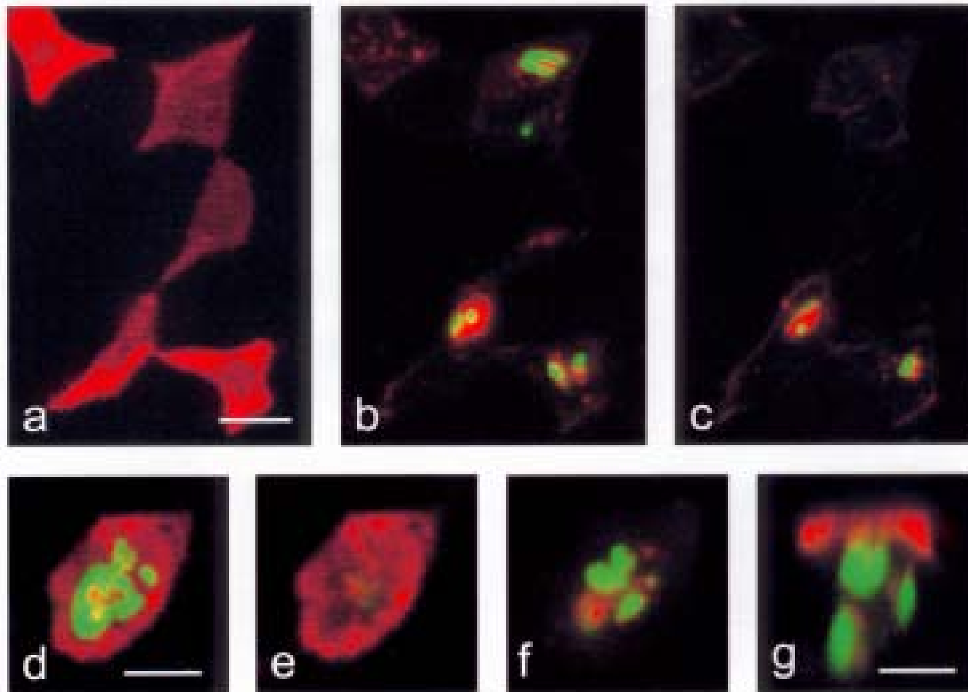

Figure 1: Confocal laser scanning microscope images of mouse Peyer's patch FAE from gut loops infected for 60 min with wild type *Y. pseudotuberculosis* strain YPIII/pIB1 constitutively expressing green fluorescent protein (GFP). The bacteria were grown in Luria Bertani (LB) broth at 26°C in a shaking incubator, and inoculated at a concentration of 2 x 10^9 CFU/ml directly into the gut loops. After tissue fixation, M-cells were localised with the lectin *Ulex europaeus* 1 (red).
(a)-(c): multiple bacteria are observed at 2 µm (b) and 4 µm (c) below the M-cell apical surfaces (a).
(d)-(g): a single M-cell is invaded by numerous bacteria. In (d), images from 0 to 8 µm depth have been projected to give a single image to show that very large numbers of bacteria are associated with this M-cell. Bacteria are absent from the M-cell surface (e), but multiple invaded bacteria are located at 3 µm below this level (f). Multiple invaded bacteria are similarly observed when this cell is viewed in vertical (x-z) section (g).
Scale bars = 5 µm.

Savidge et al., 1991; *Savidge*, 1996) or on their atypical expression of intermediate filament proteins (*Gebert* et al., 1992, 1994; *Jepson* et al., 1992) or glycoconjugates (*Clark* et al., 1993, 1994b, 2000; *Gebert* and *Hach*, 1993; *Jepson* et al., 1993a; *Giannasca* et al., 1994). Of these techniques, the positive cytochemical markers provided by antibodies to intermediate filaments and by lectins are more reliable and most used (e.g. Figure 1). However, there are some problems with the application of these markers which have been discussed extensively elsewhere (*Jepson* et al., 1996; *Jepson* and *Clark*, 1998; *Clark* et al., 2000). Specifically, each marker is only applicable to a limited range of species and/or sites due to great variation between different populations of M-cells (*Jepson* et al., 1993a, 1995a,b; *Clark* et al., 1994b; *Giannasca* et al., 1994). In addition, reliable positive markers for human M-cells are currently lacking. However, some lectins predominantly label human FAE al-

though without clear M-cell selectivity (*Jepson* et al., 1996; *Sharma* et al., 1996). Recent evidence has shown that the sialyl Lewis A antigen is preferentially expressed on a subset of human intestinal M-cells, although some expression on FAE enterocytes and goblet cells was also reported (*Giannasca* et al., 1999).

DEVELOPMENT AND REGULATION OF THE M-CELL POPULATION

There is controversy regarding whether M-cells can be formed from fully differentiated enterocytes within the follicle-associated epithelium (FAE) or whether their differentiation is pre-programmed in the crypts that supply epithelial cells to both the FAE and adjacent villi (*Gebert* and *Posselt*, 1997; *Gebert* et al., 1999; *Niedergang* and *Kraehenbuhl*, 2000). In either case, it can be assumed that local factors associated with the lymphoid tissue environment trigger M-cell development. There is now considerable evidence from studies of immunodeficient and knockout mice that B-lymphocytes are the predominant cell population involved in Peyer's patch development (*Savidge*, 1996; *Debard* et al., 1999; *Niedergang* and *Kraehenbuhl*, 2000). Although B-cells appear to promote M-cell development, the presence of small numbers of M-cells in B-cell knockout mice suggests that other cells may substitute for B-cells in promoting M-cell development (*Debard* et al., 1999; *Golovkina* et al., 1999). The role of lymphocytes in M-cell development is also supported by reports that co-culture of an intestinal epithelial cell-line (Caco-2) with Peyer's patch lymphocytes induces the epithelial cells to develop certain characteristics of M-cells (*Kernéis* et al., 1997). In this model B-lymphocyte cell lines, but not T-lymphocyte lines, were able to partially substitute for the Peyer's patch cells in inducing M-cell-like cells (*Kernéis* et al., 1997). Further analysis of knockout mice and *in vitro* models should permit characterisation of the factors stimulating M-cell development.

The finding that co-culture with lymphocytes converts Caco-2 cells into cells with characteristics of M-cells has been cited as evidence that differentiated enterocytes can also be transformed into M-cells. However, the fact that Caco-2 cells also retain crypt cell-like properties (e.g. the ability to divide) has led to others questioning whether similar transformations of enterocytes to M-cells occur *in vivo* (*Owen*, 1998b). Indeed, the bulk of evidence now indicates that the development of M-cells normally starts in the FAE-associated crypts. For example, lectin cytochemistry has shown that in mouse Peyer's patches and rabbit caecum, cells with characteristic lectin-binding signatures of M-cells are arranged in radial stripes within the FAE (*Clark* et al., 1993; *Gebert* and *Posselt*, 1997) and, in the mouse, emerge from morphologically specialised crypts (*Gebert* et al., 1999). These observations suggest that a proportion of the cells emanating from certain crypts is pre-programmed to develop into M-cells. Furthermore, a subpopulation of cells within FAE-associated crypts in rabbit caecum (*Gebert* and *Posselt*, 1997) and mouse Peyer's patch (Giannasca et al., 1994; Gebert et al., 1999) already displays lectin-binding patterns typical of mature M-cells. Despite these data we cannot exclude the possibility that, under appropriate conditions such as antigenic stimulation,

some enterocytes may retain an ability to differentiate into M-cells. For example, it has been reported that *Salmonella typhimurium* infection rapidly (12-24 h.) increases the population of alkaline phosphatase-negative cells throughout the FAE of mouse Peyer's patches, an observation consistent with new M-cells being formed from enterocytes (*Savidge* et al., 1991). Although this phenomenon has yet to be confirmed using positive cytochemical markers or functional definition of mouse M-cells, another recent report suggests that *Streptococcus pneumoniae* can induce rapid (within three hours) formation of M-cells in rabbit Peyer's patches (*Meynell* et al., 1999). In this study the presence of M-cells was inferred from measurement of increased microsphere binding and transcytosis following *Streptococcus* infection and tentatively confirmed by SEM (*Meynell* et al., 1999). *Borghesi* and co-authors (1999) subsequently confirmed by TEM analysis that newly formed M-cells appeared within one hour of *Streptococcus* infection. Interestingly, in this latter study newly formed M-cells were limited to the periphery of the dome suggesting the presence of an immature, uncommitted, epithelial cell population that retains the ability to switch to an M-cell phenotype. These observations on the development of M-cells have suggested the intriguing possibilities that M-cells may represent a highly regulated cell-population and that plasticity in the M-cell population might be exploited by pathogens (*Kernéis* and *Pringault*, 1999).

It is well established that cells with the morphological and functional characteristics of M-cells predominate in the periphery of rabbit and mouse FAE (e.g. *Savidge* et al., 1991; *Clark* et al., 1993; *Jepson* et al., 1993b,c; *Gebert* et al., 1996, 1999; *Gebert* and *Posselt*, 1997; *Borghesi* et al., 1999). The application of positive cytochemical markers has revealed that individual characteristics which are strongly expressed by peripheral M-cells disappear at different rates during maturation and migration to the dome apex (*Jepson* et al., 1993a; *Gebert* and *Posselt*, 1997), demonstrating that the M-cell phenotype is not static. It has also been suggested that the M-cell phenotype is transiently expressed and does not represent an end-point of epithelial cell differentiation. This latter view is supported by studies on mouse and chicken FAE which failed to detect apoptic cells displaying M-cell-specific cytochemical markers, apoptosis being restricted to the dome apex (*Debard* et al., 1999; *Takeuchi* et al., 1999; *Niedergang* and *Kraehenbuhl*, 2000). However, it should also be noted that other studies have not detected intermediate cell types close to the dome apex of rabbit Peyer's patch FAE: the presence of such cells would be expected if M-cells were to revert to enterocytes prior to their undergoing apoptosis and extrusion (*Borghesi* et al., 1999). Thus, significant controversies remain over the fate of M-cells, as is also the case with theories concerning the origin of M-cells and their relationship to FAE enterocytes. These issues have important implications for our understanding of the role of M-cells and the function and pathology of mucosa-associated lymphoid tissues.

INTERACTION OF MICRO-ORGANISMS WITH M-CELLS

As outlined previously, M-cells appear to be an important site of invasion by a diverse range of pathogenic bacteria, viruses and protozoa (*Gebert* et al., 1996; *Siebers* and *Finlay*, 1996; *Jepson* and *Clark*, 1998; *Sansonetti* and

Phalipon, 1999). Some pathogens, e.g. *Salmonella* and *Yersinia* (see below) selectively interact with M-cells while others, e.g. *Vibrio cholerae* exhibit tropism to the Peyer's patch FAE without clear preference for M-cells over enterocytes. In some species, including mice and rats, apathogenic segmented filamentous bacteria (SFBs) preferentially colonise Peyer's patch FAE, though without apparent preference for M-cells (*Klaasen* et al., 1992; *Jepson* et al., 1993d). In other species such as rabbit, the villi appear to be the preferred site of SFB attachment (*Heczko* et al., 2000a). Understanding the mechanisms by which micro-organisms selectively target and/or invade M-cells is a topic of considerable importance because it may ultimately permit the development of novel disease control strategies based on the prevention of initial pathogen invasion. In addition, it may prove possible to exploit microbial invasion pathways for mucosal drug and vaccine delivery.

Possible determinants of M-cell targeting and invasion

The study of cultured epithelial cells has provided a wealth of information on the adherence and invasion mechanisms employed by pathogenic bacteria (*Finlay* and *Cossart*, 1997; *Finlay* and *Falkow*, 1997). Such studies have revealed that bacteria employ diverse strategies to modulate host cell function involving, for example, intracellular signalling, cytoskeletal changes and cytokine release (*Finlay* and *Cossart*, 1997; *Finlay* and *Falkow*, 1997; *Wilson* et al., 1998). Some of these strategies are likely to be employed during the interaction of micro-organisms with M-cells, particularly since some of the events observed during bacterial/M-cell interactions appear to mimic the changes observed *in vitro*. However, it is also clear that additional mechanisms contribute to the interaction of micro-organisms with this specialised epithelial cell type. Some of these mechanisms may involve specific, microbial-mediated events whist others may be independent of active bacterial adhesive/invasive strategies and represent non-specific, M-cell-mediated transport. In recent years it has become increasingly clear that many pathogens, including *S. typhimurium* and *Yersinia* species possess multiple alternate mechanisms for M-cell invasion, a phenomenon which is perhaps unsurprising in view of the importance of the initial invasion step to the pathogenicity of these micro-organisms. Such a multiplicity of invasion mechanisms may permit the bacteria to invade the intestinal epithelial barrier under a wide variety of environmental and gut luminal conditions.

M-cells are able to transport inert particles (*Jepson* et al., 1993c,e, 1996; *Gebert* et al., 1996) and it has, therefore, been suggested that pathogens may similarly interact with M-cells via non-specific, passive mechanisms. Such mechanisms are likely to be influenced by the physical properties of the pathogen, as M-cell interactions with inert particles are influenced by the physicochemical properties of the particle preparations and also by species-related variations in M-cell surface properties (*Jepson* et al., 1993e, 1996). M-cell targeting by inert materials and bacteria may also be promoted by the reduced quantities of mucus overlying Peyer's patches and by the relatively poorly developed M-cell surface carbohydrate coat (glycocalyx) (*Gebert* et al., 1996; *Siebers* and *Finlay*, 1996; *Neutra* et al., 1999). Experiments with a range of cholera toxin B subunit conjugates have demonstrated that the glycocalyx acts as a size-selective barrier that limits access of particles, over a broad size range, to apical membrane glycolipids

of rabbit enterocytes. The reduced M-cell glycocalyx permits access of the smaller of these particles (sub-bacterial size) to the same membrane components (*Frey* et al., 1996). These data suggest that the M-cell glycocalyx, whilst thinner than that of enterocytes, may still limit the interaction between particles of bacterial size and the cell apical membranes. The precise effect of this phenomenon on bacterial interaction with cellular receptors is uncertain.

It is now clear that specific mechanisms also mediate M-cell targeting by micro-organisms, and recent studies have attempted to identify the bacterial and M-cell determinants responsible for these interactions. Several studies have examined whether bacterial proteins which mediate invasion of cultured cells also promote M-cell invasion. In some cases, these studies have demonstrated that modification of genes encoding *in vitro*-characterised invasion determinants significantly attenuates bacterial invasion via M-cells (*Jones* et al., 1994; *Sansonetti* et al., 1996; *Marra* and *Isberg*, 1997; *Penheiter* et al., 1997; *Clark* et al., 1998a,b; *Jepson* and *Clark*, 1998; *Sansonetti* and *Phalipon*, 1999). However, mutation of some bacterial genes has a less dramatic effect on M-cell invasion than that observed for cultured cells, indicating that multiple mechanisms contribute to M-cell entry. It is currently uncertain whether these include invasion mechanisms unique to the *in vivo* situation. The bacterial proteins which mediate M-cell targeting have now been identified for some bacterial species, although the identity of the corresponding M-cell surface receptors remains less certain. It has been hypothesised that components of the M-cell glycocalyx mediate M-cell targeting by micro-organisms, and that the site- and species-related variations in this glycocalyx may contribute to tissue tropism exhibited by pathogens (*Clark* et al., 1994b; *Giannasca* et al., 1994; *Jepson* and *Clark*, 1998; *Neutra* et al., 1999). This hypothesis awaits rigorous investigation. Interestingly, recent studies have investigated whether inert materials (e.g. vaccine preparations) might be targeted to M-cells using ligands that selectively bind to M-cell-specific surface carbohydrates (*Clark* et al., 2000). This approach has proved successful for targeting inert particles and/or macromolecules for uptake by M-cells in mouse Peyer's patches (*Giannasca* et al., 1994; *Clark* et al., 1995; *Chen* et al., 1996; *Foster* et al., 1998) and hamster nasal lymphoid tissue (*Giannasca* et al., 1997).

Salmonella

The interaction of *Salmonella typhimurium* with intestinal M-cells has received considerable attention in recent years, due in part to the potential importance of this bacterium as a live oral vaccine delivery vehicle. Studies utilising a mouse gut loop model have demonstrated that *S. typhimurium* selectively targets to and invades mouse M-cells (*Clark* et al., 1994a; *Jones* et al., 1994). Invasion of murine M-cells is accompanied by M-cell destruction and sloughing of the FAE (*Jones* et al., 1994; *Pascopella* et al., 1995; *Clark* et al., 1998b; *Jensen* et al., 1998; *Jepson* and *Clark*, 1998). *S. typhimurium* infection of calf ileal loops similarly results in rapid invasion and destruction of M-cells and is accompanied by widespread FAE damage (*Frost* et al., 1997). The epithelial damage induced by *Salmonella* is likely to have serious consequences in naturally infected animals as it will result in unrestricted bacterial invasion and may explain the frequent occurrence of intestinal ulcerations and perforation in typhoid patients. In addition, it is essential that *Salmonella*-based oral vaccines are designed to minimise epithelial damage.

It has been demonstrated in the mouse gut loop model that the extent of *Salmonella* invasion and associated epithelial damage is dependent on the composition of the inoculum. Bacteria suspended in Luria-Bertani (LB) broth initiated extensive FAE damage, whereas damage was negligible when bacteria were inoculated in phosphate-buffered saline (*Clark* et al., 1998b). These findings suggest that local environmental factors within the gut lumen may influence the course of *Salmonella* infections. The regulation of *Salmonella* invasion and FAE destruction is not well understood, although one study has indicated that FAE destruction, but not M-cell invasion, is promoted by the regulator SlyA (*Daniels* et al., 1996). It has also been reported that mutants lacking DNA adenine methylase fail to evoke significant M-cell cytotoxicity - a property which might contribute to their enhanced ability to promote immune responses (*Garcia-del Portillo* et al., 1999; *Heithoff* et al., 1999).

The mechanisms responsible for M-cell invasion by *Salmonella* await precise identification, although a number of factors that may contribute to this process have been identified. For example, the invasion machinery encoded by *Salmonella* pathogenicity island 1 (SPI1) which is essential for the efficient invasion of cultured cell lines is also one of the factors that contributes to M-cell invasion by *S. typhimurium* (*Jones* et al., 1994; *Penheiter* et al., 1997; *Clark* et al., 1998b). When inoculated in LB, SPI1-deficient *S. typhimurium* mutants exhibit reduced levels of M-cell invasion compared with wild-type bacteria (*Clark* et al., 1998b). However, these mutants still invade M-cells in significant numbers (*Clark* et al., 1996, 1998b), and M-cell invasion is accompanied by membrane remodelling (ruffling) similar to that induced by wild-type *S. typhimurium* both *in vitro* and *in vivo*. Consistent with these observations, SPI1 mutants readily kill mice following oral infection although they exhibit 10-100-fold higher oral LD_{50} values than wild-type organisms (*Penheiter* et al., 1997). Interestingly, SPI1 mutants consistently fail to induce M-cell damage or FAE destruction (*Jones* et al., 1994, *Penheiter* et al., 1997; *Clark* et al., 1998b). At present it is uncertain whether this phenomenon is solely a consequence of insufficient bacterial invasion or whether SPI1-encoded proteins also have a direct role in the destructive process.

It has been suggested that M-cell targeting by *S. typhimurium* is mediated by a specific adhesin, namely the long polar fimbria (LPF) encoded by the *lpf* operon (*Bäumler* et al., 1996). This proposal was initially based on the observation that *lpfC* mutants exhibit reduced colonisation of Peyer's patches and moderately increased oral LD_{50} values compared with wild-type bacteria. Further evidence that LPF may target bacteria to M-cells came from the finding that incorporation of the *lpf* operon into non-piliated *Escherichia coli* enhanced their uptake into Peyer's patches (*Bäumler* et al., 1996) and from observations on the phase variation of the *lpf* operon which demonstrated that greater numbers of *S. typhimurium* recovered from Peyer's patches were of the phase-on phenotype (*Norris* et al., 1998). It is clear, however, that LPF is not the sole adhesin involved in *Salmonella* infection, as strains carrying mutations in *lpfC* or both *lpfC* and *invA* (a critical component of SPI1) still kill mice, albeit with increased oral LD_{50} values (*Bäumler* et al., 1996, 1997). Consistent with this observation, our own preliminary studies have demonstrated that *S. typhimurium* strains carrying mutations in *lpfC* or both *lpfC* and *invA* still enter murine M-cells (*Jepson* and *Clark*, 1998), and recent studies have also

demonstrated that mutation of *lpfC* has no measurable effect on the mucosal immune response to *S. typhimurium* following oral inoculation (*Vazquez-Torres* et al., 1999). Furthermore, the distribution of the *lpf* operon is not closely correlated with the ability of Salmonella strains to invade M-cells in murine gut loops, because it is absent from *Salmonella typhi*, which invades murine M-cells in this model, and is present in *Salmonella gallinarium*, which does not (*Pascopella* et al., 1995; *Bäumler* et al., 1997). Multiple fimbrial operons contribute to *Salmonella* virulence (*van der Velden* et al., 1998), and it is now clear that neither the *lpf* operon nor SPI1 are the sole determinants of intestinal invasion by *S. typhimurium*. It is perhaps not surprising that there appears to be a degree of redundancy in the mechanisms employed by *Salmonella* to enter M-cells and to breach the intestinal epithelial barrier since this is arguably the most critical event contributing to *Salmonella* virulence.

It must be noted that M-cells constitute a minute proportion of the intestinal epithelia (estimated at around 0.01%). The intestinal surface area available for bacterial translocation will, however, exhibit a localised increase if, as in the case of *Salmonella* infection, M-cell invasion is accompanied by FAE destruction. In addition, extra-intestinal dissemination of enteropathogens such as *Salmonella* is also likely to involve translocation at other non-FAE sites. Indeed, the first reports of *Salmonella* invasion of gut epithelia described entry into guinea pig enterocytes (*Takeuchi*, 1967) and subsequent reports in species other than mice have consistently drawn attention to enterocyte invasion (*Wallis* et al., 1986; *Frost* et al., 1997; *Bolton* et al., 1999a,b). Recently it has been proposed that an M-cell-independent route of extra-intestinal dissemination of *Salmonella* and possibly other enteropathogens is mediated by CD18-expressing phagocytes. It has been suggested that these phagocytes may either engulf luminal bacteria at the apical epithelial surfaces or engulf bacteria which have gained access to the lamina propria via paracellular pathways (*Vazquez-Torres* et al., 1999; *Vazquez-Torres* and *Fang*, 2000). The proposed role of this CD18-mediated route was based on the observation that, after oral administration, dissemination to spleen and liver by an attenuated *S. typhimurium* mutant (lacking *aroA*, *lpfC*, *invA*) was reduced in CD18 knockout mice compared to congenic controls. In addition, following inoculation of ligated ileal loops, invasion deficient (lacking *aroA*, *invA*) bacteria expressing green fluorescent protein (GFP) were observed within CD18-expressing phagocytes located in the villous lamina propria (*Vazquez-Torres* et al., 1999). It was further proposed that the ability of SPI1 deficient mutants to elicit a serum IgG (but not a mucosal IgA) response might be a consequence of CD18-mediated bacterial dissemination in the absence of the M-cell mediated bacterial uptake required for a mucosal response (*Vazquez-Torres* et al., 1999). The implication that M-cells play no part in this CD18-mediated route was based on the assumption that *lpfC/invA* mutants cannot enter M-cells and seems somewhat premature since the possible existence of such a route was not investigated. Indeed, our preliminary data have demonstrated that *lpfC/invA* mutant *S. typhimurium* can enter murine M-cells following infection of gut loops (*Jepson* and *Clark*, 1998), and it therefore remains possible that M-cells contribute to the extra-intestinal dissemination reported by *Vazquez-Torres* and co-authors (1999).

Yersinia

Early studies suggested that M-cells are, at least during the early stages of infection, a major site of invasion by the enteropathogenic *Yersinia* species (*Hanski* et al., 1989; *Fujimura* et al., 1992). Detailed microscopic studies have now demonstrated that murine M-cells are preferentially invaded by *Yersinia pseudotuberculosis* (Figure 1; *Clark* et al., 1998a) and *Yersinia enterocolitica* (*Autenrieth* and *Firsching*, 1996), and that infection with the latter may result in severe damage to the Peyer's patches (*Autenrieth* and *Firsching*, 1996). The mechanisms responsible for M-cell targeting by *Y. pseudotuberculosis* have been partially defined. As with the infection of cultured cell lines (*Isberg* and *Leong*, 1990), interaction of *Y. pseudotuberculosis* with intestinal M-cells is primarily mediated by the bacterial protein invasin, which promotes both M-cell adhesion and invasion (*Marra* and *Isberg*, 1997; *Clark* et al., 1998a). In contrast to enterocytes, murine Peyer's patch M-cells express β_1 integrins on their apical surfaces (*Clark* et al., 1998a) and, since multiple β_1 integrins act as receptors for invasin-mediated interactions with cultured cell lines (*Isberg* and *Leong*, 1990), it seems likely that M-cell surface β_1 integrins similarly act as receptors for *Yersinia in vivo*. This hypothesis awaits rigorous investigation. In support of a more widespread role for β_1 integrins in M-cell targeting by *Yersinia*, it was recently reported that expression of these receptors on the apical surface of a human epithelial cell line (Caco-2) is increased by co-culture with lymphocytes (*Schulte* et al., 2000). Furthermore, *Y. enterocolitica* were shown to utilise β_1 integrin receptors in this *in vitro* model of M-cell infection (*Schulte* et al., 2000). Together these findings suggest that M-cell surface integrins may act as receptors for the *in vivo* invasion of *Yersinia* or other pathogens in a variety of host species and mucosal sites in addition to mouse Peyer's patches. This hypothesis currently awaits thorough investigation.

It is clear that the enteropathogenic *Yersinia* species also possess invasin-independent routes of intestinal invasion. This is supported by the observations that invasin deficient mutants of *Y. pseudotuberculosis* retain the capacity to adhere to and invade M-cells, albeit at dramatically reduced levels (*Clark* et al., 1998a), and to colonise the Peyer's patches (*Marra* and *Isberg*, 1997) and by results obtained using the Caco-2 cell/lymphocyte co-culture model of *Yersinia* infection (*Schulte* et al., 2000). Gut loop infection with mutants deficient in two other *in vitro* identified adhesins, namely YadA and the pH 6 antigen, failed to detect a direct role for either protein in M-cell invasion by *Y. pseudotuberculosis* (*Marra* and *Isberg*, 1997). It thus appears that *Yersinia* invasion *in vivo* can proceed via additional mechanisms to those previously identified in conventional cultured cell lines. The mechanisms and significance of these alternative mechanisms of *Yersinia* invasion remain unclear, as does the possible contribution made by enterocytes to intestinal invasion by *Yersinia* species.

Enteropathogenic and enterohaemorrhagic Escherichia coli

Intestinal infection by enteropathogenic *E. coli* (EPEC) and enterohaemorrhagic *E. coli* (EHEC) strains involve the formation of attaching and effacing (A/E) lesions, with EPEC adhering intimately to membrane pedestals under which cytoskeletal proteins accumulate (*Celli* et al., 2000). The rabbit EPEC (REPEC) strain RDEC-1, adheres selectively to rabbit Peyer's patch M-cells (*Cantey* and *Inman*, 1981; *Inman* and *Cantey*, 1984) and, in striking contrast

to other pathogens and inert particles which are readily taken up by M-cells following initial adherence, RDEC-1 adherence to M-cells does not result in their internalisation (*Inman* and *Cantey*, 1984). At present, it is unclear how RDEC-1 resists M-cell phagocytosis, although this is probably related to the ability of EPEC strains to hijack host signalling pathways and induce cytoskeletal reorganisation (*Celli* et al., 2000).

RDEC-1 is also unusual among M-cell-selective pathogens in that the primary adhesin mediating M-cell targeting, the AF/R1 pilus, has been recognised for some time (*Inman* and *Cantey*, 1984). While the distribution of REPEC binding between FAE and villous epithelium varies between strains, with FAE binding being favoured by all AF/R1 pilus-expressing strains examined in detail, REPEC strains lacking AF/R1 pili do adhere to Peyer's patch FAE, indicating that this is not the only adhesin that can mediate FAE binding (*von Moll* and *Cantey*, 1997). Indeed, Heczko and co-authors (2000b) recently reported that REPEC O103 strain 85/150, which expresses different (AF/R2) pili, also preferentially colonise rabbit Peyer's patch FAE in the early stages of infection although it is unclear whether this phenomenon involves M-cell tropism. Interestingly, Heczko and co-authors have also reported that commensal SFBs, which had previously not been described in rabbits, prevented ileal colonisation and the induction of clinical disease by REPEC O103 (*Heczko* et al., 2000a). Similarly, circumstantial evidence suggests that SFBs may also exert a negative effect on ileal colonisation by *Salmonella* in rats (*Garland* et al., 1982). The mechanisms by which SFBs may protect against enteropathogens remain unclear (*Klaasen* et al., 1992), although it has been suggested that they may exert their influence by mechanical exclusion since massive numbers of SFBs are frequently located in the distal ileum, a site favoured by many enteropathogens including *Salmonella* (*Garland* et al., 1982). SFBs must also exert their influence by additional, as yet undefined mechanisms since REPEC O103 colonises the FAE of the rabbit distal ileum, whereas colonisation by rabbit SFBs appears to be restricted to the villi (*Heczko* et al., 2000a). It is possible that SFBs may induce colonisation resistance by stimulating the mucosal immune system (*Klaasen* et al., 1993) or by modifying local environmental conditions within the gut lumen.

The study of rabbit EPEC strains has suggested that human EPEC and/or EHEC might also target Peyer's patch M-cells or FAE but until recently this possibility remained untested. Elegant studies employing *in vitro* organ culture (IVOC) of human intestine have now examined initial interactions of EPEC and EHEC with various regions of paediatric intestine (*Phillips* et al., 2000). These studies revealed that the EPEC strain E2348/69 adhered to both proximal and distal small intestine and to Peyer's patch FAE while, in marked contrast, the EHEC O157:H7 strain 85-170 adhered only to Peyer's patch FAE during the time-course of these experiments. The extent of adherence of EHEC suggested that this involves enterocytes, and the possible involvement of M-cells could not be inferred from these studies. *Phillips* and co-authors (2000) hypothesised that EHEC intimin might be responsible for the preferential binding to FAE since EHEC express a different form of intimin (γ) to that expressed by EPEC E2348/69 (intimin-α). Confirmation of the role of intimin in EPEC/EHEC tissue-specificity has subsequently been provided by infection of human IVOC with EPEC E2348/69 after replacement of the eaeα gene

(encoding intimin-α) with the EHEC eaeγ gene (intimin-γ) and by a natural intimin-γ-expressing EPEC O55:H7 strain; both of which exhibited highly selective binding to Peyer's patch FAE (Phillips and Frankel, 2000). While these data indicate that intimin does mediate FAE binding the cellular receptor remains unidentified, though since intimin is known to bind β1 integrins it is tempting to speculate that these molecules might play a role in EHEC/EPEC tissue tropism.

Shigella flexneri

Shigellosis is characterised by acute recto-colitis, and is caused by members of the genus *Shigella* invading the intestinal epithelium. *In vitro* studies have demonstrated that *Shigella* invasion of intestinal epithelial cell lines is restricted to their basolateral surfaces, and it now appears that *Shigella* invasion *in vivo* initially occurs via M-cells and at sites where epithelial integrity is compromised (*Sansonetti* and *Phalipon*, 1999). The putative role of M-cells in initial *Shigella* invasion is supported by the observations that early lesions in patients and Macaque monkeys infected with *Shigella* are located over the lymphoid follicles and from experimental studies using the rabbit model of infection (*Sansonetti* and *Phalipon*, 1999). The latter studies have demonstrated that *Shigella flexneri* selectively invades M-cells in rabbit gut loops (*Wassef* et al., 1989; *Perdomo* et al., 1994). As with *S. typhimurium*, M-cell infection by *S. flexneri* is associated with membrane remodelling similar to that seen following invasion of cultured cells (*Sansonetti* et al., 1996). In contrast to *S. typhimurium* infection, *S. flexneri* does not appear to exert a direct cytotoxic effect on M-cells. However, by 8 h. post infection, *S. flexneri* induces a massive inflammatory response which results in extensive FAE destruction (*Perdomo* et al., 1994; *Sansonetti* et al., 1996). Analysis of mutant strains revealed that both an adhesive and an invasive phenotype were required for efficient FAE colonisation (*Sansonetti* et al., 1996). Mutant strains deficient for these phenotypes induced only a limited inflammatory response, and this permitted the cellular changes associated with *Shigella* infection to be analysed in more detail (*Sansonetti* et al., 1996). An invasion deficient mutant, cured of a virulence plasmid essential for invasion of cultured cells, was rendered M-cell adherent by expression of the RDEC-1 M-cell adhesin (AF/R1 pili) (*Inman* and *Cantey*, 1984; *Sansonetti* et al., 1996). Infection with this mutant induced migration of mononuclear cells into the M-cell pockets, which was accompanied by a marked increase in the proportion of the FAE occupied by M-cells (*Sansonetti* et al., 1996). This increase in M-cell surface area resulted from an enlargement of individual cells rather than from an increase in their number as has been reported after *Salmonella* infection of mice (*Savidge* et al., 1991) and *Streptococcus* infection of rabbit Peyer's patches (*Borghesi* et al., 1999; *Meynell* et al., 1999).

The interaction of *S. flexneri* in mouse gut loops has now also been described (*Jensen* et al., 1998). During early infection bacteria are associated with the surface of mouse Peyer's patch M-cells, with some M-cells exhibiting surface protrusions. Extensive FAE destruction is apparent 90 min post infection, although it is unclear whether this is a direct consequence of bacterial invasion or, as in the rabbit, a result of bacterial-induced inflammatory responses. Relatively large numbers (>10^9 CFU/ml) of *S. flexneri* are required to induce FAE destruction compared to those required for *S. typhimurium*-induced cytotoxicity, confirming that this *Salmonella*-induced cytotoxic response

is distinct from responses induced by other pathogens.

Listeria monocytogenes

Some studies have suggested that, following oral infection of mice with *Listeria monocytogenes*, Peyer's patches constitute the primary site of bacterial infection (*Marco* et al., 1992). These observations suggest that *Listeria* might be added to the list of pathogens that utilise M-cells as a gateway into the host (*Gebert* et al., 1996). However, some studies were unable to demonstrate involvement of M-cells in *L. monocytogenes* infection following oral infection of mice (*Marco* et al., 1997) or inoculation of rat gut loops (*Pron* et al., 1998), and it has been suggested that Peyer's patches may instead serve as a preferential site of *Listeria* replication rather than invasion (*Pron* et al., 1998). In contrast to these findings, interaction between *L. monocytogenes* and mouse M-cells has been observed in gut loops infected with large numbers ($>10^9$ CFU/ml) of bacteria (*Jensen* et al., 1998). In this latter study, *L. monocytogenes* was also reported to induce extensive loss of FAE-cells although much larger inoculae of this bacterium were required to induce FAE damage than that required for *S. typhimurium* (*Jensen* et al., 1998). The inability of some studies to demonstrate a clear role for M-cells in *Listeria* infection suggest the existence of M-cell-independent route(s) of invasion. Recent studies have examined this possibility and demonstrated extra-intestinal dissemination of *L. monocytogenes* after intragastric inoculation of germfree severe-combined immunodeficient (SCID) mice and after rectal inoculation of normal mice (*Havell* et al., 1999). These observations clearly demonstrate that *L. monocytogenes* can translocate across the intestine where fully formed Peyer's patches are absent. However, this approach does not exclude the possible involvement of small numbers of M-cells in the observed translocation since these cells are present in the colon of conventional mice (*Giannasca* et al., 1994; *Gebert* et al., 1996) and in rudimentary GALT of SCID mice (*Savidge*, 1996). Further research is thus required to fully elucidate the role of M-cells, if any, in natural *Listeria* infections.

DISCUSSION

Significant progress has been made in recent years in our understanding of M-cell biology and function. However, despite these advances, many questions remain unresolved and it is hoped that the continuing refinement of *in vitro* and *in vivo* experimental tools will facilitate further characterisation of these fascinating cells. The bulk of experimental evidence now indicates that M-cells primarily originate from undifferentiated crypt cells under the influence of local lymphoid factors. However, the possibility that M-cells may represent a dynamic cell population which can rapidly respond to changes in the local levels of antigenic stimulation is fascinating and merits further investigation. Future analyses should investigate more closely the possibility that enterocytes may differentiate into M-cells, identify the range of micro-organisms which might induce such an event and determine the functional status of these newly formed M-cells. Further studies are also required to determine the fate of M-cells.

Accumulating evidence from a range of animal species now indicates that many pathogens exploit M-cells as a

route of host invasion. In recent years significant progress has been made towards characterising the mechanisms by which these pathogens interact with M-cells and in defining the consequences of these interactions. Much, however, remains to be learnt. It is already clear that bacteria employ a range of strategies to subvert M-cell function and to exploit these cells as a gateway into the host or, in the case of EPEC, to resist internalisation. In some cases, these strategies mimic those previously identified *in vitro* (*Marra* and *Isberg*, 1997; *Penheiter* et al., 1997; *Clark* et al., 1998a,b) but it is also clear that additional, previously unidentified, mechanisms contribute to M-cell invasion. These studies highlight the fact that there is some degree of redundancy in the mechanisms employed by many pathogens to breach the intestinal epithelial barrier and that individual pathogens may encode a variety of alternate mechanisms to exploit M-cell function. Similarly, there appears to be redundancy in the bacterial ligands and cellular receptors that mediate adherence to M-cells. In many cases the precise nature of these bacterial adhesins, cellular receptors and invasion mechanisms await further investigation, as does the relative contribution of these mechanisms to bacterial infectivity.

It is now recognised that many of the pathogens which invade M-cells also induce significant changes in Peyer's patch architecture including alterations in M-cell morphology or number, cellular infiltration and epithelial destruction ranging from localised M-cell disruption to complete loss of the FAE. It is not yet clear what factors regulate these responses and whether they are analogous to changes observed upon infection of cultured cells. However, the observed changes suggest that bacterial interaction with M-cells may also play a key role in the pathogenesis of important human diseases, including typhoid fever, shigellosis and Crohn's disease (*Siebers* and *Finlay*, 1996; *Sansonetti* et al., 1996), although direct evidence of the significance of M-cell infection in human disease is currently lacking. It is clear, however, that M-cells play a key role in both infection and immunity in animal infection models. It is less clear what factors determine whether microbial internalisation by M-cells results in the induction of a protective immune response or the generation of local or systemic disease. It should, however, be remembered that, while M-cells appear to represent a major route of pathogen invasion, they constitute only a minute proportion of the mucosal surface area and thus bacterial translocation may also be significant through other less efficient routes. Similarly, while M-cells are the specialised antigen-sampling cells of the mucosal immune system, other cell types also appear to contribute to this function. Much further research is still required to complete our understanding of M-cell/micro-organism interactions. Such understanding is of considerable importance, since further definition of the nature of these interactions is likely to prove invaluable in the design of mucosal vaccines and the development of effective disease control strategies against pathogens that colonise or invade mucosal surfaces.

ACKNOWLEDGEMENTS

M.A. Clark is supported by the Wellcome Trust grant 055009/Z/98/Z and M.A. Jepson is supported by a Medical Research Council Infrastructure Award. We are

grateful to the Wellcome Trust (including equipment grant 049828/Z/96/Z), Medical Research Council and Royal Society, for funding our research discussed in this review. The authors also wish to thank B.H. Hirst, N.L. Simmons and P. McParlin, (Department of Physiological Sciences, University of Newcastle upon Tyne); A.D. Leard (Cell Imaging Facility and Department of Biochemistry, University of Bristol); and T.A. Booth, (Biomedical Electron Microscopy Unit, University of Newcastle upon Tyne) for helpful discussions and contributions to our work described in this review.

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HYPOTHESIS ON THE PHYSIOLOGICAL CONTROL OF INFECTIONS BY OPPORTUNISTIC (POTENTIALLY PATHOGENIC) MICRO-ORGANISMS

DIRK VAN DER WAAIJ

Hoge Hereweg 50, Glimmen, The Netherlands

SUMMARY

A hypothesis is set up regarding the extend at which in vertebrate animals the (four) 'layers' of the defence system, which sequentially have developed in evolution and co-operate in the clearance of translocated micro-organisms. On the basis of data from several previously published studies, it is proposed that co-operation is likely to be *additive* and in most cases perhaps *synergistic*. A mouse model for *in vivo* study of intestinal translocation is proposed. This model permits sequential (tail) blood sampling for microbiological and immunological analysis after an experimental oral contamination.

INTRODUCTION

To treat/prevent opportunistic infections in the compromised patient when multi-resistance to antibiotics becomes unsurpassable, artificial maintenance of the capacity of the defence (which clears off opportunistic bacteria) at a normal level, may provide a solution, as proposed by the ISGNAS-group (*Araneo* et al., 1996). For such a new approach for preventive treatment, however, comprehensive insight of the normal functioning of the physiologic (innate and adaptive) defence is required.

In vertebrates, the host defence to opportunistic micro-organisms consists of several 'layers'; each of them may act in the defence system more or less in the sequence in which they developed in evolution (*Medzhitov* and *Janeway*, 1998). Between these defence layers, interactions occur between the defence factors to control potentially pathogenic microbes along their route from the outside world into a host organism. The layers meant are respectively:

1. Gastro-intestinal tract microflora
2. Mucosal layer of the gastro-intestinal tract
3. Innate immune system
4. Adaptive immune system of the gut as well as the systemic immune system

In order to 'survive' the selective forces in evolution these steps in evolution of the defence system *must have evolved in optimal mutual co-operation* to provide the host organism a more effective clearance system for micro-organisms. This principle of a better outcome following concerted action is known from the clinical use of antimicrobials: In case two antibiotics are used for treatment of an infection they should work *additive*[i] or better *synergistically*[ii]. *Antagonism*[iii] may occur during use of combinations of certain antibiotics in treatment.

Synergism is of great advantage when one of the antimicrobial drugs, in

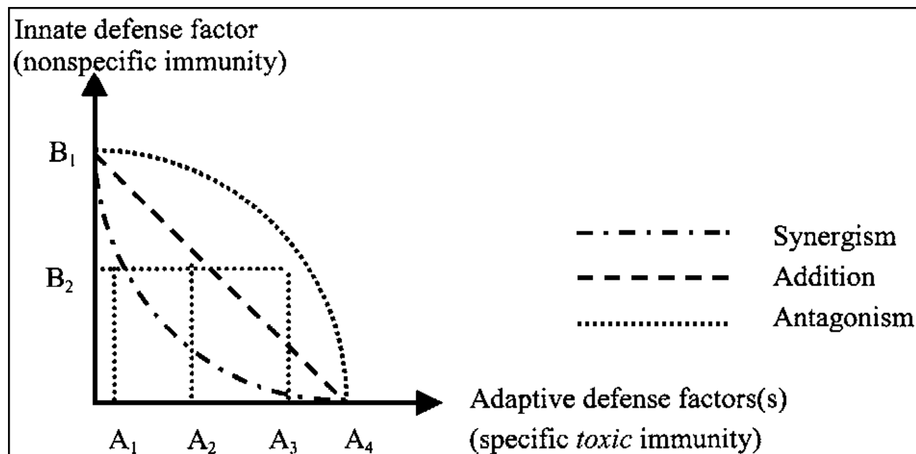

Figure 1: Graphic presentation of the influence of *synergism*, *addition* or *antagonism* between innate and adaptive immunity may theoretically have in the clearance of a certain number of micro-organisms.

a combination of two selected for treatment, is strictly indicated but *toxic*. Because of *synergism*, the toxic antibiotic can be effective in the combination in a much lower dosage than even half of the dose required when used as a single drug for treatment of the same infection. This is shown schematically in Figure 1 (dosages A_1 and B_1).

In opsonisation for clearance, complement activating antibodies such as IgG, may cause an *inflammatory reaction* and thus should be regarded as 'toxic'. These antibodies may normally not participate in the clearance of bacteria from tissues or (because of synergism with non-specific opsonins) only in low (non-toxic) concentrations.

In conclusion: If induced and in the circulation, complement activating antibodies may co-operate efficiently in low titres in the process of opsonisation and clearance with non-specific opsonins (glycoproteins) of the innate defence. It is thus hypothesised, that:

1. *In vivo* the dose ratio of opsonising adaptive- and opsonising innate defence molecules seems comparable with the dose ratio in the above given example of toxic and non-toxic antibiotics for treatment mentioned.
2. The physiologic clearance of microorganisms from the lamina propria, mesenteric lymph nodes and spleen occurs without inflammation because of synergism between innate and adaptive immunity. Would the production of sufficient innate opsonising molecules decrease, higher antibody titres and thus inflammatory reactions are to be expected.

Because proper understanding of these basic principles is considered important for understanding of the functioning of the physiologic defence, a schematic presentation is shown graphically in Figure 1.

In this theoretical example, three ways of co-operation between innate and adaptive immunity regarding the clearance of a particular translocated microorganism are shown. Absence of adaptive immunity would require a strong innate immune capacity B_1; conversely in the absence of innate defence a strong adaptive immune reaction (A_4) is necessary to clear the same number of trans-

locating microbes. In the presence of both innate and adaptive immunity and when their co-operation is *synergistic*, less innate immune capacity (B_2) and a very low level of adaptive immune support (A_1) may provide total clearance of the translocating micro-organisms. However, in case co-operation is *additive*, stronger specific immune support (A_2) is required for clearance, while a much stronger adaptive immunity is necessary when *antagonism* exists between the co-operating antimicrobial forces.

Intestinal microflora

In maturing and adult animals with an established intestinal microflora, the first act of defence may occur either inside the intestinal tract by resident bacteria or indirectly by host immune factors. The host immune system is modulated by molecules released by intestinal bacteria which stimulate/inhibit. Resident bacteria may in addition have advantages in competition for nutrients while some may release substances which are directly active to newcomers in the gut; i.e. substances which block attachment receptors for opportunistic bacteria on mucosal cells as well as ingested particles which would enhance proliferation of the newly ingested microbes. The mechanism which controls colonisation by newly ingested bacteria and fungi is called *colonisation resistance* (*van der Waaij* et al., 1971).

Mucosal layer of the intestinal tract

From time to time bacteria which, regardless the colonisation resistance (CR) reach the mucosal layer and adhere to it, may penetrate the epithelial cells to reach the underlying lamina propria. This penetration into the lamina propria, a process named *translocation* (*van der Waaij* et al., 1972; *Berg* and *Garlington*, 1979; *Berg*, 1981; *Wells* et al, 1988), is often followed by a further transfer to the lymphatic organs and the liver. High concentrations of an opportunistic bacterial species in the intestines as well as certain virulence factors promote translocation (*Christensen* and *Beachey*, 1984; *Miller* and *Cossart*, 1999). Some pathogens can encapsulate which hinders phagocytosis, express that capacity often only after they have reached host tissues (*Bassler*, 1999).

Immune system

The immune system consists phylogenetically of two systems:

1. *Innate* immunity, which has developed in evolution in lower animal species, and
2. *Adaptive immunity,* which is confined to phylogenetically younger groups of animal species and becomes only highly specific (T-cell controlled) in the vertebrates.

Because, the adaptive part of the immune system has developed relatively late in evolution, it is conceivable that this 'new system' had to optimally co-operate with the phylogenetic older non-specific innate part. In animal species with a thymus-controlled immune system, a previous contact of a micro-organism with the adaptive immune system, can have resulted in either *defensive immunity*, like by antibody formation (or cytotoxic T-cells) induced in the lymphnodes or/and spleen, or a negative feedback i.e. *oral tolerance* (specific non-responsiveness) predominantly induced in the Peyer's patches.

THE CONTRIBUTION OF EACH OF THE FOUR DEFENCE LAYERS

Gastrointestinal ecosystem

The gastro-intestinal ecosystem represents a very potent defence system; forming the first barrier to the outside world, it could be regarded as the most important part of the defence. Phylogenetically it is the oldest part of the defence system of man and animals. Although rather constant in composition, the intestinal microflora may vary during life and therewith in efficiency as well. The composition of the gastro-intestinal-tract microflora does not only differ between animal species, as it may also differ significantly between individuals of a species and even between individual animals of an inbred strains (*van der Waaij and van der Waaij*, 1990). The way the intestinal microflora exerts a protective role is still largely unclear and so is the composition of the microflora; let alone the composition of an ideal ('guaranteed protective') microflora.

In vertebrates, the host organism appears to co-operate in several ways with the intestinal ecosystem improving its protective effect. The host organism provides nutrients by secretion of mucus and other secretory products as well as immunologically in a (T-cell dependent) as yet unclear way. Specific antibodies may play a decisive role. The outcome of the grand total of these very complex (*synergistic*) interactions between host and resident microflora is named *colonisation resistance of the digestive tract* (CR) (*van der Waaij* et al., 1971; *van der Waaij* and *Berghuis*, 1974; *van der Waaij* et al., 1977).

In a *conventional environment*, many if not most of the daily ingested opportunistic micro-organisms may generally not reach the gut mucosa to colonise it as a result of CR-activity. If colonisation of the gastro-intestinal tract mucosa nevertheless occurs, it will mostly not result in sufficient numbers (intestinal concentration (IC) for noticeable *translocation*[iv].

Translocation (TL) is related to the intestinal concentration of the bacterial strain involved (*Medzhitov* and *Janeway*, 1998; *van der Waaij* et al., 1971; *van der Waaij* et al., 1972; *Berg* and *Garlington*, 1979). Furthermore, TL may differ between strains of the same microbial species (difference of "invasiveness") (*Berg* and *Garlington*, 1979; *Berg*, 1981). The experimental design and technique used permitted the study of the faecal (intestinal) concentration and the CR following various different oral doses, as well as the occurrence of TL of the strain at daily intervals after contamination *in vivo*. In addition, mice were before contamination daily treated orally with non-absorbable antibiotics in order to very strongly lower their CR. In the presently reported study, techniques were unfortunately not yet available to monitor the NSO and SO.

The immune system (innate and adaptive)

There is strong evidence, that certain non-specific ligands between bacteria and phagocytic cells may play a role in the control of translocation of micro-organisms (*Russell*, 1995). Recent studies show that the adaptive immune system is normally not, or only with low antibody titres, involved in the response to intestinal non-pathogenic and opportunistic micro-organisms. The various non-specific ligands, active in innate defence, are therefore likely to co-operate with each other and when necessary, with specific antibodies in a *synergistic* way. *Synergism* between innate components and (if developed) specific immunity may make a clearance of trans-

located bacteria by the non-specific system more efficient; a point which requires confirmation by *in vivo* studies (see later ISGNAS research priorities).

In serum samples of healthy human subjects, specific IgG (and IgM) antibodies to, on the average, 72% (median range 10-73%) of their own endogenous faecal microflora have been found. Many of these circulating antibodies may find their way out into the intestinal tract lumen as they have also been found on many bacteria in washed fresh faeces where as many as 30% is *in vivo* IgG coated and 45% is coated with IgA (*van der Waaij* et al., 1994; *Jansen* et al., 1993; *Apperloo-Renkema* et al., 1993). It seems likely that these antibodies are in the circulation (in low titres) to support innate clearance of translocating bacteria; secretion of antibodies with the mucus into the intestines may play an important direct role in the intestinal CR. The circulating anti-bacterial antibodies are not only binding to endogenous faecal bacteria of a host organism, since a fraction appears active against endogenous microflora components of other (unrelated) subjects (*Apperloo-Renkema* et al., 1993).

Criticism to the role of specific antibodies in the clearance of translocating bacteria is based on the fact that certain bacteria may bind antibodies non-specifically, by binding the Fc-part of antibodies subjects (*Apperloo-Renkema* et al., 1993). Such non-specific binding, however, concerns predominantly a few gram-positive bacterial species (staphylococci) and as this binding is non-specific; consequently, it does not *selectively eliminate antibodies specific to this small subset of non-specific binding bacteria.* The effect of non-specific binding of antibodies *in vivo* is unclear.

HYPOTHESIS

To my knowledge, so far no prospective study on the role of non-specific opsonising (NSO) factors in clearance of bacteria with or without specific opsonisation (antibodies) has been undertaken and published. The present hypothesis is consequently based on previously published observations. It is conceivable, that in the lamina propria *non-specific opsonisation and in some cases in combination with antibodies enhance phagocytosis for clearance of translocating intestinal bacteria.* A wide variety of bacterial products can trigger inflammation (*Henderson* et al., 1996), yet the clearance of small numbers of translocating micro-organisms by NSO is likely to occur mostly without inflammation. Translocation may be a daily occurring event in individuals who live in a conventional environment.

Depending on their concentration in the gut, bacteria may pass the mucosal lining in numbers related to their capacity to adhere and penetrate (a virulence factor). Another port of entry of the host organism in the gut is the M-cell layer overlaying a Peyer's patches. There, they may induce either IgA or (occasionally) T-suppressor cells for tolerance (or both?) (*Brandtzaeg* et al., 1999). Both may represent a (feedback?) mechanism which controls 'pathogenic immunity' (i.e. inflammation). It is likely that the proposed *synergism* between NSO factors and *specific opsonisation* (SO) represents a more important mechanism in the non-pathogenic clearance of bacteria (without inflammation) than *intestinal tolerance*.

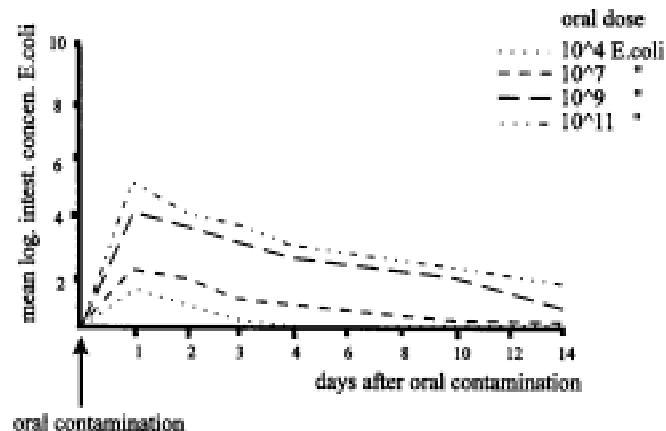

Figure 2: Relation between oral dose and mean log. intestinal (faecal) microbial concentration in mice (standard deviations of the mean were not calculated because of samples with 'zero' counts in each set of samples) (*van der Waaij* and *van der Waaij*, 1990).

Following ingestion of high(er) numbers or ingestion of more virulent bacteria, equipped with special tools to penetrate the mucosal lining, sufficient antigenic material may arrive in the lymphatic organs to induce specific immunity, either by stimulating existing (innate?) poly-reactive IgM isotype antibodies or by induction of circulating antibodies of the IgG isotype. Would the number or the pathogenicity of translocating bacteria be higher than can be managed by the 'synergistic clearance system' a certain 'threshold' may become passed which results in a stronger specific antibody production; their clearance will be predominantly due to adaptive (specific) immunity. Inflammation due to activation of complement factors must in such cases be expected. Interacting mechanisms in *the normal control of opportunistic micro-organisms* are:

1. Oral dose **(0D)** of opportunistic microbes
2. Colonisation resistance **(CR)** of the digestive tract
3. Intestinal concentration **(IC)** of opportunistic microbes reflecting the numbers which may reach the mucosa
4. Translocation **(TL)**
5. Non-specific opsonisation **(NSO)** and specific opsonisation **(SO)** to enhance phagocytosis and killing; i.e. *clearance* of TL micro-organisms.

A RETROSPECTIVE ANALYSIS OF MOUSE DATA REPRESENTING MOST OF THE FLOW OF EVENTS AFTER INGESTION OF AN OPPORTUNISTIC MICRO-ORGANISM

As a first step to test our hypothesis, evidence was sought for the assumed co-operative interactions between 'defence layers'. The following brief review of data provides certain indications for 'inter-layer co-operation':

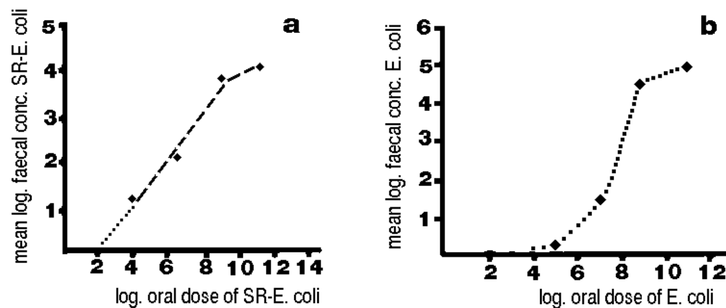

Figure 3: Relation between oral dose and faecal concentration (data from *van der Waaij and van der Waaij*, 1990 [a]; and from *van der Waaij* and *Berghuis*, 1974 [b])

The relation between an oral dose of opportunistic microbes, the colonisation resistance and the intestinal concentration reached by contaminants

The relation between the oral dose of opportunistic bacteria (a strain of *SR-E. coli* as an example) and the resulting intestinal concentration in mice during the next days, is quoted from previous publications (*van der Waaij* and *Berghuis*, 1974; *van der Waaij* et al., 1977) and shown in Figure 2.

The curves in Figure 2 show, that during two weeks after contamination, the daily relation between an oral dose of *E. coli* and their mean log concentration in faeces, is not linear. This is most clear in the first days after contamination. In Figures 3a and 3b data from two different series of experiments in mice are shown. However, on the basis of these data, it cannot be concluded, that the curves are really S-shaped is seems likely by their appearance. Furthermore, it should be made clear that the curves in these figures are derived from experiments with only one *E. coli* strain in one particular mouse strain (ND2 mice). Results may differ when other *E. coli* strains are used or other *Enterobacteriaceae* species and other (gram-positive) opportunistic bacteria such as *Enterococcus faecalis* (*van der Waaij* et al., 1972). However, these differences may not be essential, as the general (S-shaped) trend in the first days is the same in all.

The non-linear shape (S-shape?) of the curve, which may represent the relation between dose and effect, could be the result of a switch in the mechanism involved in the obtaining that effect. In the curve shown in Figures 3a and 3b, the lower oral doses could have been controlled by predominantly the intestinal microbial ecosystem, whereas following higher numbers of ingested bacteria, the immune system of the host organism may have been involved in the outcome. No experiments have been performed as yet to further investigate this prospectively.

Passing of the aforementioned 'innate-adaptive-immunity threshold' -inviting the adaptive immune system to action- could either be due to a *deficient defence system,* unable to clear translocating bacteria which normally would have been cleared optimally (like may be the case in the compromised patient), or to certain *virulence factors* of the translocating bacteria. These virulence factors permit them to escape NSO so that their clearance relies completely on SO.

The switch in the clearing system from predominantly innate to predominantly specific immunity could be made likely, by plotting the *"infective dose"*

and its *"effect"* graphically. The 'threshold', which determines the shift, from predominantly innate to predominantly specific immunity, may show it self by a change in the slope of such a curve from gradually increasing to gradually decreasing; the curve then has more or less an S-shape. On the basis of observations made in our reviewed previous studies (*van der Waaij* and *Berghuis*, 1974; *van der Waaij* et al., 1977), it is concluded that indeed antimicrobial defence factors may co-operate intensely in the process of controlling colonisation by newly ingested micro-organisms. In this S-shaped relation between oral dose and the intestinal concentration of the 'contaminant', the *E. coli*, there is evidence of a contribution of another additional mechanism from above the oral dose level of 10^8 *E. coli* on. This additional defence mechanism prevents (or ads to the control) a rise of the intestinal concentration to above 10^6 *E. coli*/g. This additional mechanism which may act in the CR, could be specific immunity as a control mechanism which maintains the intestinal concentration at 10^6 bact./g is absent in congenitally thymus-less mice once the reach an age of about 6 weeks when passive maternal antibodies have disappeared (*van der Waaij* and *Heidt*, 1990).

AN *IN VIVO* MODEL FOR MICROBIAL INFECTION, COLONISATION, TRANSLOCATION, AND CLEARANCE

Because the effect of CR on various doses appears to be non-linear but perhaps S-shaped, it was aimed to determine experimentally the graphical correlation between dose and effect, by determining the clearance of translocated bacteria *in vivo*. A graphic relation between invading numbers and their clearance might provide further insight in the physiologic clearance process of TL bacteria. To measure factors involved in the clearance *in vivo*, however, the clearance had to be defective (incomplete) for some time after oral infection, permitting a low degree of bacteraemia for several hours, in order to have some 'prove' for the occurrence of TL. Incomplete clearance furthermore, would provide some insight in the flow of events in a relatively compromised individual. In addition, blood sampling in the course of the critical first days after oral challenge would, in addition to culturing, permit the use of blood for chemical analysis of NSO and study of SO factors (antibodies). A study of this kind has been performed in the seventies as a pilot study for a grant application, unfortunately the grant was not awarded and further investigations had to wait.

Materials and Methods

Selection of E. coli strain for TL

For our experiments, a 'rather invasive' neomycin, streptomycin and ampicillin resistant *E. coli* strain was selected. Selection of this strain was carried out with a technique described by *Schabinsky* (1965). Schabinsky developed this technique in fact for another application namely to study the effect of antibiotics on i.p.-injected *E coli* by determining the number of colony forming units in the tail tip blood at several intervals.

For our purpose we selected an *E. coli* strain, which appeared in the tail blood of mice after the lowest infective dose (by *i.p. injection),* for further use. To minimise the chance that immunologic contact between the strain and the experimental animals had ever occurred, only strains of human (patient)

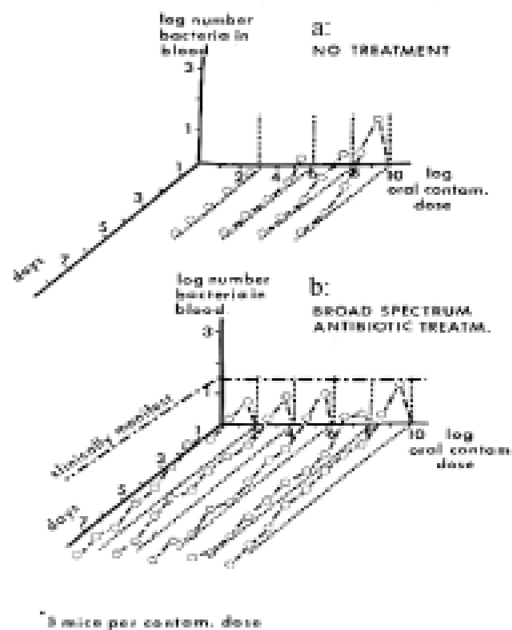

Figure 4: Mean number of E. coli colony forming units cultured from the blood of non treated mice (a) and antibiotic treated mice (b).

origin from the hospital were tested. Of the strain we selected, 10^3 *E. coli* i.p. per mouse; the strain was resistant to neomycin, streptomycin and ampicillin (NSA). The strain may have developed resistance to innate defence factors common to man and mice (*Rhen* et al., 2000).

In a pre-study with this *neomycin/streptomycin/ampicillin resistant strain* (*NSA-E. coli*) in germfree mice, it was found that following *oral* doses of 10^3 or higher, this *NSA-E. coli* strain could be cultured in varying numbers (several samples per day were positive; some were negative) from the tail blood for several days (possibly longer; not studied) after oral contamination. These oral doses of 10^3 of these rather invasive bacteria as well as higher contamination doses did not cause clinical signs and symptoms in these mice. This finding could be reproduced so that it was decided to perform a study with this strain in conventional mice. It can not be excluded that the relatively high virulence of the strain, causing its appearance in the tail blood, was due to an escape from efficient clearance as a result of resistance (developed in the patient?) to one or more innate defence factors (*Schabinsky*, 1965).

Translocation studies in conventional (control) and antibiotic decontaminated mice

In the experiments summarised hereafter, groups of five conventional or decontaminated female ND2 mice (12 weeks of age) were orally contaminated with various doses (Figures 4a and 4b) of the *NSA-E. coli* strain. Tail blood was collected *thrice* daily after disinfection of the tail tip. For the first collection of blood, the very end (small piece) of the very end of the tip of the tail was cut off. Each time a subsequent blood sample was required, bleeding started soon after some massage of the tail (automatically involved in the tail disin-

fection procedure). The drip of blood collected after tail disinfection was smeared (plated out) onto a neomycin/streptomycin-McConkey agar plate and incubated overnight at 37°C. Counting of the colonies grown at the site of the blood streak was supposed to give information about the number of organisms that were present in the blood sample. The highest count per mouse/day was used to calculate the mean number of *E. coli* colonies of each group of five mice.

In experiments in non-treated *control* mice, a fraction, proportional to the oral dose of the *NSA-E. coli,* may have reached sufficient high numbers in the gut contents to translocate. Following the highest doses, this *E. coli* strain may in the first 3 days have been able to 'bypass' (overload?) the normal sequence of events which lead to efficient clearance which may have caused the positive blood cultures. The initial 'escape' from efficient clearance may have been due to *the virulence (invasiveness and resistance to subsequent clearance) of the E. coli strain.*

Results

Oral dose -TL relation in control mice

In Figure 4a, the mean number of *E. coli* colony forming units (CFU) cultured from the blood of control mice contaminated with different oral doses are presented. Standard deviations of the mean are not calculated because of several negative cultures on most days; particularly in the control group of mice.

Oral dose -TL relation in 'antibiotic decontaminated' mice

In Figure 4b, the same results are presented obtained in mice which were 'antibiotic decontaminated' by oral treatment with neomycin and bacitracin-pimafucin respectively (1 g/l and 0.1 g/l drinking water).

Discussion

The results show a dose related mean number of colony forming units (CFU's) of *E. coli* in the tail blood of the mice at daily intervals after oral contamination in the control group. Following comparable oral doses, the numbers of CFU's in tail blood were significantly higher after the lower oral doses in the decontaminated mice (low CR). In addition in the week of observation all groups had mice with (low) positive blood cultures, which were not found in the non-treated mice. None of the mice in both group clinical signs of illness (hunched back ruffled fur or clear loss of appetite).

A *tentative conclusion* of the findings of this *TL-study* could be that, on the log-log scale in which the results are shown, a non-linear relation appear to exist between the oral dose and the occurrence of TL in the first days after oral contamination, both in non-treated as well as in the antibiotic decontaminated mice. This relation showed **no** S-shaped form, suggesting that following the highest oral doses (above 10^6 bacteria) no other (additional) mechanism came into action to control the situation as was above concluded to exist between oral dose and CR.

In the non-treated (control) group of mice in the present experiment, a logarithmic curve could perhaps be drawn through the mean number of *NSA-E. coli* CFU's in the blood collected at day one. In the antibiotic decontaminated group on the other hand, no relation between oral dose and number of *NSA-E. coli* CFU's existed. Resistance to innate defence factors, essential for the clearance of the *NSA-E. coli* after translocation makes the outcome of the experiment the resultant of the action in first barrier the CR. Specific immunity may have taken care of the intra-intestinal concentration of the contaminant as

well as after translocation. In the first (about four) days after contamination, however, no specific immunity may have existed in these mice, since the *NSA-E. coli* strain, with which they were contaminated, was of patient origin so that it is unlikely that a previous contact with the immune system of the mice in this experiment had occurred.

Several days after oral contamination, either TL decreased or the clearance efficiency increased significantly:

a. In the non-treated control group, it is likely that a gradual decrease of TL with elapsing time was responsible for this finding. The TL decrease may have been the consequence of a decrease of the *NSA-E. coli* concentration in the gut perhaps due to an increase of the CR by specific immunity.

b. In the decontaminated group however, even a week after contamination TL appeared to occur albeit at a strongly reduced level. As the intestinal concentration of the *NSA-E. coli* may not have decreased as strongly as in the control group, as the decontaminated group lacked concerted action of an intestinal microflora. Development of specific immunity may also have developed in these mice. Specific immunity (antibodies?) may have had some lowering effect on the intestinal numbers as well as have enhanced clearance after TL. Yet, a combination of both effects of specific immunity (not investigated) may explain the reduced numbers of *E. coli* in the tail blood after the first days.

RESEARCH PRIORITIES FOR THE STUDY OF EXISTENCE OF CO-OPERATIVE INTERACTIONS BETWEEN DEFENCE FACTORS

Multiply-resistance to antibiotics is increasingly causing failures in the treatment of infectious diseases. There is no doubt about the point that ways must be searched to stop this increase of resistance before it gets completely out of control. Assuming that new antibiotics may provide the ultimate answer, an international study group (International Study Group on New Antimicrobial Strategies (ISGNAS-foundation) was founded 1992 and seeks a solution(s) for the resistance problem. ISGNAS recognises the potential importance of several new avenues of research and strongly stimulates research on these subjects (*Araneo* et al., 1996). A major avenue is seen in research that leads to treatment modalities to maintain in immunocompromised patients, the defence to opportunistic bacteria and fungi at a normal (physiologic) level. Maintenance of the defence normal by either stimulation or supplementation should go along physiologic lines. To this end, precise knowledge of the mechanism(s) and factors involved in the normal physiologic control of opportunistic bacteria. The ISGNAS-group strongly stresses the importance of the urgent need of comprehensive studies on the mechanisms that lead to TL as well as those involved in the physiologic clearance of TL micro-organisms. It is felt that the *in vivo* mouse experiments reported in this paper, may provide a model for a detailed study of factors in the microflora as well in the host organism. The model permits microflora modulation as well as blood sampling, albeit in micro-amounts, for chemical and microbiological analysis.

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[i] *Additive* activity means that the outcome is the sum of the effects of each of the factors. This means that for example 50% of both doses provide the same effect as 100% of each of them.

[ii] *Synergism* means that smaller doses - in the example less than 50% - of each compound are required to obtain that 100% effect.

[iii] In *antagonism* the outcome of a combination is smaller than one would expect by summing up the effects of each of them alone.

[iv] In this paper translocation is defined as: *the penetration of the mucosal epithelial lining into the underlaying lamina propria by living micro-organisms.*

NUTRITION AND RESISTANCE TO DISEASE

STIG BENGMARK

Lund University, Lund, Sweden

EPIDEMIC OF CHRONIC AND DRUG-RELATED DISEASES

My decision to become a surgeon was made in the early fifties. At that time pharmaceutical medicine had made some remarkable progress and created a great enthusiasm for the future of therapy with pharmaceutical drugs, an enthusiasm similar to the expectations for the future of gene therapy often met today. There were those, who forecasted that within a few years most diseases, if not all, would successfully be controlled by pharmaceuticals. It is a fact, that I was so affected by this that I felt a real hesitance to choose surgery as my profession, fearing that within few years this form of therapy would be redundant. I would at that time have had difficulties to accept suggestions that as we enter a new century and a new millennium, surgery would be the leading therapy for organ failures such as those of the liver, lung, heart, kidney, intestine but also for diabetes, arteriosclerosis and obesity.

Pharmaceutical medicine, particularly that based on synthetic drugs, has in general failed to meet the high expectancies from 50 years ago, sometimes to the extent that many, especially in the US, seriously question its importance for human health and some turn back to herbal medicine. It is obvious that the Western world as we enter a new millennium suffer more than ever of an epidemic of chronic (Table 1) and of drug-induced (Table 2) diseases. Adverse drug reactions (ADR), e.g. side effects, which occur despite taking the drugs in dosage as prescribed, has become a serious problem as drugs be-

Table 1: Chronic diseases frequent in the Western world, of which most are increasing in incidence (after: *Carper*, 1997)

- One million get cancer each year – in lifetime 40 % of the population
- 60 millions suffer from Hypertension
- 40 millions suffer from Arthritis
- 23 millions suffer from Migraine

Increasing in incidence are:

- Asthma and other allergies
- Chronic fatigue
- Coronary Heart Disease
- Congenital malformations
- Diabetes
- Immune deficiency
- HIV
- Neurodegenerative diseases
- Obesity
- Overwhelming infections

Table 2: Negative consequences of use of pharmaceutical drugs (after: *Johnson* and *Bootman*, 1995; *Bates* et al., 1997; and *Carper*, 1997)

Each year do:
- about two millions get adverse effects to drugs and
- 106,000 die in adverse drug effects (compare accidents 91,000)
- 61,000 get drug-induced Parkinsonism
- 16,000 are involved in automobile accidents due to drugs
- 163,000 get drug-reduced memory
- 32,000 get hip fractures caused by drug-induced falls
- 6 millions abuse prescription drugs and
- more than 100,000 die in overdose of drugs
- 97,000 die in "medical accidents"

comes increasingly stronger, and it is reported that about seven percent of hospital patients suffer ADR. Each year do only in the US 106000 persons die in ADR, which is more than in accidents (91000) (*Lazarou* and *Pomeranz*, 1998). It has been calculated that the total costs for treatment of ADR only in the US is equal to or eventually exceeds the total costs for care of diabetes and cardiovascular disease, when added together (*Bates* et al., 1997; *Johnson* and *Bootman*, 1995). Also the use of antibiotics is becoming increasingly controversial and there are those who suggest that we are in the process of leaving the antibiotic era (*Roszkowski* et al, 1988). Restricted use of antibiotics is increasingly recommended not only because antibiotics induce antibiotic resistance of some pathogens, but also because they reduce the preventive commensal flora and also temporarily reduce or eliminate macrophage activity (*Henderson* et al, 1996).

ARE ALL DISEASES INFECTIOUS?

We are increasingly aware that health is much depending on interactions between micro-organisms and the human body but also depending on a harmonic balance between microbes and resistance to disease. Robert Koch was in 1876 the first to prove that a disease was caused by a particular micro-organism (anthrax) (*Stanier* et al., 1963). Since the introduction of modern techniques such as polymerase chain reaction (PCR) the establishment of an association of a specific infectious agent and a disease have been made possible without the fulfilment of the so called Koch postulates. This has lead to a revolution in the understanding of disease, showing that infectious agents can be the causes of, precipitating factor for, or risk factors for various diseases that not previously were considered to be caused by transmissible agents. Consequently it has been suggested that eventually all diseases are infectious (*Lorber*, 1996). Not only are there strong indications that diseases such as peptic ulcer disease (*Helicobacter pylori*; *Cover* and *Blaser*, 1995) and arteriosclerosis/cardiovascular disease (*Chlamydia pneumoniae*; *Leinonen* and *Saikku*, 2000) but it is also suggested that various forms of cancers, psychiatric (mental depression), inflammatory, and degenerative diseases are suggested to be infectious.

It is not easy to define health and

well-being, but it is likely to occur as the result of a dynamic interplay between factors that in the body control numerous important processes such as growth, cell proliferation, repair systems, apoptosis, stress response, appetite, energy balance, metabolic rate and numerous other important processes (*Frame* et al., 1998). It is suggested that health and well-being is depending on harmonious interaction in the body of more than two 2 million different chemical molecules. Only of fats have more than 250 thousand been identified in the human body. It is suggested that our Palaeolithic forefathers on annual basis consumed between four and five hundred various plants, a consumption, which modern man to a large extent has reduced to a few such as potato, rice, corn and wheat. In addition do we cook, fry, freeze and dry our food, which is known to destroys numerous important food ingredients (*Schroeder*, 1971), including lactic acid bacteria, but also induce production of mutagenic substances (*Lankaputhra* and *Shah*, 1998). It is against the background of this information the importance of unipharmacy and of synthetic drugs are increasingly questioned, and suggested that "polypharmacy" in the form of proper nutrition and in the form of various herbs and spices should be important to human health and well-being. Many of the numerous health-promoting nutrients such as short chain fatty acids (SCFAs), amino acids and polyamines, vitamins and antioxidants etc. are released in the large intestine by bacterial enzymes (for further information see: *Bengmark*, 2000a).

PARADOXICAL MALNUTRITION COMMON IN WESTERN COUNTRIES

There are those who suggest that all Westerners are malnourished in the sense that they do not receive the large variety of molecules with the food as our ancestors did. Two rather recent studies, one performed in the UK (*McWhirter* and *Pennington*, 1994) and the other in the US (*Gallagher-Allred* et al., 1996) suggest that about half the population are either severe malnourished or at the border of malnourishment. These persons are not malnourished in the traditional sense (as seen in starvation); they show instead signs of overfeeding as they are often obese (paradoxical malnutrition). Clearly it is in this group that we see a significantly increased morbidity and mortality. These individuals show clear signs of metabolic dysfunction and of disease clustering. When subject to surgical operations they often develop complications such as sepsis, thrombosis and also severe adhesion formation. They do often suffer hypertension, arthritis, dyslipdaemia, glucose intolerance and insulin resistance, a condition given the name of metabolic syndrome X (MSX), and suggested to occur in about 20% of the population in Western countries, in about 50% of hospital patients and in almost every patient suffering from severe complications to surgery. Overconsumption/abuse of diary products and red meat (C12-C16 saturated fatty acids and trans fatty acids) and of refined sugar, plus an obvious underconsumption of fresh fruits and vegetables, is suggested to be the cause. Among the food ingredients, which have been suggested to reduce or prevent MSX are various fibres, polyunsaturated fatty acids, phytosterols (rich in soya and in rye), garlic, and fermented products containing LAB.

LESS SATURATED FAT, MORE FIBRE AND LACTIC ACID BACTERIA

It was suggested in 1985 that our genes, which during millions of years of human life on earth are adapted to the pre-agricultural lifestyle do badly tolerate the dramatic change in lifestyle, including eating habits, which has occurred in more recent years (*Eaton* and *Konner*, 1985). It is an interesting hypothesis that this should be the reason why humans are increasingly sensitive to disease and should eventually explain why we suffer an epidemic of chronic diseases. A study of a population uninfluenced by Western dietary habits gives support to such an assumption (*Lindeberg*, 1994). At the island of Kitava in New Guinea do the inhabitants not eat diary products, margarine, lard, oils, refined sugar, alcohol and cereals, which constitutes more than 70% of the Western diet. Such diseases as sudden cardiac death, stroke, retrosternal angina are here virtually absent despite the fact that the majority of the population on this island often smoke and saturated fat from the coconut is a frequent staple food (*Lindeberg*, 1994; *Lindeberg* et al., 1994, 1997, 2000). Here no signs of metabolic syndrome X, which is a serious infliction to about 20% of Westerners, were found. On examination this population is characterised by leanness, low diastolic blood pressure, low values of plasminogen activator inhibitor-1 (PAI-1) (*Lindeberg* et al., 1997) and low serum insulin (*Lindeberg* et al., 2000).

PALAEOLITHIC FOREFATHERS CONSUMED LARGE AMOUNTS OF LACTIC ACID BACTERIA

Our Palaeolithic forefathers ate much less saturated fat, protein, and sodium salt. Instead they consumed up to ten times more of fruit and vegetable fibre, ten times or more of vitamins and other antioxidant, about fifty times of more of omega-3 fatty acids and most interestingly billions of times more of non-pathogenic bacteria, mainly lactic acid bacteria (LAB) such as *Lactobacillus plantarum, Lactobacillus pentoceus, and Lactobacillus paracasei.* Their foods were often stored in the soil, where it became naturally fermented. Furthermore, the food consumed by our palaeolithic forefathers was mainly raw and uncooked. Instead it was often fermented and rich in lactic acid bacteria (LAB).

The food eaten in Western countries has during the last hundred years become increasingly sterilised/pasteurised. The last traces of naturally fermented food (sauerkraut) disappeared from the daily diet in most Western countries during the last fifty years. But, the ability of fermentation to preserve nutrients is much superior to “modern” technologies for processing and storing foods such as drying, freezing, cooking and canning, as it preserves important but sensitive food ingredients much better (examples: various vitamins, antioxidants such as glutathion, and amino acids such as glutamine), ingredients which most often are destroyed by Western methods for handling and treatment of the food.

FIBRE-FERMENTING LACTIC ACID BACTERIA ARE IMPORTANT

Fibre-fermenting bacteria such as *Lactobacillus plantarum* are still dominating among the LAB colonising the intestine of rural Asians, Africans and most likely also South Americans. *Lactobacillus plantarum* is the dominating species in fermented foods such as sour dough, sauerkraut, natural wines and beers and in most third world staple foods such as African *ogi, kenkey* and *wara* (*Olasupo* et al., 1995). Although several of these fibre-fermenting LAB do no longer colonise the gut of Westerners, they are still often found in vegetarians. A study performed in the USA showed that about 65% of vegetarians to be colonised with *Lactobacillus plantarum* compared to only 25% of the omnivorous (*Finegold* et al., 1983). A recent study performed in Sweden suggest the largest LAB taxa found on rectal mucosa in healthy humans are *Lactobacillus plantarum, Lactobacillus rhamnosus,* and *Lactobacillus paracasei* ssp. *paracasei* isolated in 52%, 26% and 17% respectively (*Ahrné* et al., 1998). Commonly milk-born LAB such as *Lactobacillus casei, Lactobacillus reuteri* and *Lactobacillus acidophilus* was found on the colonic mucosa only in a minority of individuals, 2%, 2% and 0%. It is reported that cosmonauts on return to earth from space flights have lost most of their commensal flora: *Lactobacillus plantarum* to 100%, *Lactobacillus casei* to almost 100%, *Lactobacillus fermentum* to appr. 50%, only to mention a few (*Lencner* et al., 1984), but also increased their flora of potentially pathogenic micro-organisms, changes attributed to the poor food eaten in the space and to stress. It is suggested that many Westerners also on Earth have an "astronaut-like" lifestyle, which could explain their, compared to our forefathers and to rural Africans and Asians, deranged intestinal flora.

NOT ALL LACTIC ACID BACTERIA SURVIVE GASTRO-INTESTINAL PASSAGE

It is important to remember that there is a great variety among the LAB in their ability to survive passage through the gastro-intestinal tract and to influence the immune system. A recent study compared in a gastro-intestinal model four different LAB: *Lactobacillus plantarum* (E98), *Lactobacillus paracasei* (E510), *Lactobacillus rhamnosus* (E522) and *Bifidobacter* animalis (E508), all administered in a dosis of 10^8 (*Miettinen* et al., 1998a). All strains were reduced in number, but the survival was much better for *Lactobacillus plantarum* (remaining 10^7) than for *Lactobacillus rhamnosus* (remaining 10^2). Most strains showed after the passage a weak ability to induce cytokine, but *Lactobacillus plantarum* demonstrated despite its reduction in numbers, an increased ability to induce production of cytokines such as TNF-α and IL-6, suggesting that this particular LAB is activated during the passage through the gastro-intestinal tract.

TWO PRINCIPALLY DIFFERENT DIGESTION SYSTEMS

Digestion is depending on two different digestion system, one based on digestive enzymes produced by eukaryotic cells and secreted as saliva, gastric, pancreatic and intestinal secretions, the other based on microbial enzymes produced mainly in the large intestine. It is obvious that the microbial system is much more complex and much richer in various enzymes. An indication of this is that the microbial genome is calculated to contain about four to five times as many genes as the human genome (about 300,000 versus 60,000). It is in the large intestine that complex carbohydrates and proteins are broken down and hundreds of thousands if not millions of nutrients set free and absorbed. Among these are short chain fatty acids, amino acids, polyamines, various other fatty acids, vitamins, antioxidants, growth factors and coagulation factors, and messenger molecules such as cytokines and nitric oxide produced.

It is increasingly recognised that an important part of the food should be of a type which reaches the large intestine more or less untouched, e.g. to large extent fruits, vegetables, pulses, tubers, a type of food often referred to as colonic food. Health authorities in various countries do also recommend that about 70% of the food eaten should be of the type destined for the colon. While the importance for health of daily supply of prebiotics/plant fibres is well documented in the literature, there is not yet enough evidence in the literature to support the necessity of daily consumption of probiotics. It seems often difficult to understand that a few grams of daily consumption of LAB can have a significant influence on human health when the large intestine in a normal and healthy individual contains one kilogram or more of live bacteria. However, it cannot be excluded that daily intake of LAB is of the greatest importance for the upper gastro-intestinal tract, the stomach and the small intestine, which are poorly colonised by LAB (*Lencner* et al., 1984).

MICROBIAL DYSBALANCE AND REDUCED RESISTANCE TO DISEASE

The microbial content of the colon of Westerners is quantitatively and qualitatively much different from that of rural Africans and, most likely, also from that of our Palaeolithic forefathers. There are good reasons to suggest that all Westerners have a dysbalance of the commensal flora, and that this could be associated with the observed reduced resistance to disease. Not only is the flora larger in rural Africans (about 2 kg versus about 1 kg in Westerners), it is also much richer.

Among the pathogenic bacteria associated with MSX are *Helicobacter pylori* and C*hlamydia* pneumoniae (*Laurila* et al., 1997, 1999). There are good reasons to speculate that the main reason why these microbes cause morbidity is a reduced resistance to disease, parallel to a microbial dysbalance, which leads to abnormal short-term (acute phase) and long-term (chronic phase) responses (*Bengmark*, 2000a). It appears in this connection to be of the greatest interest that LAB are shown to inhibit not only the growth of *H pylori in vitro* but also to exhibit strong antagonistic activity against *H. pylori in vivo* (*Kabir* et al., 1997; *Coconnier* et al., 1998).

Gastric production of NO is suggested to be crucial for control of the

Table 3: Content of arginine (mg/100 g) in some arginine-rich foods, compared to common Western foods such as hamburgers and French fries

Food		mg/100 g
Gelatine		6,000
Pumpkin seeds		4,030
Soya protein		3,760
Pea nuts		3,600
Sesame seeds		3,330
Soya beans		2,730
Almonds		2,500
Sunflower seeds		2,400
Brazil nuts		2,390
Peas, lentils		2,050
Shrimps		2,000
Baker's yeast		2,000
Parmesan cheese		1,560
Meat, fish		1,500
HAMBURGERS		950
Cereals	appr.	500
FRENCH FRIES		140
KETCHUP		125
Vegetables	appr.	100
Pulses	appr.	100
Fruits	appr.	50

pathogenic flora in the stomach, and acidified nitrite is shown to be effectively eliminate *Candida albicans, Escherichia coli, Shigella, Salmonella, H. pylori* but also conditions such as amoebic dysentery and chronic intestinal parasitism (*Duncan* et al., 1995). Adding 1 mM of nitrite *in vitro* of to an acidic solution (pH 2) will within 30 minutes produce a complete kill of *H. pylori*, which is not seen when acid alone is administered ($p<0.001$) (*Dykhuizen* et al., 1998). Gastric NO production does not occur in germfree animals, or when some pharmaceutical drugs influencing gastric secretion, especially H_2-blocking agents or proton inhibitors, are administered. It is also significantly less when antibiotics are supplied.

It is suggested that in the oral cavity species such as *Actinomyces* spp. and *Veillonella* spp., but not the most frequent species *Streptococcus* spp., exhibit a strong capacity to reduce nitrate and to produce NO (*Smith* et al., 1999). This process is both in the oral cavity and in the stomach influenced by the level of the pH in the oral cavity and a sucrose rinse results in a significant decrease in intra-oral generation of NO.

A link between endogenous nitric oxide production, MSX and occurrence of arteriosclerosis and thrombosis has been suggested (*Petrie* et al., 1996; *Pollard*, 1997), and dietary supply of the NO donor molecule L-arginine has been shown to reduce oxidative stress and preserve endothelial function in hypercholesterolaemic rabbits (*Böger* et al., 1998). NO-donating molecules such as arginine (Table 3) and nitrate/nitrite (Table 4) are rich in some foods such as some vegetables and fruits (*Andersson*, 1985). It is likely that also NO produced by bacterial action is important not only to the control of microbial overgrowth, but also to regulation of mucosal and

Table 4: Content of nitrate (mg/kg) in some nitrate-rich vegetables (after: *Andersson*, 1985)

Fennel	3,200
Lettuce	2,900
Celery	2,700
Mangold	2,600
Dill	2,400
Spinach	1,900
Beetroot	1,700
Nettle	1,600
Radish	1,300
Chinese cabbage	1,100
Savoy cabbage	1,100
Leek	700
Rhubarb	700
Chives	670
White cabbage	620
Squash	580
Broccoli	490
Horse radish	390

splanchnic (digestive tract) blood flow and to stimulation of gastro-intestinal motility (*Bengmark*, 2000b).

Rhubarb is rich in nitrate and when tried as a rhubarb decoction in experimental pancreatitis a significant reduction in the rate of microbial translocation to mesenteric lymph nodes and to pancreatic tissue (treated 25% vs. controls 100%), in mortality (1/8 vs. 5/8 animals) and in serum endotoxin levels (treated: 5.41±3.6 pg/L vs. controls: 61.36±28.3 pg/L, $p<0.001$) was observed (*Chen* and *Ran*, 1996). The authors did also observe that the gut motility otherwise seriously inhibited in pancreatitis was "significantly improved by administration of rhubarb."

HOMEOSTASIS/EQUILIBRIUM IN BODY SYSTEMS ARE IMPORTANT

Equilibrium in various body systems was regarded as extremely important to health by ancient medicine both in the East and the West. The French physiologist Claude Bernard introduced in the 19th century the concept of homeostasis to describe the importance of balance in the body of water and electrolytes. Today this concept has received a much broader meaning. Much support as a matter of fact that health is depending on numerous cellular systems and bodily processes in equilibrium: eukaryotic and prokaryotic cells, growth/regeneration and apoptosis, omega 3 and omega 6 fatty acids, pro-inflammatory and anti-inflammatory cytokines, just to mention a few.

It has in recent years often been suggested that a balance between Th1-lymphocytes, primarily associated with cellular immunity, and Th2-lymphocytes, mainly associated with humoral immunity is essential to health and well-being. Reduced microbial stimulation during early infancy and childhood, especially in developed countries, has been associated with the increasing

prevalence of allergy in children and young adults (*Björksten*, 1994). Reduced microbial stimulation is associated with slower postnatal maturation of the immune system, a delayed development and lack of balance between Th1 and Th2 immunity (*Lucey* et al., 1996).

Swedish infants have been reported to have a different gut flora than both Pakistani (*Adlerberth* et al., 1991) and Estonian children (*Sepp* et al., 1997). Some *Lactobacillus* species promotes Th1-type response and inhibits Th2-type immune response through stimulation of IFN-γ and IL-12 production (see below), hereby inhibiting allergic reactions (*Murosaki* et al., 1998). Stimulation of human peripheral blood mononuclear cells (PBMC) with various *Lactobacillus rhamnosus* and *Lactobacillus bulgaricus* strains leads to induction of Th1 type cytokines IL-12, IL-18, and IFN-γ (*Miettinen* et al., 1998b); and supply of *Lactobacillus casei* (*Shida* et al., 1998) and *Lacobacillus plantarum* (*Murosaki* et al., 1998) respectively to a total inhibition of antigen-induced IgE secretion both in ovalbumin- and casein-primed mice, an effect not observed when *Lactobacillus johnsonii* is tried.

SOME LACTIC ACID BACTERIA PROMOTE APOPTOSIS

Programmed cell death, apoptosis, is an important process, which leads to control of diseases such as cancer but also infections, especially those of viral origin. Some foods, such as diary products, rich in fat and in insulin-like growth factor 1 (IGF-1) and other tropic hormones and often also xeno-estrogens (from pesticides) are known to delay apoptosis (*Outwater* et al., 1997; *Westin* and *Richter*, 1990). On the other hand foods rich in fibres such as oat, wheat, rye, and chicory (inulin) will significantly increase apoptosis and prevent diseases such as cancer (*Hong* et al., 1997). It has been observed in experimental animals that feeding beans increases the production of short chain fatty acids (SCFAs) sevenfold (*Key* and *Mathers*, 1995), and that feeding fibres such as oligofructans (inulin) inhibits induction of colonic preneoplastic lesions (*Reddy* et al., 1997). As LAB produce SCFAs and SCFAs are known to promote apoptosis (*Heerdt* et al., 1994; *Marchetti* et al., 1997) and inhibit neoplasm, it should be reasonable to assume that LAB have an important role in cancer prevention.

SOME LACTIC ACID BACTERIA ACTIVATE MACROPHAGES

Saturated fat is known to inhibit the important process of phagocytosis e.g. the ability to engulf, kill and eliminate invading micro-organisms and/or defective cells, but also to eliminate toxins, mutagens and other poisonous substances. Also many chemicals including drugs and antibiotics do significantly inhibit apoptosis, effects, which has not been investigated and considered, as they should. Phagocytosis is known to be significantly enhanced by low molecular weight peptides obtained from species of the indigenous gastro-intestinal tract microflora such as such as *Bacteroides sp., Clostridium sp., Propionibacterium sp.* and from *Lactobacillus sp*. (*Pulverer* et al., 1990; *Kilkullen* et al., 1998), as well as of live bacteria including LAB, although not

all. As an example it has been observed that *Lactobacillus fermentum* is unable to increase macrophage activation (Kato et al., 1988).

CYTOKINE RELEASE INFLUENCED BY LACTIC ACID BACTERIA

The explosion in cytokine research in recent years and the availability of commercial kits to study cytokine reactions has lead to a flood of studies where reactions in various cytokines are observed in trauma and disease, but also after supplementation of LAB. There seems to be support for the assumption that the cytokine profile observed after oral administration of LAB reflects the direction and efficacy of the humoral response, and that different LAB, when supplied, influences this effect in different ways. Most of the attention is so far given to the cytokine production by monocytic cells such as macrophages, but mononuclear eukaryotic cells are also important sources of cytokines. It has become increasingly evident that tissues such as intestinal mucosal cells (*Eckmann* et al., 1995) and prokaryotic cells such as commensal flora and/or supplemented probiotic bacteria (*Ogle* et al., 1997; *Henderson* et al., 1997) secretes a spectrum of chemo-attractants and cytokines or cytokine-like molecules, often called bacteriokines.

Supplementation with some LAB seems to significantly influence the expression of cytokines by the cells, but the cytokine response is very different depending on the strain of LAB, which is supplied. It is regrettable that research has concentrated on the effects of milk-born LAB, but there is today a growing interest specifically in LAB with ability to ferment plant fibres, which are expected to have much stronger immunological effects. Among such LAB are bacteria such *Lactobacillus plantarum, Lactobacillus paracasei* ssp. *paracasei, Lactobacillus pentoceus* but also various *Lactococcus, Pediococcus* and eventually also *Enterococcus* species.

STRAIN-RELATED RESPONSE TO LACTIC ACID BACTERIA

St. thermophilus stimulates macrophage and T-cell cytokine production to a greater extent than *Lactobacillus bulgaricus, Bifidobacterium adolescentis* and *Bifidobacterium bifidum*, but a significant variability in effect has been observed between four different strains of *S. thermophilus* tried (*Marin* et al., 1998). Heat-killed *Lactobacillus acidophilus* (LA 1) has *in vitro* been shown to increase the production by mouse macrophages of IL1-α (appr. 300%) and TNF-α (appr. 1000%), an effect said to be of considerably greater magnitude than that produced by other tried *Lactobacillus* and *Bifidobacteria* (*Rangavajhyala* et al., 1997). The activity of 2´-5´synthetase, an expression of interferon-gamma (IFN-γ), in blood mononuclear cells of healthy subjects is 24 hours after a LAB-containing meal shown to be significantly increased (appr. 250%) (*Solis-Pereyra* et al., 1997). Significant increases in cytokine activity compared to controls are also observed when human mononuclear cells are incubated in the presence of the yoghurt bacteria; *L. bulgaricus (BUL),* and *S. thermophilus (Ther),* alone or in combination (Yog); INF-γ: *Bul* 775 %,

Ther 2100%, Yog 570 %, TNF-α: *Bul* 1020%, *Ther* 3180 % Yog 970%, and in IL-1β: *Bul* 2120 %, *Ther* 1540%, Yog 1920 %, all indicating a significant immuno-activation after supply of LAB.

Lactobacillus casei, when administered intrapleurally in tumour-bearing mice induces significant production of cytokines such as IFN-γ, IL1-β, TNF-α, paralleled by inhibited tumour growth and increased animal survival (*Matsuzaki* et al., 1997). Supply of this LAB to diabetes-prone and alloxan-treated animals delays also the onset of diabetes (*Matsuzaki*, 1998). It is of interest to observe that in experimental animals nitric oxide (NO)-donating molecules such as L-arginine and sodium nitroprussid when supplied will restore antioxidant status to almost normal and to prevent alloxan-induced beta-cell damage (*Mohan* and *Das*, 1998).

LACTIC ACID BACTERIA INDUCE GUT Ig-A PRODUCTION

Adaptive immunity at mucosal surfaces provides an immunological barrier to foreign matter, particularly pathogenic micro-organisms, allergenic food proteins and carcinogens. Immunoglobulin A (IgA) and to some extent immunoglobulin M (IgM) are dominant among the intestinal secretion immunoglobulins. Selective IgA deficiency is the most common immunodeficiency in Westerners. Decreased IgA levels are often compensated for by increased production of IgM, which is why clinical abnormalities often are difficult to recognise. IgA does not participate in the pro-inflammatory and cytotoxic responses that are readily activated by other immunoglobulins such as complement activation, nor in antibody-directed cytotoxic responses (*Kagnoff*, 1993).

Large quantities especially of IgA are transferred each day from the lamina propria to the lumen of the gut. It has been calculated that about 80% of all the body's Ig-producing immunocytes are localised in the gut (*Brandtzaeg* et al., 1989). The synthesis of IgA is highly dependent on T-cells and several cytokines produced by activated lymphocytes, which influence different steps in the IgA differentiation pathway (*Kiyono* and *McGhee*, 1994). Changes in nutrition, physical activity, sleep, mood, age, gender, circadian rhythm, drug use, medical illness and other innate changes are known to influence lymphocyte function and the Ig-production and hereby also resistance to disease. It has as an example been observed in liver transplantation patients that deficiency in IgA, but not in IgG and IgM, is associated with significantly increased morbidity and mortality in sepsis by opportunistic infections after major surgery, but also with increased rejection after liver transplantation (*van Thiel* et al., 1992).

LAB may during fermentation release components that possess immunomodulatory activity. However not all LAB may possess that ability. As an example, only 3/120 *Bifidobacteria* strains isolated from human faeces - two *Bifidobacterium breve* and one *B. longum* - had when studied in tissue culture the ability to induce production by Peyer´s patches of large quantities of IgA (*Yasui* et al., 1992). Supply of LAB such as *Lactobacillus* GG in Crohn´s disease is reported to significantly increase the IgA immune response (*Malin* et al., 1996). LAB are also reported to enhance the IgA response to rotavirus (*Kaila* et al., 1992). Intake of *Lactobacillus acidophilus* will

Table 5: Results of treatment with *Lactobacillus plantarum 299* after liver transplantation in man* (after *Rayes* et al., 1999)

Patient group	Treatment	30-day infection rate
Group 1	Selective bowel decontamination (tobramycine, cefalaxine and metronidazole) for 4 weeks. 2 litre enteral nutrition without fibre from the second postoperative day.	6/15 (40%)
Group 2	2 litre enteral nutrition with inulin fibre and heatkilled Lactobacillus plantarum from the second postoperative day.	4/15 (27%)
Group 3	2 litre enteral nutrition with inulin fibre and live *Lactobacillus plantarum* 299 from the 2nd day.	2/15 (13%)

*Enteral nutrition via naso-jejunal tube from day 2, peri-operative antibiotics (tobramycine, cefalaxine, and metronidazole) to all patients, prophylaxis against HSV (acyclovir), PcP (co-trimoxazole, cyclosporine or tacrolimus).

a >4 fold increase in IgA response, when challenged by *S. typhi* (*Solis-Pereyra* et al., 1997), and supplementation of *L. reuteri* (R2LC) or *L. plantarum* (299 V, DSM 9843) to rats with methotrexate colitis to significantly increase small and large intestinal IgA secretion to elevate the numbers of both CD4 and CD8 T-cells (*Mao* et al., 1997).

STRONG CLINICAL EFFECTS OF LACTIC ACID BACTERIA IN VARIOUS DISEASES

Numerous studies have proved the efficacy of LAB in various induced diseases in rats such as acetic acid colitis, methotrexate colitis, intra-abdominal infections, pancreatitis, acute liver injury etc. – for review see *Bengmark*, 1998a,b,c, 1999, 2000a,b,c,d; *Bengmark* et al., 2000. A recent study in rats documented efficiency of *L. plantarum* to preserve the gut mucosal barrier and to prevent colitis in Interleukin-10 knockout mice (*Kennedy* et al., 2000). Another recent published study demonstrated improved healing of colonic anastomoses after administration of *L. plantarum* to rats (*Colucci* et al., 2000).

So far only few controlled clinical studies with LAB and fibres have been performed but several studies are on the way. However, a most interesting study in liver transplantation patients was recently published (Table 5) (*Rayes* et al., 1999). The one-month sepsis rates were with selective decontamination 40%, enteral supply with inulin and oat fibres plus heat-killed Lactobacillus plantarum 299 27% and the same fibres + live *Lactobacillus plantarum 299* 13%. *Lactobacillus plantarum 299* was also in recent study found to significantly improve natural immunity, nutritional status and to improve growth in children with congenital HIV (*Cunningham-Rundles* et al., 2000).

IMMUNOLOGICAL EFFECTS MORE IMPORTANT THAN NUTRITIONAL

Two recent studies compared short-term enteral and parenteral supply to patients after larger operations (liver resection: *Shirabe* et al., 1997) and acute severe disease (pancreatitis: *Windsor* et al., 1998). They found no difference when measuring nutritional parameters but very significant differences when measuring immune-related parameters, paralleled by significant reduction in sepsis and sepsis-related complications in the enterally nourished groups. From these studies and others it has become increasingly evident that the immunological effects of nutrition is far more important than those obtained by measuring traditional nutritional parameters such as energy and nitrogen balance. Much support that immunological effect of nutrition is an important key to the understanding and control also of chronic diseases. As we enter a new millennium there seem to be a fast increasing interest in the role of nutrition to control disease within the medical professions as well as among the consumers. Government and other health authorities are also increasingly involved and publish dietary guide recommending dramatic life-style changes. It is my believe that the recommended alternative life-style and food habits should not only consist in a significantly increased consumption of fibre- and antioxidant rich fruits and vegetables, but also an increased consumption of especially fibre-fermenting lactic acid bacteria.

There is in modern medicine a tendency that the sickest patients, those in intensive care, will get the poorest foods, often only provided as semi-synthetic powders. This must immediately be changed and the sickest patients also receive fresh juices of fruits and vegetables but also of lactic acid bacteria.

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MICROBIAL TRANSLOCATION IN THERMAL INJURY

J. WESLEY ALEXANDER

University of Cincinnati College of Medicine, Department of Surgery, Cincinnati, Ohio, USA

SUMMARY

Cutaneous injury by thermal burns in experimental animals produces a highly reproducible acute model for studying the barrier function of the intestine wherein the magnitude of microbial translocation is directly associated with the extent of injury. Translocation after burn occurs throughout the intestinal tract with extreme rapidity, reaches an early peak within hours, and then has a less intense prolonged phase (for up to 20 days). Blood flow and oxygenation in individual villi and oxygenation appear to play important roles. Translocation occurs primarily through intact epithelial cells and M-cells but passage through mucosal ulcerations may also be important. The loss of barrier function to microbial products may be even more important than translocation of intact microbes since it appears that the products from approximately 100 microbes pass across the intestinal barrier for every one microbe that can be detected by culture. Loss of the barrier function is clearly associated with the hypermetabolic response in experimental animals. Importantly, a number of therapeutic interventions can improve gut barrier function against bacteria. These include lumenal nutrients, especially arginine, glutamine and the omega-3 fatty acids, growth factors such as G-CSF, GM-CSF, sucralfate and basic fibroblast growth factor, prostaglandin analogues, interferon-gamma, heparan sulphate and enalapril. Adrenocortical steroids will increase translocation but this can be counteracted by dietary arginine or glutamine. Thus, burn injury has provided a highly reproducible model for studying the mechanisms and pathological events following translocation as well as the development of treatments that may have useful clinical application in improving outcome following injury.

INTRODUCTION

Thermal injury provides a multifactorial clinically relevant model for studying barrier functions of the intestine in diseased states since there is altered nutritional intake by the afflicted subject, reduced blood flow to the intestine, and altered intestinal flora because of treatment, especially with antibiotics. Furthermore, the size and depth of the burn as well as its location can be varied at will in experimental animals, and all of the commonly used experimental animals can be used for such studies. The magnitude of translocation is directly related to the extent of burn with the maximum effect achieved with a 30% full thickness injury (*Gianotti* et al., 1993). However, larger burns further

impair the ability to kill the translocated organisms. The extent of translocation is also related to a variety of other variables, including the types and numbers of organisms within the intestinal tract, the nutritional status, the immunological status of the host, and the genetic background of the individual. Translocation of organisms occurs with similar intensity throughout the small and large intestine but there is better killing of the translocated organisms in the lower intestine (*Fukushima* et al., 1994). Translocation occurs with extreme rapidity after burn, with large numbers translocating to the mesenteric lymph nodes as early as five minutes (*Eaves-Pyles* and *Alexander*, 1998). Significant but delayed translocation to the liver occurs over a four-hour period whereas translocation to the spleen occurs at an intermediate rate. This is consistent with the primary route of translocation occurring by the lymphatics rather than the portal venous system as has also recently been suggested by Deitch's group who have shown that ligation of the main lymphatics draining the intestine decreases the extent of lung injury associated with translocation (*Sambol* et al., 2000). After 24 hours, the rate of translocation across the intestinal tract progressively diminishes for up to 5 days after which there is a lower but persistently elevated number of organisms that translocate across the intestine compared to control animals for up to at least 20 days (*Eaves-Pyles* and *Alexander*, 1998). The rapid translocation of organisms from the intestinal tract is consistent with the observations of Arnold and his colleagues in the 1920s in a non-burned canine model (*Arnold* and *Brody*, 1928).

Burn injury increases the translocation of all organisms we have studied. However, the pattern of translocation in organs and tissues is different. *Staphylococcus epidermidis* and *Escherichia coli* preferentially translocate to the mesenteric lymph nodes whereas *Klebsiella pneumoniae*, *Pseudomonas aeruginosa,* and *Serratia marcescens* preferentially translocate to the liver and spleen (*Eaves-Pyles* and *Alexander*, submitted).

Early translocation appears to correlate with the relative blood flow to individual villi with an increased translocation occurring in villi that have reduced perfusion (*Gianotti* et al., 1993). This suggests that blood flow, and therefore oxygenation, to the individual villi may be an important factor determining the extent of translocation. This hypothesis is supported by experiments which show that hyperoxia will decrease translocation from the intestine after burn injury (*Gennari* and *Alexander*, 1996) and that progressive lowering of the pH using *in vitro* models involving cultured cacao 2 cells is associated with an increased extent of internalisation of *E. coli* (unpublished data). Vaso-active substances undoubtedly play a role, and it has recently been shown that elimination of neutrophils will improve intestinal barrier function (*Fazal* et al., 2000) suggesting that cytokines such as IL-8 may play an important role.

MODELS AND QUANTIFICATION OF TRANSLOCATION AFTER BURN INJURY

The most commonly used method for detection and quantification of translocating organisms is by culture of the organisms from normally sterile tissues and organs (*Maejima* et al., 1984). However, the values obtained depend upon several important factors that may not be easily controlled. These include

1) the barrier function of the intestine itself, 2) the numbers of organisms within the lumen that are exposed to the mucosal surface and 3) the ability of the host to kill the translocated organisms, making interpretation of the results somewhat difficult if only viable organisms in the tissues are counted. We have tried to circumvent this problem by using micro-organisms labelled with radioisotopes and also by infusing a quantified number of viable organisms into the stomach at the time of burn injury or at other selected intervals. Two isotopes have been used by our laboratory for these studies. ^{14}C glucose in the culture medium becomes incorporated widely throughout the organism. It has the disadvantage that it may be excreted via metabolic by-products as well as being released whenever the organism dies. In contrast, ^{111}In oxide becomes attached to macromolecules within the organisms and is not released until the organism is killed. Macrophages, which take up the bacteria, then incorporate the isotope. Our experiments suggest that for every viable organism that is detected by culture from normally sterile tissues and organs, there are 10 intact organisms that translocate to these tissues (90% kill rate) but the metabolic products of approximately 100 organisms reach the tissues as measured by ^{14}C radioactivity (*Alexander* et al., 1991). It is perhaps of great importance that the toxic products from 100 organisms reach the tissues for every 1 organism that is detected by viable counts since toxins may be central to inducing clinical illness. Obviously, consistent relationships do not always exist and such estimates may be changed markedly by the type of organism and the innate ability of the host to kill the microbes. Nevertheless, it would appear that the extent of "translocation" far exceeds that which can be estimated by bacterial cultures. The biological effects of these products from dead organisms may be a much more important factor than the physiological and pathological effects of failure of gut barrier function. Even more sensitive methods of detection of translocation include measurement of ß-glucuronidase in tissues (unique to *E. coli*) and the use of PCR for genes unique to different bacteria. With this technique, it has been shown that the genetic markers for bacteria appear much more frequently than viable organisms in the subjects who have received a thermal injury (*Kane* et al., 1996).

HOW DO ORGANISMS TRANSLOCATE?

There are four potential sites where organisms can cross the epithelial barrier. These are through M-cells, through epithelial cells, through ulcerations, or through the tight junctions. For several years, we have studied this process by using electron microscopy and have established clear relationships for the first three pathways but have never observed either bacteria or *Candida albicans* translocating through tight junctions. *Candida albicans* has been the easiest organism to study, and this has been primarily in Thiry-Vella loops so that the effects of local nutrients could be avoided (*Alexander* et al., 1990). In loops that are infused with a suspension of *C. albicans*, attachment occurs early after injury to M-cells, but more commonly to epithelial cells, as shown by scanning electron microscopy. After contact with the microvilli of epithelial cells, the microvilli appear to undergo disruption and lysis with movement of the candidal body into the microvillous layer. Some of the disrupted villi be-

come attached to the cell wall of the *Candida* and can remain there during passage of the *Candida* through the epithelial cell. The candidal body rapidly crosses the cell membrane with repair of the membrane. Then it passes through the cell that appears to be undisturbed and without lysosomal degranulation. Finally, when the candidal body reaches the basement membrane, it is released into the lamina propria still surrounded by the cytoplasm of the cell. There, they are taken up by macrophages or may enter the lymphatics or venules without prior phagocytosis. Interestingly, Flory, the discoverer of penicillin, made similar observations using a light microscope in 1933 (*Flory*, 1933). Since the extent of translocation is measured by determining the number of microbes and/or their products that pass through the epithelium, it cannot be determined how many actually pass through the intact intestinal cell and how many enter through ulcerations. However, numerous studies have shown that ulcerations may occur at the villous tips and that these become a site for the attachment of the microbes and undoubtedly their penetration.

That *Candida* can penetrate the intact mucosa of a healthy individual was clearly shown by the striking experiment of *Krause* and his colleagues (1969) who demonstrated the existence of fungaemia and funguria after oral administration of a suspension of *Candida albicans* by a normal human volunteer. Blood cultures were positive for *Candida* between 3 and 6 hours after ingestion, and the subject was symptomatic between 2 and 9 hours.

THE RELATIONSHIP OF TRANSLOCATION TO THE HYPERMETABOLIC RESPONSE AFTER BURN INJURY

In one study (*Gianotti* et al., 1994), guinea pigs were given a gastrostomy and then subjected to a 40% total body surface thermal injury one week later. They were then randomly assigned to receive complete enteral diets by gastrostomy or lactated Ringer's in an equal volume. Both were started immediately post-burn and continued for 48 hours. The animals were then gavaged with 10^{10} ^{14}C *E. coli* at 48 hours post-feeding. Resting metabolic expenditure was determined four hours post-gavage and the animals were killed for measurement of radioactivity in the portal blood and ileal mucosal, intestinal mucosal weight, plasma cortisol and urinary vanillylmandelic acid (VMA). There was an inverse relationship between the numbers of bacteria in the intestinal mucosa and the mucosal weight (R=0.676) with the animals given enteral nutrition having a higher intestinal weight and lower numbers of bacteria in the mucosa. Furthermore, the numbers of bacteria in the mucosa, as measured by radioactivity, correlated directly with the numbers of bacteria in the portal blood (R=0.88). There was also a direct relationship between the numbers of bacteria in the blood and plasma cortisol. Both plasma cortisol and the urinary VMA were related to the degree of hypermetabolism (R=0.84 and 0.73 respectively). Not unexpectedly, there was also a strong relationship between the radioactivity in the blood and the portal blood and the increase in the metabolic rate compared to baseline (R=0.86). All of these relationships were statistically significant and showed that starvation increased translocation which then triggered a hypermetabolic response. These studies also support the clinical benefit of early and aggressive enteral feeding following burn injury (*Chiarelli*, 1990).

It has been difficult to demonstrate

that the degree of translocation has an effect on mortality. However, *Fukushima* et al (1992) were able to determine that the radioactivity from ^{14}C *E. coli* infused into the stomach at the time of burn injury in a mouse was detectable by sampling of the blood from the retroorbital plexus four hours after injury. The amount of radioactivity (translocation) that occurred at 4 hours related to both the overall survival and the length of time to death, with larger amounts of translocation being associated with earlier death. *Inoue* et al. (1991) also made similar associations using *Candida albicans* in burned guinea pigs.

EFFECTS OF TREATMENT ON TRANSLOCATION AND OUTCOME FOLLOWING BURN INJURY

Numerous therapeutic agents have been studied for their ability to improve survival in burned animals that have been made susceptible to intestinal translocation by burn injury. Many successful agents have different effects. Sucralfate and basic fibroblast growth factor seem to improve survival by improving the barrier function and decreasing translocation without an effect on killing (*Gianotti* et al., 1993). Prostaglandin E_1 analogues, misoprostal and einosoprost also improve the barrier function but inhibit killing of the translocated bacteria (*Gianotti* et al, 1993). Interferon gamma (*Gennari* et al., 1994), G-CSF (*Eaves-Pyles* and *Alexander*, 1996) and heparan sulphate (*Gennari* et al., 1994) all improve survival, primarily by improving the ability of the host to kill the translocated bacteria. GM-CSF improves both barrier function and killing of translocated bacteria (*Gennari* et al., 1994). Enalapril, an ACE inhibitor, decreases translocation, possibly by improving blood flow to the intestine (*Gennari* et al, 1996). IL-6 increases translocation and is associated with a higher mortality and this can be blocked by an anti-IL-6 antibody (*Gennari* et al., 1994).

Importantly, glutamine, arginine and the omega-3 fatty acids all improved survival in animals having translocation associated with burn injury. The effect of glutamine is primarily by improving the barrier function although it may improve the killing of bacteria (*Gianotti* et al., 1995) whereas the effect of arginine is primarily on clearance mechanisms of the translocated bacteria via a NO associated mechanism (*Gianotti* et al., 1993). The omega-3 fatty acids improve both barrier function and bacterial killing (*Gianotti* et al., 1996). Glutamine, arginine and DHEA have positive effects in burned animals additionally receiving steroids (*Gennari* et al., 1997).

CLINICAL OBSERVATIONS

Complete enteral diets containing the immunonutrients, arginine and the omega-3 fatty acids found in fish oil, have been shown to improve outcome in burn patients by decreasing the incidence of infections and shortening hospital stay, compared to diets not containing these (*Gottschlich* et al., 1990). In non-burn subjects, the immunonutrient diets which would decrease the rate of translocation have also been beneficial in shortening hospital stay, decreasing wound infections, and decreasing the incidence of multiple organ

failure (*Alexander*, 1998). The probable mechanisms are by inhibition of over-exuberant inflammatory responses that are at least partly related to translocation of microbes and their products associated with injury and/or malnutrition.

CONCLUSIONS

Burn injury has provided a suitable model for studying gut barrier function. Studies in burned animals have led to an understanding of how microbes translocate, the pathological consequences of translocation, the development of treatments that decrease translocation and concepts which have had successful clinical application such as the improvement in outcome from using enteral nutrition to improve barrier functions of the intestine.

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OLD HERBORN UNIVERSITY SEMINAR ON TRANSLOCATION: MINUTES AND REVIEW OF THE DISCUSSION

NICO A. BOS and PAUL NIEUWENHUIS

Department of Cell Biology, University of Groningen,
Groningen, The Netherlands.

DISCUSSION PARTICIPANTS (in alphabetical order):
J. Wesley Alexander, Stig Bengmark, Rodney Berg, Nico A. Bos, Ingeborg Bovee-Oudenhoven, Brad Allen Feltis, Peter J. Heidt, M. Veronica Herías, Nasir Hussain, Paul Nieuwenhuis, Mark Jepson, Reinhard Pabst, Volker Rusch, Gerhard Volkheimer, Dirk van der Waaij, and Torkel Wadström.

INTRODUCTION

The discussion was organised around different topics concerning with translocation. The used definition for translocation was "Passage of viable bacteria from the gastro-intestinal tract to the mesenteric lymph nodes and other possible organs" (*Berg* et al, 1979). However, also dead particulate matter may "translocate", even particles of up to 150 mm.

The discussion was centred on the following topics (Figure 1):

1. Intestinal content: Gut flora/ inert particles
2. Site of translocation: structured elements or anywhere along the enterocytes?
3. After translocation: innate and specific immune system in the lamina propria
4. Systemic factors influencing translocation

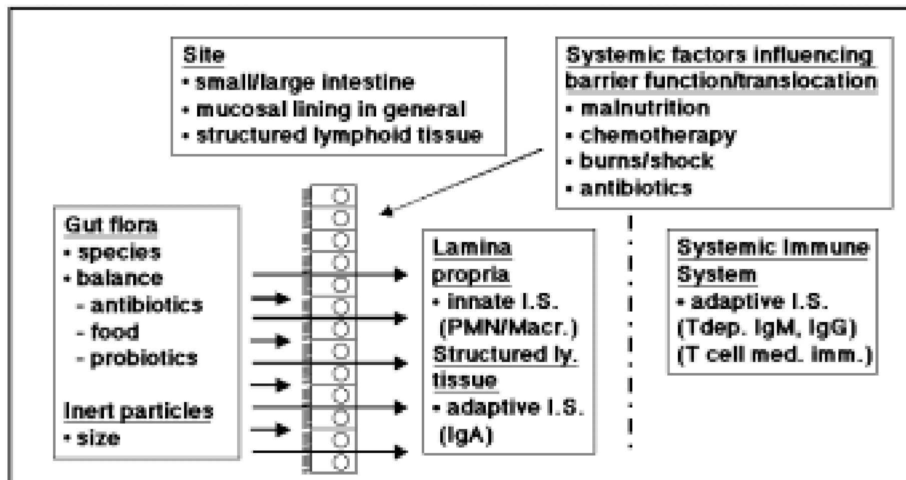

Figure 1: Factors involved in intestinal translocation

INTESTINAL CONTENT

The discussion first focused on the observations done by Dr. Volkheimer, that large particles (7-150 micrometer) such as starch can be transported very quickly from the gut lumen to different parts of the body. Most observations were done in humans and some in dogs. Next to the presence in lymph, the bloodstream, particles were found in kidney, in the urine, bile and within the peritoneal cavity. Passage to the peritoneal cavity was discussed and lymph vessels that end into the peritoneal cavity was suggested (Alexander), but denied by others (Pabst).

The site of passage can vary from the stomach to the large intestine. In gastric translocation the site of entry might be related to the mucus excretion sites. Within the small intestine the tips of the villi seem to be mostly involved, which might be related to turnover of the epithelium.

Drugs influencing the peristaltic movement, such as nicotine and adrenaline clearly have an effect on the passage frequency, which is about 1: 50,000 particles under normal conditions.

Another surprising observation discussed was that such particles are not caught within the mesenteric lymph nodes, but proceed beyond that. This contradicts the general opinion of the filter function of the (mesenteric) lymph nodes. Currently we have no explanation for these observations.

In addition, Hussain discussed possibilities to use this process for drug delivery. There is a clear distinction between particles <500 nm which are processed through M-cells, in contrast to the very large particles. Surface properties of particles might be manipulated to direct their uptake in Peyer's patches or within the villi. Lectins can promote particles to stick to the surface of different parts of the gut. In addition to lectins, bacterial ligands such as invasin promote inert nanoparticle uptake. More importantly, these ligand-conjugated nanoparticles were more readily absorbed through normal enterocytes rather than M-cells. Lactose that binds to rat mammalian intestinal lectins was found not to promote the uptake of polystyrene particles (Hussain), probably because this might render the surface hydrophilic. A balance should be found between hydrophilicity for quick passage through the mucus layer, versus hydrophobicity for good uptake into cells. Delivery of gut-labile molecules, such as peptides, vaccines and DNA might also be delivered by the oral route via encapsulation using biodegradeable particles. The relevance of *in vitro* models such as the Caco-2 cell lines to this process was discussed. Surface properties of Caco-2 cells are very different from intestinal epithelia *in vivo* (Jepson). Mucus-secreting intestinal epithelial cell lines also exist (HT-29), but the mucus produced by this cell line may also be very different from the mucus of the *in vivo* situation. It is also possible that epithelial cells themselves change the uptake of certain particles by producing factors upon binding of particles.

Studies concerning uptake of small particles have been done from 45 minutes and on, while passage of starch particles was within five minutes. Experiments should be done with earlier time points.

Otherwise, collecting of lymph through thoraxic duct canulation might give insight into the route of particles (Pabst).

Other factors like total parenteral nutrition (TPN) might be important in uptake by changing the amount of mucus present. This might also be one of the

reasons why TPN enhances translocation of bacteria. Another explanation might be the increase in the number of M-cells after TPN.

D. van der Waaij presented some data by *L. van der Waaij* et al. (1996) which show that patients with inflammatory bowel disease (IBD) have a significantly increased percentage of coating by immunoglobulins; IgA (range 25-80%) and IgG (range 10-40%); the percentage of *in vivo* IgG coating of endogenous faecal bacteria is normally much lower (range 2-10%). This difference in coating incidence could be the result of enhanced translocation in IBD. The only direct evidence for enhanced translocation, however, comes from patients where mesenteric lymph nodes have been removed during operation, which show an increase in enteric bacteria in the MLN.

SITE OF TRANSLOCATION

Berg opened the discussion with presenting some data on the site of translocation. In the experiment with *E. Coli* C25 the ileum and caecum seem to be most prominent sites for translocation. Removal of the caecal patches did not change the rate of translocation. Also ligation of the ileal Peyer's patches did not influence the number of *E. Coli* observed in the MLN. These experiments suggest that the site of translocation is not limited to the lymphoid areas, but can occur with the same efficiency along the enterocytes. Its was suggested to repeat the experiments with the currently available knock-out mice such as the LTB knockout, that are not able to make lymphoid follicles (Pabst).

Another issue is the possibility of bacteria to survive inside an intestinal tract. In general the more bacteria are sensitive to oxygen, the less they translocate. In a comparative study where SPF animals were antibiotic treated and then given 10^9 bacteria, Berg and colleagues showed that obligate anaerobes almost do not translocate, Gram-positive bacteria on average are intermediate (~20 bacteria/MLN), while *Enterobacteriaceae* translocate to about 50 bacteria/MLN. The initial rate of translocation of bacteria as observed by radioactive labelling of the bacteria was, however, the same for the different kinds of bacteria (Alexander). Of course, anaerobic bacteria will have a disadvantage in the aerobic environment of the host.

Then the role of virulence factors of bacteria was discussed. Berg stated experiments were Entero-pathogenic *E. coli* (EPEC) and Entero-invasive *E. Coli* (EIEC) showed low penetration in the MLN translocation assay. This suggested that virulence factors not necessarily cause enhanced translocation. Salmonella does have specific factors involved in enhanced translocation and survival inside the host. Salmonella for instance encodes specific invasion machinery, recognises matrix proteins and is able to survive inside macrophages. Type 1 fimbriae on *E. Coli* can be involved by turning off the production of the fimbriae while passing through the mucus and turn it on again for epithelium attachment (Herias).

The composition of the mucus in different layers can also be of influence. Rodents for instance have a more hydrophobic mucus layer. Alexander referred to experiments by *Katouli* et al. (1994) in intensive care patients in which translocated vancomycin resistant bacteria were compared with the caecal bacteria by biotyping. In these experiments there was no clear correlation between the biotypes observed in caecal

isolates and the translocated species. This suggests that there is a selective advantage of some species over others in their ability to translocate (Alexander) and also within the same species (*E. coli*) there are some strains differing in certain traits that facilitate translocation (Herias, referring to studies by Bark and Katouli (1993), where they found different biochemical phenotypes present among translocating *E. coli* strains).

AFTER TRANSLOCATION

Berg presented data about the effect that a *Propionibacterium acnes* vaccine had on translocation of *E. Coli* after decontamination. In conventional mice this vaccination reduced the rate of translocation. When, however germfree (GF) mice that were recently conventionalised were used, the vaccination had no effect on the rate of translocation, as it was similar to GF mice. When the same experiments were repeated in neonatal GF mice that were conventionalised at less than 1 week after birth, the ex-GF mice reacted as conventional mice and responded to the vaccination. These results suggested that the indigenous flora had to be established before 1 week after birth.

Also in tolerance experiments the same observations were done. A conventional animal can be tolerised to ovalbumine by oral administration, while this is not possible in germfree mice. When germfree mice are neonatally conventionalised they can be tolerised as adults, while conventionalisation at adult age does not result in facilitating tolerisation (Nieuwenhuis). Other examples of the importance of the gut flora in the early shaping of the immune system come from experiments comparing the occurrence of allergies in different countries with similar ethnic background but differing in economic and hygienic conditions. For example, comparing Swedish children with children form Estonia showed that children of Estonia had a lower intestinal worm infestation than Swedish children (Waldström, Herias). Herias, referring to the studies by *Björksten* et al. (1999), suggested that this might be related to the higher incidence of asthma and allergy among Swedish children. Sweden has a higher index of allergic disease than Estonia, and these differences could depend on a reduced microbial stimulation during early life that could result in a slower postnatal maturation of the immune system, especially the TH1/TH2 balance (shift from TH2 to TH1).

The role of the structured lymphoid tissue was next discussed. There is an intimate contact between lymphocytes and the epithelium. M-cells that overlay the dome of structured lymphoid tissue such as Peyer's patches contain some lymphocytes of which the function is unknown. Also the role of IEL's that are in close contact with the epithelium elsewhere still remains unclear. The origin of M-cells was discussed by Jepson. There seems to be an early predetermination, since early M-cell markers are already present within the crypts where the M-cells differentiate (*Gebert* and *Posselt*, 1999; *Gebert* et al., 1999; *Giannasca* et al., 1994). The number of M-cells can be regulated. Cytokines derived from B-cells might be involved, since IgM-KO mice have less M-cells (*Debard* et al., 1999; *Golovkina* et al., 1999; *Niedergang* and *Kraehenbuhl*, 2000) and co-culture of Caco-2 cells with B-lymphocytes induces formation of cells with some properties of M-cells (*Kerneis* et al., 1997). Also *Salmonella*

typhimurium and Streptococcus pneumoniae have been reported to increase the number of M-cells (*Borghesi* et al., 1999; *Meynell* et al., 1999; *Savidge* et al., 1991). The fate of M-cells is unclear. They may die or differentiate into enterocytes (*Borghesi* et al., 1999; *Debard* et al., 1999; *Niedergang* and *Kraehenbuhl*, 2000). M-cells are also found in the epithelium covering isolated follicles. The functional relevance of the great heterogeneity of markers for M-cells in different species is still unknown.

The role of IgA was discussed by Bos. In general sIgA within the gut lumen is thought to enhance exclusion of bacteria from the gastro-intestinal tract by prevention of attachment. Otherwise, Lamm and colleagues have shown that excretion of IgA-immune complexes via the poly-Ig receptor might also be part of the natural clearance of bacteria. Although commensal bacteria are also very often coated with IgA, they are maintained at steady levels. Pathogenic bacteria, however, are completely excluded from the gut. The affinities and specificities of the involved IgA molecules might be important in explaining such different effects of IgA. Bos showed experiments where there is a clear IgA response after mono-association of GF mice with the commensal, strictly anaerobic Segmented Filamentous Bacterium (SFB) (*Talham* et al., 1999). The numbers of SFB in the small intestine show a sharp rise directly after weaning, which disappears after some weeks. In breeding experiments with SFB mono-associated mice, where the mother and/or the pups were impaired in IgA production, this temporary localisation does not occur. This example illustrates the effect that IgA can have on the composition of the gut flora.

The different methods of detection of translocation were next discussed. According to the definition of Berg et al., the culturing of live bacteria from mesenteric lymph node is still the standard assay. Labelling of micro-organisms is very useful for tracing the bacterial load within different organs, but the viability of these micro-organisms within the host is unknown. The detection of NOx within the urine, is a measurement of macrophage activation. In some infection models there is a good correlation between the NOx levels and the level of translocation, but in situations where the infectious agent is unknown, this might not be true. Detection of micro-organisms by PCR in blood samples is very sensitive, but very often inhibitors of the PCR reaction results in false negative results. The endotoxin level within the blood is also a good indicator for Gram-negative sepsis.

SYSTEMIC FACTORS INVOLVED IN TRANSLOCATION.

Alexander discussed the role of nutrition in burn patients. Good results were obtained by feeding patients immediately an "immune enhancing diet" containing extra arginine, glutamine and fish oil. These additions seem to downregulate the inflammatory response. Arginine influences the level of factors such as Insulin-like growth factor 1. Starvation and malnutrition alone do not increase the chance of translocation. In combination with hypovolaemic shock there is an increased translocation. Lack of critical factors such as Vitamin-A might lead to increased translocation.

Total parenteral nutrition (TPN) also leads to increased translocation. Addition of solid particles or fibres to an oral diet decreases translocation. Addition of dried food does not help. Fresh fruits

might be very beneficial because of the amount of the amino acid taurin present, which fills up macrophages.

The balance between natural clearance and translocation can be disturbed by local malnutrition of the colon. Bacterial digestion allows LPS to be released and absorbed onto particles derived from the environment. This might lead to products that might influence cytokine levels, opsonisation and antioxidants. LPS in solution is relatively innocuous to the epithelium when compared to LPS absorbed onto a solid surface (Hussain). Also the composition of the mucus is influenced by the food intake.

In patients that where implanted with biomaterials, such as Teflon, most implant related infections were not blood borne. This suggests that in those cases most infections originate from the time of operation.

In critically ill patients there is some evidence for gut-derived infections. Selective decontamination with non-absorbable antibiotics against Gram-negative bacteria has obtained variable success rates. To determine how successful the selective decontamination has been, one should culture the stools for Gram-negative bacteria. The disturbance of the flora by this method might lead to overgrowth of certain species, such as enterococci and an enhanced risk for translocation.

The mechanisms of a Ca^{++}-rich diet to prevent translocation were discussed by Bovee-Oudenhoven.

Dietary Ca^{++} decreases the soluble concentration of intestinal bile acids and fatty acids. Precipitation of these surfactants prevents damage to the intestinal epithelium, decreases proliferation of epithelial cells (risk marker for colon carcinogenesis) and strengthen the mucosal barrier function (*Bovee-Oudenhoven* et al., 1999*; van der Meer* et al., 1997).

Calcium carbonates are also used for the reflux-syndrome. The long-term effects of the increased intake of these kinds of Ca-salts are unknown. Possibly, a high intake of carbonate neutralises gastric acid, which is important for host defence against food borne infections.

Furthermore, Ca^{++} seems to decrease the number of Gram-negative bacteria, while the number of Gram-positive bacteria such as Lactobacilli can be increased. However, also other Gram-positive bacteria such as Listeria monocytogenes may increase in number, which could be regarded as a negative side effect. When the daily human intake of Ca^{++} is less then 500 milligram, addition of Ca^{++} might be beneficial to improve resistance.

Bengmark stressed the importance of the fermentation by intestinal bacteria. Micro-organisms ferment 20-25% of our food, which results in release of a great number of nutrients. The processing of food (cooking, drying, and freezing) can lead to a great loss of nutrients.

Lactobacillus plantarum is very active in these fermentation processes and has been used as probiotic.

The modern food style does not favour the presence of this bacterium within the digestive tracts since only 5% of Swedish students have this bacterium in their stools, while in Africa 100% of the autochthonous population delivered positive cultures from their stools. Also people eating vegetarian diets have an increased presence of *L. plantarum*.

Preliminary data in liver transplantation patients, show that addition of 10^{10} live *L. plantarum* to their diet in combination with selective bowel decontamination leads to less patients with infections (2/15) compared to SDD alone (6/15) or SDD + dead bacteria (4/15) (*Rayes*, 1999).

Since with the modern food style

lactobacilli very often are only transient members of the gut flora, alterations of food habits might ultimately lead to a better establishment of such fermenting bacteria

Food additives such as galactose, fructose and mannose-oligosaccharides might lead to better fermentable carbohydrates for such bacteria.

CONCLUSION

Translocation can be regarded as an illustration of the close interaction between the gut microbiota and the host. Many aspects of these interactions are still poorly understood. A better understanding is needed of both the physiology of this dynamic equilibrium and the pathological consequences when the dynamic balance between the gut microbiota and the host is disturbed. Factors influencing this balance can be of crucial importance for managing general health conditions.

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