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15. PROBIOTICS: BACTERIA AND BACTERIAL FRAGMENTS AS IMMUNOMODULATORY AGENTS

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PROBIOTICS AND DEFINITIONS: A SHORT OVERVIEW

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SUMMARY

Probiotics receive increasing attention in the medical field. This is partly due to the recognition that microbial resistance to antibiotics presents a serious world-wide problem. In addition, fast growing insight into host-microbe interactions opened new routes in the development of rational alternatives in therapy and prevention of diseases as result of the complex procaryotic-eucaryotic evolutionary symphony. Consequently, long existing aspects of probiotics came into focus, and already provide exiting prospects. However, there is no consensus about an appropriate definition of the term probiotic. The International Study Group on New Antimicrobial Strategies (ISGNAS) proposed the distinction into medical, pharmaceutical, and alimentary probiotics. It is mandatory to accept clear-cut definitions and link these to accordingly differentiating regulations to foster probiotics in therapy and prevention, where suitable, and to optimise antibiotic measurements, where necessary.

The majority of recent publications refer to more or less the same sources concerning the definition of the term probiotic. It is attributed to *Lilly* and *Stillwel*l to have coined the term first in 1965 (*Conway*, 1996a, 1996b, 1996c; *Fuller*, 1989, 1992; *Havenaar* and *Huis in't Veld*, 1992; *Ouwehand* et al., 1999). *Lilly* and *Stillwell* defined probiotic as "a substance produced by one microorganism stimulating the growth of another microorganism" and understood a probiotic as opposite to an antibiotic (*Lilly* and *Stillwell*, 1965). A totally different view was introduced by *Parker* (1974): "Organisms and substances which contribute to intestinal microbial balance". *Fuller* followed this line and defined in 1989 a probiotic as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance" (*Fuller*, 1989). *Havenaar* and *Huis in't Veld* broadened this scope 1992 and associated a probiotic with the description: "A viable mono- or mixed culture of microorganisms which, applied to animal or man, beneficially affects the host by improving the properties of the indigenous microflora" (*Havenaar* and *Huis in't Veld*, 1992). In 1996 *Conway* claimed: "Today it is generally agreed that a probiotic is a preparation of live microorganisms which, applied to man or animal, beneficially affects the host by improving the properties of the indigenous microbiota". The same year *Sanders* issued her view: "Probiotics, simply defined, are microbes consumed for a health effect. The term probiotic is used in food applications. The term biotherapeutic is used in clinical applications" (*Sanders*, 1996).

Today, numerous definitions of the term probiotic are existing (*Conway*,

1996a, 1996b, 1996c; *Fuller*, 1989, 1992; *Hanson* and *Yolken*, 1999; *Havenaar* and *Huis in't Veld*, 1992; *Ouwehand* et al., 1999; *Tannock*, 1999). Unfortunately, in contrast to *Conway's* claim that it is generally agreed that a probiotic is a preparation of live microorganisms which improve the indigenous microbiota, there is no consensus among the experts. In fact, the definition of the term probiotic is still controversial as experienced earlier (see *Fuller* et al., 1995).

Most of the proposed definitions for probiotics centre around the ingestion of viable microorganisms with the purpose to modulate the host's intestinal microflora, with the exception of *Lilly's* and *Stillwell's* connotation and concentration onto growth factors (*Lilly* and *Stillwell*, 1965). It is generally neglected that there are other compartments in man and animals colonised by symbiotic microbes such as the oral cavity, the skin or the vagina, for instance. Furthermore, despite noticing of immunological effects of probiotic microbes, an eventually most important mode of action, immunomodulation, is not explicitly part of any definitory concept. Additionally, non-viable microorganisms or microbial components are not considered. In fact, there are publications on effects of microbial preparations onto compartments other than the intestine (e.g., see *Heidt* et al., 1999), as well as there are studies on the effects of viable and non-viable microorganisms and microbial components (e.g. see: *Ottendorfer* and *Zimmermann*, 1997; *Ouwehand* et al., 1999; *Panijel* and *Burkhard*, 1993; *Rusch* et al., 2001). Finally, the term probiotic was not first introduced by *Lilly* and *Stillwell* in 1965.

In the electronic era scientific knowledge is confined to databases reaching back not too far. Since most scientists today rely upon databases, tracking of older work or of publications in languages other than English is difficult or even impossible. Back to the roots. During one of the more recent Old Herborn University Seminars, *Christoph Persin* drew the attention of the author to an article written by *Werner Kollath* in an old German journal. *Kollath* was a renowned nutritionist (*Koerber* et al., 1999). He wrote in 1953: "High value food should supplement low value food. In order to make such food supplements palatable to people one may denote all organic and inorganic complexes as *probiotics* in contrast to harmful *antibiotics*. All these factors, probiotics, are common in vegetable food as vitamins, aromatic substances, enzymes or possibly other substances connected with vital processes in accordance with *Santo* and *Rusch*. This connotation links probiotics with food ingredients, however, includes yet another aspect indicated in the last words of the quotation. This aspect was cultivated further in a group of physicians engaged in natural medicine with whom *Kollath* was associated. Out of this group evolved a more focussed view of probiotics, as expressed by *Vergin* in 1954: "It is more important to perceive that *antibiotics* affect our autochthonous microorganisms living together with us in a biocoenosis or even in a symbiosis and thus deplete us of essential *probiotics*. *Werner Kollath* proposed the term *probiotics*. Consequently, *probiotics* are the opposite of *antibiotics*." The quoted "other substances connected with vital processes" as expressed by *Kollath* lead to the author's father, *Hans Peter Rusch*, who was convinced that physiological bacteria and their constituents delivered via vegetables are an important health factor. The lead is further continued to physicians joined in an *Association for Microbial Therapy* in the early fifties of the last century. This group was dedicated to explore therapy

with physiological microbes and presided by *Hans Kolb*, *Helmut Mommsen*, and *Hans Peter Rusch*. Quotations reveal a clear therapeutic approach in connection with probiotics. "Antibiotic therapy causes flora damage. In such cases we administer cultures of symbionts. In this way, deleterious effects of antibiotics are prevented by *probiotic therapy*" (*Kolb*, 1955). "Symbioflor provides us with a biological antiseptic. This kind of disinfection has the advantage of affecting pathogenic but not physiological microorganisms, and in addition enhances cellular functions. Thus, *Mommsen* designates bacteriotherapy in contrast to antibiotic therapy as probiotic" (*Rusch* sr., 1956).

Out of past and present it becomes obvious that it is mandatory to distinguish different categories of probiotics. This is due to the mode of action of probiotics, the aims of administration of probiotics and their mode of administration as well as claims in relation to food and drug legal regulations. Consequently, the International Study Group on New Antimicrobial Strategies (ISGNAS, see *Araneo* et al., 1996 and *Rusch* et al., 1996) developed a concept for the detailed definition of probiotics in three categories: 1. Medical probiotics (drugs), 2. Pharmaceutical probiotics (food supplements), and 3. Alimentary probiotics (food) (ISGNAS 1998). A *medical probiotic* is a microbial preparation which contains live and/or dead microorganisms including their components and products determined to be employed as a drug for therapeutic purposes. A *pharmaceutical probiotic* is a microbial preparation designed for manufacture of food supplements. An *alimentary probiotic* is a microbial preparation designed for use in food fermentation or food production. The mode of action includes immunomodulation, host microflora modulation, and the modulation of metabolic processes.

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DEFENSINS AND INNATE IMMUNITY OF THE MAMMALIAN SMALL INTESTINE

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SUMMARY

The release of gene-encoded antimicrobial peptides by epithelial cells is thought to contribute to innate mucosal immunity. Defensins are a predominant class of such antimicrobial peptides in mammals. These cysteine-rich cationic peptides have antibiotic activity against a wide range of bacteria and other microbes. In the mammalian small intestine, Paneth cells at the base of the crypts of Lieberkühn secrete defensins and other antibiotic proteins. These Paneth cell antimicrobials are proposed to have several overlapping functions. First, they likely help to protect the epithelial stem cells from noxious microbes. The stem cells, which reside at the neck of the crypt, are responsible for continual renewal of cells necessary for maintained integrity of the surface epithelium lining the villi and crypts. Second, defensins and other Paneth cell products likely interact with bacteria that exist in the intestinal lumen. Based on relative sensitivity to these antimicrobials, the composition of the enteric microbial flora might be influenced. Third, enteric defensins may regulate the numbers of colonising microbes in the small intestine. Forth, enteric defensins may contribute to defence from food and water borne pathogens in the intestinal lumen. Further defining the contributions of Paneth cell defensins to innate defence should improve our understanding of normal small bowel function. Given that microbial products stimulate Paneth cell secretion, it is possible that the mechanism of action of probiotic agents may, in part, involve modulating Paneth cell secretion.

INNATE HOST DEFENCE OF THE SMALL INTESTINE

The mucosal surface of the mammalian small intestine is remarkable from the perspective of host defence. Its life-sustaining physiological function requires that these sites maintain direct contact with the external world via the lumen. The surface area of this mucosa is amplified anatomically by its many folds, villi and microvilli that increase the luminal absorptive area (Figure 1). The structural adaptations that have evolved to satisfy requirements for adequate nutrient absorption also predict a host vulnerability, because they increase the opportunity for microbes to establish invasive infections. In addition, the requirements of efficient nutrient absorption also place limits on the barrier components of host defence. Unlike many other surface epithelia, the intesti-

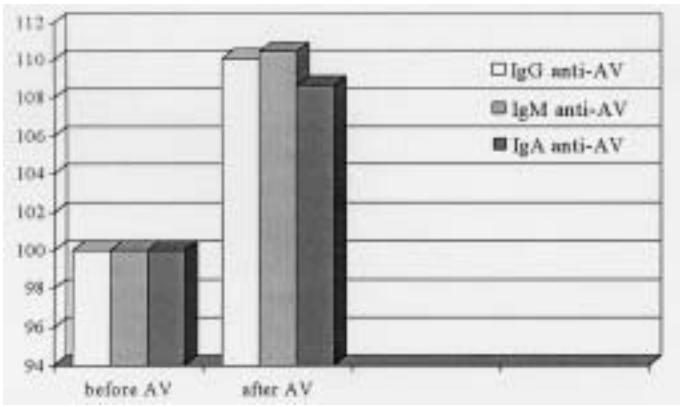

Figure 1: Microbial challenges in the alimentary tract - a schematic diagram.
The alimentary tract of humans and other non-ruminant mammals are confronted with microbial challenge (designated as dots) from abundant quantities of microbes in a heavily colonised colon and from variable quantities of microbes in food and water sources. In contrast, the small intestine has relatively low level of colonisation. The surface area of the small intestine is large, amplified by mucosal folds, villus projections and microvilli. Paneth cells are found along the length of the small intestine. These secretory epithelial cells, armed with defensins and other antibiotic peptides, reside in the crypts of Lieberkühn.
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nal epithelia are comprised of only a single layer of cells. While the complications that often follow antibiotic therapies offer support for the notion that colonising symbiotic microbes contribute to host defence against pathogens, in the absence of effective defence mechanisms even these microbes could rapidly multiply and overwhelm the mammalian host. Thus, the epithelium of the small bowel must execute its digestive and absorptive roles while inhibiting most microbes from establishing themselves as significant resident populations.
The architecture of the small intestinal epithelium is characterised by villi and crypts (Figure 2). This epithelium is replaced continually by a process involving stem cell mitosis, cellular differentiation and migration, apoptosis and ultimately exfoliation. Stem cells, which reside in the neck of the intestinal crypts, replicate to maintaining a continuous supply of new epithelial cells required to repopulate the villi and crypts (*Potten*, 1998; *Booth* and *Potten*, 2000). An interruption of this stem cell replication would have important consequences for the maintenance of the normal digestive epithelium and could generate portals of entry for luminal bacteria. Therefore, mechanisms that protect crypts against bacterial overgrowth and infection are also key elements of small intestinal defence.

Innate immunity (also termed natural immunity) encompasses a complex array of defence mechanisms (*Janeway* and *Travers*, 1997). At mucosal surfaces the innate defence system generally employs two broad and overlapping strategies that are central to effective defence: minimise microbial adherence and create a hostile environment for potential pathogens. In the small bowel, examples of innate defences include:

i) *physical processes*, such as peristalsis and the shedding of epithelial cells,

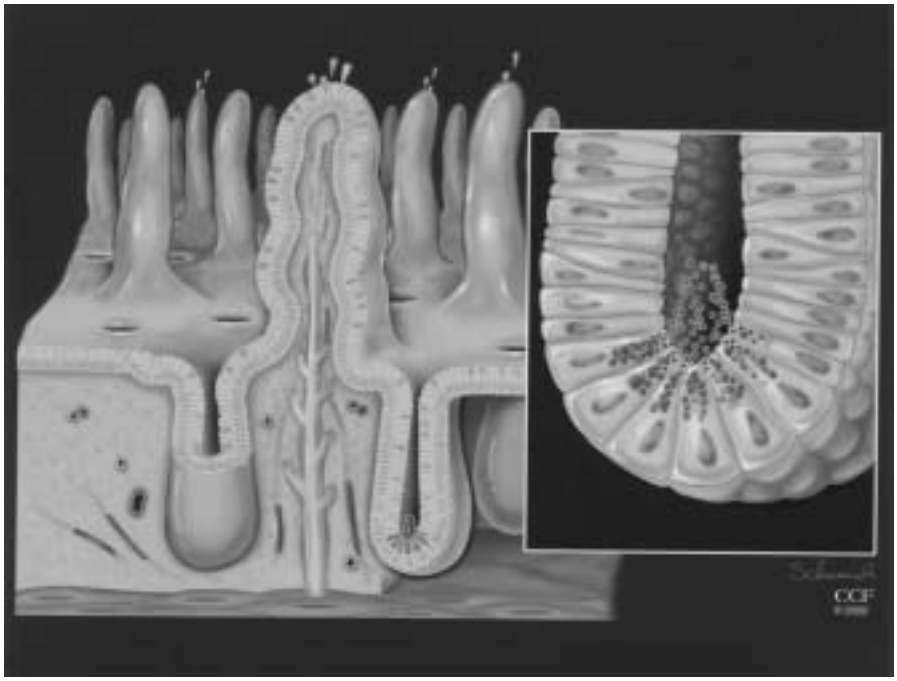

Figure 2: Small intestinal crypt architecture.
An artist's view of the small intestinal villi and crypt relationship (*Ganz*, 2000). Stem cells reside at the neck of the small intestinal crypt and divide. Some of their progeny cells migrate upward toward the villi or while others migrate deeper towards the base of the crypt. Those cells migrating towards the villus tips undergo cellular differentiation into absorptive enterocytes, goblet cells or entero-endocrine cells. The life span of these villus cells from their origin in the crypt, through migration and differentiation, until apoptotic death and exfoliation into the lumen is approximately 2-5 days. The other cells, which descend towards the crypt base, differentiate into Paneth cells. Paneth cells have life span of several weeks. Inset. Paneth cells release secretory vesicles into the narrow lumen of the crypt (*Ganz*, 2000). These secretions contain α-defensins, lysozyme and secretory phospholipase A_2. This potent antimicrobial cocktail is proposed to have several host defence functions as discussed in the text.
Illustrated by D. Schumick, Department of Medical Illustration, Cleveland Clinic Foundation, ©2000, Cleveland Clinic Foundation as published by *Ganz* (2000), with permission.

ii) *chemical barriers*, such as mucus and various peptides and proteins, and
iii) *cellular processes*, such as phagocytosis.

In the small intestine, activities associated with the process of digestion may also contribute to innate mucosal defences. For example, gastric acidity, digestive enzymes, and bile salts may contribute effectively to the inhibition of microbial growth. Innate host defence mechanisms interface with the acquired (also termed clonal or adaptive) immune responses mediated by lymphocytes (*Fearon*, 1997; *Medzhitov* and *Janeway*, 1997). Clearly, mammals commit extensive resources to lymphocyte mediated adaptive immune functions in the intestinal compartment. Prominent examples include humoral immunity via B cell-mediated release of secretory IgA which traverses the epithelium to the gut lumen, and cell-mediated immunity via intraepithelial T-cells of villi (*Neutra* et al., 1996). However, in contrast to the lymphocyte-mediated immune system,

where an effective response involves both gene rearrangements and clonal selection developed over a period of days, the innate system remains ever-ready or immediately inducible. Therefore, innate host defence mechanisms, including both physical and chemical factors, are thought to provide immediate protection against the threat of colonisation and infection by deleterious microorganisms. This essay will focus on one component of innate immunity, defensins, a group of antimicrobial peptides that contribute to defence in the small intestine and other body surfaces. For more in-depth discussion of defensins the interested reader is directed to several reviews (*Martin* et al., 1995; *Lehrer* et al., 1998; *Ganz*, 1999).

ANTIMICROBIAL PEPTIDES IN INNATE HOST DEFENCE

Antimicrobial peptides contribute to host defence in a wide variety of settings, including plant seeds, arthropod haemolymph, and mammalian phagocytes and epithelia (*Zasloff*, 1992; *Boman*, 1995; *Hancock* et al., 1995; *Lehrer* et al., 1998; *Huttner* and *Bevins*, 1999). Unlike many other antibiotics in nature, which are secondary metabolites, antimicrobial peptides are encoded by conventional genes. There is considerable structural diversity in the many dozens of antimicrobial peptides, ranging from simple, alpha-helical linear molecules to molecules with beta-sheet conformation and multiple disulphide linkages. However, most of these peptides are cationic and amphipathic and they are generally active at micromolar concentrations against a broad range of microbes. Disruption of the target microbial membrane function is a typical feature of the mechanism action of most peptides investigated to date, and in some cases pore formation, membrane depolarisation, disruption of bacterial energy metabolism and interference with biosynthetic pathways have been observed.

Experimental evidence for the specific roles of antimicrobial peptides in host defence has been provided by experiments that assess the impact of ablation or augmentation of antimicrobial peptide production. Augmentation of antimicrobial peptide production in plants has been shown to increase their resistance to plant pathogens (*Epple* et al., 1997; *Fritig* et al., 1998). Experiments in Drosophila have demonstrated that ablation of pathways that induce the production of antifungal peptide drosomycin dramatically reduce survival after fungal infections (*Lemaitre* et al., 1996). Similar experiments in transgenic mice are a subject of intense effort in several laboratories. The multiplicity of antimicrobial peptides and redundancies in the innate and adaptive immune systems in mammals has presented challenges to the design of these experiments.

DEFENSINS

In mammals, defensins are one of the major families of antimicrobial peptides (*Lehrer* et al., 1998; *Ganz*, 1999). Characteristically, these peptides are 18 to 42 amino acids in length, have predominance of β-sheet conformation, are cationic in net charge and contain six cysteines that participate in three intramolecular disulphide bonds. Based on the spatial distribution of the cysteine residues, defensins are classified into three major groups termed α-, ß-, and

θ-defensins. The α-defensins were first identified in phagocytic leukocytes (*Ganz* et al., 1985) and later identified in mouse and human Paneth cells (*Jones* and *Bevins*, 1992; *Selsted* et al., 1992). In humans, two such Paneth cell α-defensins have been identified, HD-5 and -6 (*Jones* and *Bevins*, 1992; *Jones* and *Bevins*, 1993; *Mallow* et al., 1996; *Porter* et al., 1997; *Porter* et al., 1998). Analysis of α-defensin gene structure in several species reveals that all haematopoietic α-defensin genes have three exons, but the epithelial alpha-defensin genes have two. The ß-defensins were first isolated from trachea and neutrophils of cattle (*Diamond* et al., 1991; *Selsted* et al., 1993) and later in the mucosa of the airway, tongue, colon, kidney, skin, and gingiva in humans and in other species (*Diamond* and *Bevins*, 1998). In the digestive tract, ß-defensins have been found in the gingival epithelia, the tongue and the colon (*Schonwetter* et al., 1995; *Krisanaprakornkit* et al., 1998; *Tarver* et al., 1998; *Shi* et al., 1999). The single known θ-defensin, RTD-1, is a 2 KDa macrocyclic peptide that is found in phagocytic leukocytes of the Rhesus macaque (*Tang* et al., 1999). RTD-1 consists of an 18 amino acid, covalently closed circular polypeptide chain that is stabilised by three disulphide bonds. In humans, the genes that encode defensins are clustered in a few hundred kilobase segment of the short arm of chromosome 8 (8p23) (*Sparkes* et al., 1989; *Harder* et al., 1997; *Liu* et al., 1997), and the corresponding homologous genes are similarly clustered in the rodent genomes (*Ouellette* et al., 1989; *Huttner* et al., 1997; *Bals* et al., 1998; *Morrison* et al., 1998; *Jia* et al., 2000).

ANTIMICROBIAL PEPTIDES OF PANETH CELLS

In most mammals, including humans and rodents, Paneth cells occupy a position at the base of the crypts of Lieberkühn (*Trier* et al., 1967). Paneth cells have distinct morphology. They are intensely eosinophilic, with ultrastructural hallmarks of secretory cells, including an extensive endoplasmic reticulum and Golgi network (*Trier*, 1963). They secrete large secretory granules apically into the crypt lumen. Paneth cells are found from the duodenum to the ileum. Unlike other epithelial cells of the small intestine, which are short lived, Paneth cells have an average life span of approximately three weeks. Since Paneth cells develop prenatally during normal human ontogeny (*Mallow* et al., 1996) and are present in mice reared under germ-free conditions (*Ouellette* et al., 1989), their ontogeny does not depend on luminal bacteria or dietary constituents.

Identification of Paneth cell proteins has provided key insights into the biological role of these cells. Paneth cells of rodents and humans produce lysozyme (*Erlandsen* et al., 1974; *Peeters* and *Vantrappen*, 1975), secretory phospholipase A_2 ($sPLA_2$) (*Senegas-Balas* et al., 1984; *Harwig* et al., 1995), and α-defensins (*Jones* and *Bevins*, 1992; *Selsted* et al., 1992; *Porter* et al., 1997, 1998; *Cunliffe* et al., 2001), well-established antimicrobial proteins and peptides.

ANTIMICROBIAL ACTIVITIES OF PANETH CELL DEFENSINS

Mouse and human Paneth cell α-defensins are potent antimicrobial agents with selective activities against several varied microbial cell targets. For exam-

ple, HD-5 is active against a variety of bacterial species, including *L. monocytogenes*, *E. coli*, *S. typhimurium*, as well as the yeast-like fungus *C. albicans* (*Porter* et al., 1997). *In vitro* assays of cryptdins show that they are similarly microbicidal against *E. coli* ML35, *Staph. aureus*, and *S. typhimurium* (*Selsted* et al., 1992). In a structure-function analysis of mouse Paneth cell α-defensins, trophozoites of *Giardia lamblia* are were highly sensitive to two defensins, but far less sensitive to two others. Peptide amino acid residue position 15 was implicated in this activity, because the active defensins contain Arg at position 15 whereas the inactive defensins contain a Gly at this position (*Aley* et al., 1994).

PROPOSED FUNCTION(S) OF PANETH CELL DEFENSINS

The proposed physiological roles of small intestinal defensins may be grouped into 4 overlapping processes (Figure 3).

I) Enteric defensins may provide protection of the crypt and its stem cells from microbial invasion and parasitisation. Estimates from studies of isolated mouse crypts indicate that the concentration of defensins in the crypt at mM levels. In view of the well-documented antimicrobial activity of many defensins at concentrations in the μM range, it is reasonable to propose that an important function of Paneth cell defensins is crypt protection.

II) Enteric defensins may contribute to the factors that are selective for commensal bacteria. Because of selective sensitivity to their antimicrobial activity, the microbiological makeup of the intestinal flora may, in part, be governed by enteric defensins.

III) Enteric defensins may regulate the numbers of colonising microbes in the small intestine. Secretions from Paneth cells, which are present in highest numbers in the distal small intestine, contribute to an antimicrobial milieu that might prevent the abundance of colonic microbes from colonising the distal the small intestine in similar numbers. This function might help explain in part the perplexing observation that the bacterial load in the small intestine is estimated at 10^4 to 10^6 folds fewer microbes per gram of tissue than in the adjacent colon.

IV) Enteric defensins may contribute to defence from food and water borne pathogens in the intestinal lumen. Newly colonising microbes, including pathogens, will be confronted enteric defensins, and other Paneth cell antimicrobials. Given that parasympathetic (cholinergic) neural activity regulates numerous digestive functions throughout the alimentary tract, and that cholinergic stimulation also elicits Paneth cell secretion, we speculate that neurally mediated cholinergic stimulation that accompanies oral ingestion might induce Paneth cell secretion. This could equip the intestinal lumen with anticipatory effector molecules, including defensins, that could counter noxious microbes ingested my mouth.

These 4 possibilities are suggested to be overlapping and not mutually exclusive.

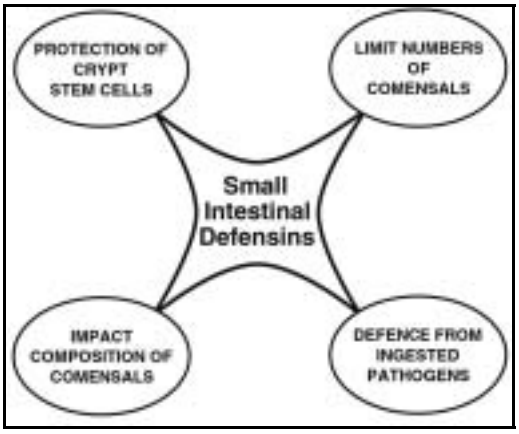

Figure 3: Four proposed functions of Paneth cell defensins.

CONCLUSIONS

Many lines of evidence support that Paneth cell defensins are key to innate immunity of the small bowel. These peptides may have additional physiologic roles. Paneth cells contribute actively to mucosal immunity by sensing bacteria and releasing microbicidal peptides at effective concentrations. The biosynthetic and processing pathways, the receptors for pattern recognition, and the signalling pathway(s) associated with apical secretion will require further study to understand this axis more completely. Given that microbial products stimulate Paneth cell secretion, it is possible that the mechanism of action of probiotic agents may, in part, involve modulating Paneth cell secretion.

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MOLECULAR BASIS FOR THE CROSS TALK BETWEEN PATHOGENS, INTESTINAL CELLS, AND THE GUT MICROFLORA

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SUMMARY

A rational and logical use of probiotic for therapeutic or nutritional tasks require a better understanding of the language used by bacteria and host cells to communicate. This cross-talk is mainly based on the exchange of molecules in both directions that control crucial metabolic steps. To start to learn this language we analysed, as an example, the glycosylation process in intestinal cells living in the presence of soluble factors produced by a bacterial species from the microflora, namely *Bacteroides thetaiotaomicron*. *In vitro* experiments carried out on the intestinal cell line HT-29 indicated that these bacteria communicate with their target cells through a remote control process that was able to modulate surface galactosylation. We demonstrated that this control was at a post-translational level. *In vivo* experiments confirmed that a similar process should be at work in mice. Germfree (GF) mice feed with the soluble factor, or mono-contaminated with the corresponding live bacteria or with a complete microflora were shown to change the surface glycosylation pattern of intestinal cells in a species-specific, segment-specific and cell-type specific manner. These data open a new window to analyse the molecular bases for the cross talk between eucaryotic and prokaryotic worlds.

INTRODUCTION

Bacteria that reside in the gut are well know to exert several effects that are good for health, either in maintaining a favourable balance or in helping to fight against diseases. Although these beneficial effects have been recognised for a long time, their mechanisms remain mainly unknown. Numerous studies have attempted to understand how gut microflora works. Based on the research of some groups, including our Unit, we will review here some of the lines of research that are currently followed to use microflora more rationally and in a more efficient way. We will first summarise the available data that accurately describe the cross talk between host cells and microflora. We will then more precisely focus on one topic where microflora is expected to play an important role, namely infectious diseases.

The cross talk between the intestinal barrier and gut microflora seems to involve various levels of regulation. During these “discussions”, either some

individual bacterial species and/or microflora as whole and/or specific cell types from the gut may change their "point de vue". Physical contact between host cells and bacteria is not obligatory and it is now recognised that a new family of molecules, the modulins, should play a key role by mediating a remote control on the cross talk. Recent data suggest that the remote control of host cells by bacteria or of bacteria by intestinal cells may concern the whole range of biologically active molecules. This means that transcriptional, post-transcriptional, translational and/or post-translational control of bacteria or cell functions may be involved.

We have been interested for several years in the regulation of intestinal cell functions. We have developed several cell models that have allowed us to start to understand some of the molecular mechanisms that stay behind the cross talk. Human intestinal cultured cells represent a vast repertoire of phenotypes in which it is possible to select a set of specific intestinal functions. We have also developed experimental systems in which we have been able to check *in vitro* the effects of whole bacteria or of specific components of the microflora. In vivo experiments, using the huge potential of gnotobiotic animals, have been also developed.

The ways by which pathogens invade intestinal cells are numerous and pathogens have learned a lot on mammalian cell biology to overcome the natural defence of the host. Microflora also interfere with pathogen-host cells cross talk. Although a number of distinct processes are here at work, it is clear that some common themes emerge in the complex cross talk between pathogens, microflora and the gut. We were especially interested by one of these themes, namely the role of host glycosylation processes in the modulation of pathogen entry. Using first an *in vitro* approach, we identified a new "modulin", produced by a major component of the microflora, namely *Bacteroides thetaiotaomicron* (BETIM) that was able to specifically modulate a galactosylation process in a model of goblet cells. We have also shown that the modulin was active through a post-translational event in which the activity of galactosyltransferase was up-regulated with no change at the trancriptional level. Further we demonstrated that this up-regulation was associated with an increased capacity to resist rotavirus infection. *In vivo* experiments have demonstrated that modulin, individual bacteria or the whole microflora were also able to modulate intestinal cell glycosylation with a species-specific, cell type-specific and tissue-specific pattern.

THE CROSS TALK BETWEEN THE INTESTINAL BARRIER AND GUT MICROFLORA INVOLVES VARIOUS LEVELS OF REGULATION

Since Metschnikoff discovered that bacteria might have beneficial effects, the mechanisms by which microflora and probiotics exert their effects are still a matter of intense research and generate a huge amount of debate. For example it is still unclear whether some effects require that bacteria remain live or not and which fraction contains the activity. Another still unresolved question is to know whether the bacteria must maintain a tight contact with the mucosa or not. These questions are however highly relevant to define a logical approach for a clinical and/or a nutritional use of microflora-derived components.

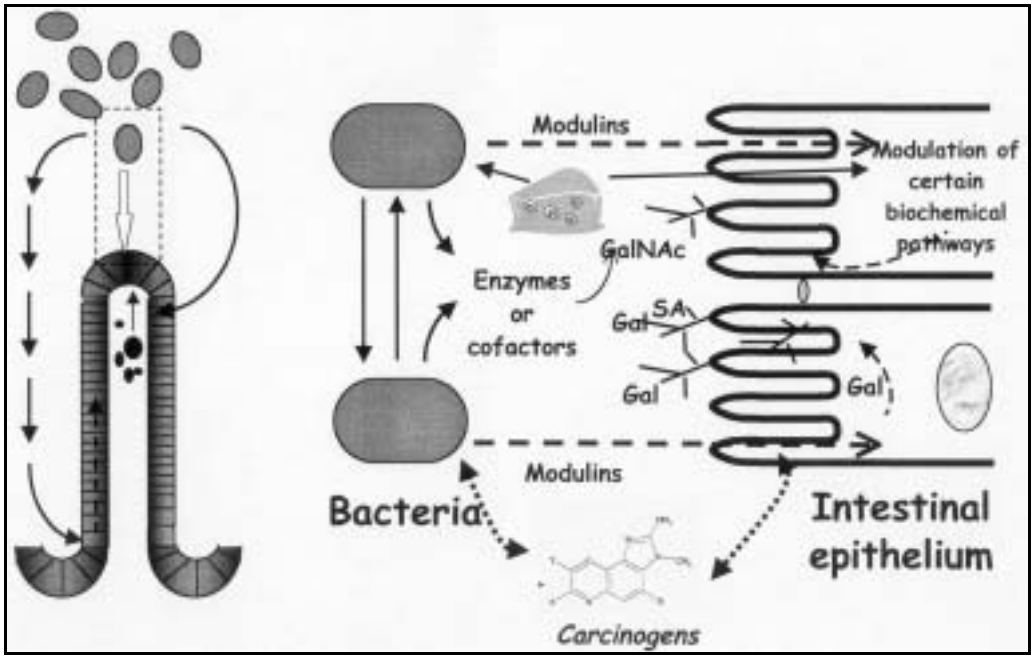

Figure 1: Host-microflora cross-talk, an overview.

Since few years, however, some works have started to exploit the revolution of genomics, proteomics and cell biology. These studies strongly suggest that besides effects mediated by the "mechanical" contact between bacteria and their host cells, the cross talk between the eukaryotic and prokaryotic worlds may be mediated through a remote control process that use soluble molecules produced by either of or the two partners (Figure 1). The concept of modulins emerges from these studies and should be central in the next few years to understand the molecular bases of the cross talk. Modulins should define those molecules produced by resident bacteria able to modulate the host cell function or to promote their cross-talk with bacteria. This functional definition doesn't tell us anything on the molecular nature of these modulins. Efforts have to be made in the next future to carefully define the chemical composition of these molecules and to know whether they belong to one or more families.

From these first studies it appears that several levels of control may be involved in the cross-talk control. As summarised in Figure 2, if we only consider the way by which bacteria may communicate with intestinal cells, three main mechanisms have to be discussed. Bacteria produce numerous compounds, including enzymes that may act directly on the surface of epithelial cells to modify their biochemical composition and/or biophysical properties. The knowledge of the full genome of an increasing number of bacteria will certainly help to define and classify these compounds. Among the bacterial enzymes, some are known for a long time and have been used to characterise the bacterial species. One should now consider how these enzymes might modulate the host cell surface. It is also clear now that bacteria produce other compounds that enter target cells or are incorporated in their membranes where they will exert at least two types of

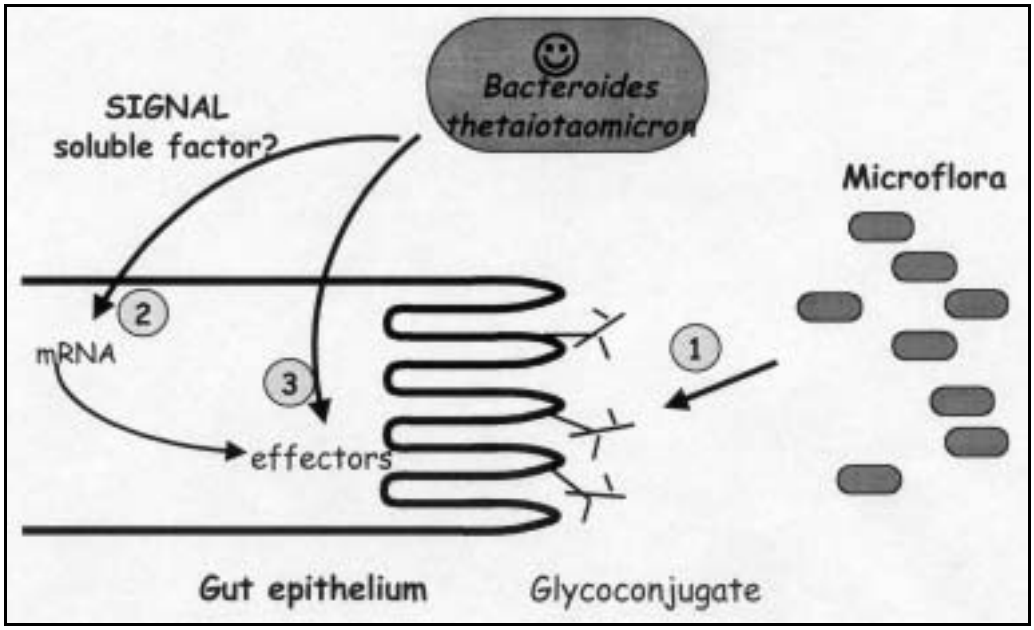

Figure 2: Putative regulation levels of the cross-talk.

effects. One is a transcriptional effect that will perturb protein biosynthesis of the host cells. In a recent paper the micro-array technology has been used to start to define the pathways and molecules whose biosynthesis may be transcriptionaly controlled. Interestingly this concern almost all the mammalian functions thus indicating that the final phenotype of mammalian cells strongly depends on the presence of the microflora. The other type of effect is a post-translational effect that will interfere with the maturation and the intracellular trafficking of another set of glycoproteins. This point is less documented and we have started to focus our research on a particular cell function, namely protein and lipid glycosylation to know more about this regulation level.

NEW EXPERIMENTAL MODELS EXPLOIT THE PROPERTIES OF CULTURED CELL LINES AND GNOTOBIOTIC ANIMALS

The cellular glycosylation process is the main post-translational event. It is of crucial importance because it concerns more than 80% of the proteins synthesised by a cell. Glycosylation will add to the complexity of the molecule, will change the capacities of the modified proteins to interact with other molecules, with cellular structures and with pathogens or microflora. For example, mucus is mostly composed of highly glycosylated proteins and mucus biophysical properties will depend on the nature of the glycans.

A survey of the literature recently published on the interactions between pathogens, microflora and intestinal cells surprisingly indicate that data derived from *in vitro* and *in vivo* experiments do not provide concordant results. These discrepancies concern both the nature of the sugars involved in the interactions and the effect of the bacteria on the sugar composition of the target

Table 1: Carbohydrates specificity of several pathogenic and non-pathogenic bacteria and viruses

Bacterial species	Carbohydrate	Reference
Clostridium difficile	Galα(1-3), Galβ(1-4)	*Krivan* et al., 1986
Entamoeba histolytica	Galactose	*Chadre* et al., 1988
Escherichia coli	Galactose, NeuNAc	*Mouricout*, 1987
Fusobacterium nucleatum FN-2	Galactose	*Shanitzki* et al., 1997
Helicobacter pylori	Fucose	*Guruge* et al., 1998
Lactobacillus acidophilus	Carbohydrates	*Greene* and *Klae*, 1994
Lactobacillus casei	galactose	*Yamamoto* et al., 1996
Lactobacillus fermentum	Carbohydrates	*Conway* and *Kjelleberg*, 1989
Lactobacillus plantarum	Mannose	*Adlerberth* et al., 1996
Lactobacillus reuteri	β-Galactose	*Mukai* et al., 1998
Listeria monocytogenes	NeuNAc	*Maganti* et al., 1998
Listeria monocytogenes	Galactose	*Cowart* et al., 1990
Propionibacterium	Gal(β1-4)Glc	*Pulverer* et al., 1994
Pseudomonas aeruginosa	Galactose, Fucose	*Gilboa-Garber* et al., 1994
Salmonella	Mannose	*Lee* et al., 1996
Streptococcus pyogenes	Galactose	*Kumar* et al., 1996
Vibrio cholerae	Fucose	*Gardel* and *Mekalanos*, 1996
Rotavirus	Sialic acid	*Dai* et al., 2000

cells. Therefore it is now needed to develop model systems both *in vitro* and *in vivo* that will allow to directly compare the effect of a given species or of a mix of endogenous bacteria or probiotics.

We have been involved since several years in the production and characterisation of *in vitro* models that reproduce part of the differentiated phenotypes of normal intestinal cells. Several cultured intestinal cell lines may be used to mimic a given phenotype such as goblet cells or enterocytes. For example the HT-29 cell line which display a mostly undifferentiated phenotype when grown in standard conditions may differentiate either in enterocytes or in goblet cells depending on the culture conditions. Other cell lines will develop a well differentiated enterocytic phenotype when grown in standard conditions, whereas some others will display a phenotype that resemble colonocytes in that they will be polarised but they will not express brush border enzyme activities. Interestingly, it has not been possible until now to induce a cell line to differentiate into a Paneth cell phenotype. It should be noted however that all cell lines used in these studies derived from human colon cancer cells, since until now experiments using normal intestinal cells over long period of time have been unsuccessful.

In the meantime, several laboratories have developed relevant *in vivo* models. Gnotobiotic animals, especially mice represent the most used model for studies on the interactions between bacteria and intestinal cells. Several studies have been published using these models but it should be mentioned that a full characterisation of the properties of these animals is still needed. For example the glycosylation pattern of the gut of germ-free mice was unknown since a very recent period. Several questions remain to know whether gnotobiotic animal may mimic all the functions of normal animals.

MICROFLORA, PROBIOTICS AND INFECTIOUS DISEASES: *IN VITRO* AND *IN VIVO* EXPERIMENTS POINTS TO A MAJOR ROLE FOR HOST CELL GLYCOSYLATION

Numerous pathogens as well as bacterial species from the microflora interfere with the host cells through specific glycans. It is also thought that bacteria may modify the glycosylation pattern of the host cells. We therefore elaborate a strategy to know whether it will be possible to manipulate the composition of intestinal cell glycosylation by using specific bacterial species in order to obtain a specific effect. To this end we used the mucus producing intestinal cell line, HT-29 MTX which have been previously characterised. We make this choice because these cells do express at reasonable levels a large repertoire of glycosyltransferases. We grow these cells in the presence of bacterial products. We selected *Bacteroides thetaiotaomicron* (BETIM) as a bacterial species for two reasons. The first one derived from the knowledge of the composition of the microflora that indicates that this bacterium is one of the most representative species in the colon. The second one is that this bacterium was previously used in *in vivo* experiments and we wanted to make comparison between the two systems. We also decided to study the effect of the products secreted by this bacteria rather than the whole bacteria because our task was to demonstrate a kind of remote control and also because we previously experienced that growing mammalian cells together with bacteria is possible but difficult and do not allow to check various conditions. We set up an experimental system in which HT-29 cells were grown for 2 weeks in the presence of a medium previously conditioned with BETIM. To explore the glycosylation pattern we used a panel of 10 lectins that recognise most of the peripheral sugars founds in mammalian glycoconjugates. These lectins were labelled with fluorescein in order to follow their distribution using immunofluorescence or to quantify their surface expression by using flow cytometry. We also used biotinylated lectins to identify the proteins that will express the different glycans by western blot. To summarise, we found that BETIM was able to specifically increase the level of expression of galactose with no modification of the other peripheral sugars. We further demonstrated that the soluble factor produced by BETIM was not a neuraminidase that may remove the peripheral sialic acid and expose more galactose. We have also shown that the soluble factor was unable to change the level of mRNA of the main galactosyltransferases, indicating that it was not acting at a transcriptional level. We finally demonstrated that the soluble factor was able to significantly increase the activity of galactosyltransferases. Together these results are the first to directly demonstrate that a soluble factor is able to modulate the activity of a glycosylation enzyme of the host cell through a remote control process. The exact nature of the soluble factor is currently under investigation. Preliminary results indicate that it is a small molecule (< 8kD), it is thermosensitive, and it is not a lipid.

Whether this soluble factor may also be active *in vivo* was investigated in a second series of experiments. We first established a baseline for the glycosylation pattern of GF mice, which was unknown. This was done by using the above-described strategy using fluorescent lectins. Each segment of the gut (duodenum, jejunum, caecum, ileum, colon) was analysed and within each segment we analysed the label of each

cell type (absorbing cells, mucus secreting cells, Paneth cells, crypt cells) both from a quantitative and qualitative point of view. We found that each lectin has a restricted distribution and that this distribution was dependent of the segment and the cell type considered. For example, Paneth cells were characterised by an intense label with WGA and DSA but the other lectins were not detected within this cell type. We also found that most of the lectins used labelled a well-defined compartment within each cell type. For example, WGA that mostly recognised α-GlcNac was essentially localised in the Golgi apparatus of enterocytes and labelled mature mucus vesicles, whereas GSI that mostly recognise Gal β1-3 labelled the Golgi apparatus and the membrane of immature mucus vesicles. Interestingly we found that this distribution of glycan expression was different in GF and conventional (CV) animals. However the differences were again restricted to some lectins. For example, WGA expression in enterocytes was reduced in CV mice compared to GF mice and the localisation of this lectin was also modified from a Golgi label in GF mice to a brush border label in CV mice. According to the lectin considered, we observed switch-on or switch-off of given sugar labels and also localisation changes.

Feeding animals with either the soluble factor of BETIM of live BETIM also promoted specific changes in lectin labelling. Interestingly in all the situations analysed the soluble factor and the live bacteria resulted in similar changes, except in two cases (expression of UEA fucose specific lectin in small intestine goblet cells and expression of RCA-I galactose specific lectin in large intestine goblet cells) where the soluble factor was unable to reproduce the effect of the live bacteria. This is interesting since in the work made by Bry in Gordon's lab, UEA was shown to be up-regulated in BETIM feeding animals (as in our hands) and since in our *in vitro* work we showed that RCA was up-regulated by the soluble factor, which is not the case *in vivo*. It is not clear however whether the active molecules present in the soluble factor may pass the stomach without being partially degraded. This also point to the fact that it should not be possible to extrapolate data obtained in the mice to human since it is well known that they do not display the same glycosylation capacities.

CONCLUSION AND PERSPECTIVES

Our results, together with those recently published by other groups indicate that we are just at the beginning of a new era where one may start to think to manipulate either the host or the microflora to interfere with their cross talk in a therapeutic or nutritional perspective. However, before being able to do so, several questions have to be answered.

As mentioned earlier in this review, the definition of the content of the new modulin family remains to be done. Are there related molecules, how many members, are there species specificities, are the compounds released constantly or in a regulated manner? Our *in vivo* experiments that demonstrated the induction of specific changes argue for the presence of a small number of factors involved in the control of host cell glycosylation, but this remain to be clearly established. The biochemical nature of the soluble factor also remained to be refined in order to further consider the possibility to use it as a

prebiotic. It is not know if there is a signalling pathway for the post-translational regulation we have identified similar to the one demonstrated for the fucose regulation at the transcriptional level as described by L. Hooper et al. Other studies will have to study the bioavailability of the soluble factor and particularly its resistance during the transit through the stomach. There is no data on the dose-response curves for these effects. There is no indication on the amount of bacteria needed to produce an active concentration of soluble factor, nor whether this is modulated by intra-intestinal process, such as the quorum sensing for example. Finally it is of crucial importance to develop challenge tests in order to clearly demonstrate that change in host cell glycosylation may be instrumental.

Much more work is now needed to answer these questions but it is now clear that we have a strategy to study the cross talk in a more logical way. Whether this will help us to define a new therapeutic or nutritional strategy remain to be clarified.

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IS THERE A TIME WINDOW OF THE IMMUNE SYSTEM AS A LEARNING SYSTEM?

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SUMMARY

In rodents there is evidence that during the first week of life orally administered antigens induce immune responses rather than tolerance. Thereafter tolerance also appears and this can be due to the seeding of late thymic migrant T cells proven to have profound regulatory potential and are crucial for the development of tolerance to certain self-structures. These T cells express CD25 and CTLA-4 and may produce suppressive cytokines like IL-10 and TGF-ß.

Maternal anti-idiotypic antibodies can induce responses in the foetus but also in the neonate via the milk. Idiotypic priming directed against one antigen on a microbe seems to enhance the response to other antigens on the same microbe as well.

In man there is no apparent evidence for any early window in the neonates' immune system indicating that an analogous development of regulatory T-cells must happen during foetal life. The human neonate is capable of responding quite well to vaccines directly, but due to the delay in appearance of a broad repertoire of protective antibodies and T cells the maternal transplacental IgG antibodies and the passive protection provided via the milk are important. The milk also contains a number of components and signals, which can activate various systems resulting in a long-term enhancement of the immune and neuro-endocrine systems.

INTRODUCTION

The neonate is in a very special situation when leaving its sterile, protected environment in utero to be delivered into a milieu heavily contaminated with microbes. The new-born is coming out next to the mother's anus which normally render them colonised with the mother's intestinal flora. This is often prevented today in industrialised countries due to misplaced hygienic measures the unnatural positioning of the woman. This has resulted in late occurrence of i.a Gram-negative bacteria in infants stool and a striking early appearance and frequent persistence for months of toxin-producing *Staphylococcus aureus* (*Lindberg* et al., 2000). In a natural birth situation Gram-negative bacteria appeared very soon after the delivery (*Adlerberth* et al., 1999).

The immune system of the new-born may be only a few percent or less of that in an adult (*Adkins*, 1999) The major stimulus to its rapid growth after birth is the exposure of the extensive mucosal membranes of the intestinal tract to the colonising microbes, finally resulting in that 2/3 of the whole immune system is localised to the intestinal tract. Against this background it is reasonable to assume that the changed intestinal flora of infants today in industrialised countries may have consequences for the development of the immune system in early life, with possible consequences in later life. Especially it seems that intestinal colonisation with Gram-negative bacteria may be necessary for the development of an adequate capacity to respond with immunological tolerance to environmental antigens.

It is clear that several components of the innate and adaptive immune systems are deficient at birth. This is illustrated by a reduced and/or different functional capacity for instance of neutrophils (*Levy* et al., 1999). Since experimental animals often used for studies of the early capabilities of host defence, like mice and rats, are less developed at birth than man the subsequent presentation will first discuss such animals and then man.

At this stage, however, it should be mentioned that deficient functions of specific T cells in mice, as well as man, may only partly be a matter of number of cells rather that their functional capacity (*Adkins*, 1999). If the relative deficiency of T cell-produced cytokines in the neonate is compensated for adequate responsiveness is claimed to be seen. With adequate stimuli, like the BCG vaccine, the often expected Th2 response of the human neonate can be transformed into a Th1 response (*Marchant* et al., 1999; *Vekemans* et al., 2001). With certain other vaccines or infectious agents *like Plasmodium falciparum* variably reduced Th1 and also Th2 responses are seen in infants (*Clerici* et al., 1993; *Prescott* et al., 1998). In addition to deficient Th1 function and T cell cytotoxicity in both human and murine T cells, the latter are more focused towards Th2 functions (*Adkins*, 1999).

IMMUNE RESPONSIVENESS IN THE NEONATAL RODENT

In the 1980-ies (*Hanson*, 1981) and (*Strobel* and *Ferguson*, 1984) published the observation that feeding ovalbumin (OVA) within the first week of life resulted in an antibody as well as T-cell response, whereas later feeding induced immunological tolerance reviewed by *Strobel* (1996). Recently it was found that naïve CD8 cells from the thymus were tolerised to a skin-expressed MHC class I antigen reaching the skin only in neonatal and not adult animals. Thus tolerance could be expressed early (*Alferink* et al., 1998). In humans very similar numbers of CD25+ regulatory T cells occurred in the thymus and cord blood as in mice and rats from 5 days of age, presumably cells which can prevent auto-immune diseases (*Wing* et al., 2002). Neonatal colonisation of rats with an OVA-*producing Escherichia coli* strain resulted in immunological tolerance to OVA, the 06 lipopolysaccharide (LPS) of the *E. coli* and its type 1 pili. This was noted when tested at 12 and 13 weeks of age, after immunisations with these antigens at 6 and 12 weeks after the neonatal colonisation (*Karlsson* et al., 1999). In adult rats the colonisation instead increased the immune responses, both the antibody levels and delayed type hypersensitivity to OVA. However, the relative increase of the response to the 06 LPS in the adult

animals was much lower after adult colonisation. The neonatal colonisation also resulted in bystander tolerance against an unrelated antigen, human serum albumin, indicating that at least part of the tolerance was a result of suppression mediated regulatory T-cells. This was confirmed in further studies (*Lundin* et al., 1999). However, it was also noted that oral tolerisation of rats mainly led to active suppression and bystander tolerance in adult rats, whereas anergy was predominant in young rats (*Lundin* et al., 1996).

Neonatal animals responded with antibody production to ng-levels of anti-idiotypic antibodies, whereas higher doses were less effective. This was shown in mice using monoclonal idiotypes and anti-idiotypes against the *E. coli* K13 polysaccharide capsule (*Stein* and *Soderstrom*, 1984). This is especially notable since polysaccharide antigens do not normally induce antibody responses in young animals or children, although polysaccharide-protein conjugate vaccines are well known to do so. Using idiotypic mimicry the K13 polysaccharide was thus transformed into a protein antigen which made it an effective immunogen in the neonate. A maternal transfer of idiotype and anti-idiotype seems to give a better priming for an antibody response than the anti-idiotype alone as shown using poliovirus antibodies in germfree piglets (*Lundin*, 1998).

A remarkable effect on the new-born rat's immune system was noted using monoclonal anti-K13 anti-idiotypic antibodies testing two consecutive generations (*Lundin* et al., 1999). Two days old neonatal female rats were fed mg doses per orally of the anti-idiotype. Six weeks later they were colonised with the OVA-producing *E. coli* 06:K13. No significant effects were noted in these rats on the immune response to the bacterium. However in their offspring a clearly enhanced antibody response was obtained after colonisation with the same strain at 6 weeks of age. The antibody responses to the OVA, as well as the 06 and K13 antigens were increased, more in the group given 10 mg than the one given 1 mg of the anti-idiotype. Concurrently, the proliferative response of spleen cells to OVA and the bacteria was lowered.

Giving new-born mice anti-idiotypes against the *E. coli* K13 and a viral antigen via the mother's milk also resulted in an enhanced serum antibody response in the offspring (*Stein* and *Soderstrom*, 1984; *Okamoto* et al., 1989).

In mice and rats there is a limited placental transfer of antibodies from the mother to offspring, but the milk brings a number of factors from the mother, including antibodies. In a recent study we have investigated the possible effect of the fatty acids in the mother's diet on the offspring's immune response. It was noted that a diet deficient in n-6 and n-3 essential fatty acids (EFA) compared to a control diet enriched in EFA resulted in an increased ratio of saturated/unsaturated fatty acids in the milk of the rat dams (*Korotkova* et al., 2001). The effect of feeding the dams OVA during early lactation on the subsequent immune response of the rat pups was measured. It was found that the pups of the dams on the diet deficient in EFA and exposed to OVA responded significantly less to immunisation with OVA compared to the pups of the dams on the control diet enriched in EFA (*Korotkova* et al., submitted for publication). They produced less delayed type hypersensitivity reactions and less IgG, IgM and IgE antibodies to the OVA. Thus it seems that tolerance to a food protein may be transferred via the milk, but less so by mothers on a diet with a higher n-6 and n-3 fatty acid intake.

It was also noted that the diet deficient in EFA to the dams reduced the

leptin levels in the serum of their pups (*Korotkova* et al., 2001). The hormone leptin has a structure similar to the IL-1 cytokine and is known to stimulate Th1 reactivity (*Lord* et al., 1998). It is not clear whether this can influence the immune responsiveness of the offspring, although leptin was recently found to stimulate CD4 as well as CD8 lymphocytes via their JAK/STAT pathway (*Sanchez-Margalet* and *Martin-Romero*, 2001).

THE IMMUNE RESPONSE IN THE HUMAN NEONATE

Secretory IgA and IgM antibodies to *E. coli* O antigens and to poliovirus antigen were found in amniotic fluid, meconium, urine and saliva from human neonates (*Mellander* et al., 1986). These antibodies could not have come from the mothers who only transfer IgG antibodies, but presumably appeared as a result of stimulation with anti-idiotypic antibodies from the mother. This was further substantiated in a new-born of a hypogammaglogulinaemia mother lacking IgA and IgM and only given IgG prophylactically (*Mellander* et al., 1986).

The neonate is deficient in a number of ways as to its immune system both as to size and quality (*Schelonka* and *Infante*, 1998). There is a smaller bone marrow reserve, a reduced serum complement function, more immature T lymphocytes and a deficient capacity to respond to bacterial capsular polysaccharide virulence antigens.

The new-born is usually colonised with an intestinal flora where anaerobes slowly take over to become totally predominant (*Adlerberth* et al., 1999). That flora together with the small fraction of facultative anaerobes like *Escherichia coli* provide a colonisation resistance limiting colonisation with other potentially pathogenic microbes (*van der Waaij*, 1999).

The innate immune system is deficient initially in the neonate with e.g. granulocytes having a small storage pool and being only slowly produced after exposure to bacteria or cytokines like G-CSF. The neutrophils show diminished migration, phagocytic capacity and killing. This may relate to a reduced actin polymerisation in response to chemotaxis, a defective defensin production (*Merry* et al., 1998; Salzman et al., 1998) and a lack of bactericidal/permeability increasing protein (*Levy* et al., 1999). Other important characteristics of cells participating in innate immunity, like dendritic cells, monocytes/macrophages, natural killer cells and mast cells, such as presence and functional capacity of Toll-like receptors (TLR) has apparently not been studied in the human neonate. These cells are very important in the initiation of adaptive immune responses especially by enhancing antigen presentation. It needs to be studied when the new-born has cells equipped to recognise and react to all potential pathogens by Pattern Recognising Receptors like TLR's, thus producing the cytokines, which promote microbial uptake by dendritic cells making them mature to efficient antigen presenting cells. The new-born's dendritic cells are less efficient because initially their capacity to produce IL-12 is impaired (*Goriely* et al., 2001; *Liu* et al., 2001).

Initially the great majority of CD4+ cells are CD45RA+ which gradually are replaced by CD45RO+, whereby memory function appears. Development of cytotoxic T cells has been reported to be less efficient than later. B cells are initially producing fewer isotypes, with little following IgM. The T cells pro-

duce IL-2 adequately, but only 50% of GM-CSF, TNF and IL-10, and 10% of IFN-γ and IL-4 compared with adult cells according to some reports (*Schelonka* and *Infante*, 1998). The CD4/CD8 ratio remains high up till 2 years of age. Recent work debates earlier data and suggests that human cord blood lymphocytes can efficiently produce Th1 and Th2 responses after polyclonal activation, although this may nor reflect the natural situation (*Chipeta* et al., 2000).

These still somewhat contradictory studies may be best understood in the light of some quite efficient vaccine responses in the neonate. This is noted for neonatal BCG vaccination even in a problematic African surrounding (*Marchant* et al., 1999) and the same has been found also with a first dose of oral poliovirus vaccine given on the day of birth as recommended by WHO. The latter may, however, become inefficient if the infant is simultaneously breastfed since the milk contains high levels of poliovirus neutralising secretory IgA antibodies (*Zaman* et al., 1993).

The human neonate is able to manage several functions of host defence, but because of the initially less functional innate immune mechanisms and the yet inexperienced and therefore not fully expanded adaptive immune system it is clearly at increased risk of infections.

WITH SOME HELP FROM THE MOTHER

The two well-known modes of support from the mother, as IgG antibodies via the placenta and various defence factors via her milk, are of major importance for the protection of the infant. However, we now recognise that at least the latter of these two modes of transfer of passive immunity also is a system of quite complex active signalling to the infant.

The transfer of IgG via the placenta is assumed to occur via Fcg receptors. This may not be the only mechanism involved because it seems that both specificity and affinity of the antibodies affect their transfer although these are characteristics of the Fab portion of the antibody molecule (*Avanzini* et al., 1998).

The mother's milk is certainly important by providing proper nutrient and efficient passive protection against infections. Actually enhanced breastfeeding by 40% in the poor parts of the world would more than half the mortality in diarrhoea and pneumonia according to WHO. These two diseases are the two major causes of death in young children. Beyond that there is now an increasing interest in the fact that the milk provides a number of components and signals, which actively influence the long-term outcome of the child. One could be anti-idiotypic antibodies which as has been mentioned above have induced antibody responses in breastfed offspring (*Stein* and *Soderstrom*, 1984; *Okamoto* et al., 1989).

Another mechanism seems quite surprising. Several studies have shown that leukocytes, including lymphocytes from human milk are taken up and are capable of transferring immune responsiveness for instance to vaccines (for review see *Hanson* et al., 2001). In fact it seems that the breastfed infant develops tolerance to the mothers HLA, so that the cells can be accepted and taken up. Evidence for that comes from the observation that renal transplantation with a mother donating a kidney to her child (even as an adult), causes significantly less rejection if she has breastfed that child (*Campbell* et al., 1984).

Breast fed individuals also has fewer cytotoxic cells reacting against maternal than paternal cells (*Zhang* et al., 1991).

The major protein in mature milk is lactoferrin. It efficiently kills bacteria, viruses and fungi and prevents urinary tract infection in an animal model (*Haversen* et al., 2000). It also blocks production of inflammatory cytokines like IL-1, TNF, IL-6 (*Mattsby-Baltzer* et al., 1996). This may help the new-born to dampen the effects of the sudden microbial exposure on the intestinal mucosa after birth diminishing production of these inflammatory and catabolic cytokines.

The milk contains numerous cytokines, soluble cytokine receptors, hormones etc. It is likely that they can play many different roles in the infant. Thus breastfed infants respond significantly better than non-breastfed against the *Haemophilus influenzae* type b (Hib) polysaccharide-protein conjugate vaccine with IgG2 antibodies to the Hib polysaccharide. They also respond better to Hib infections (*Silfverdal* et al., 2002). It might be that the IFN-γ required for IgG2 antibody production comes from the milk, which often contains significant amounts.

A better functional immune system of the breastfed child may explain why breastfeeding seems to protect against celiac disease (*Ivarsson* et al., 2002). There are suggestions that protection may also exist against certain auto-immune diseases, as well as Crohn's disease and ulcerous colitis, but this is based on few studies and must await confirmation (*Hanson* et al., 2001).

There is a continuous debate whether of not breastfeeding provides long term protection against asthma and allergy. A recent meta study supports a protective capacity, which is even more pronounced if there is a family history of atopy (*Gdalevich* et al., 2001) and so do a few other recent studies (*Oddy* et al., 1999; *Kull* et al., 2001). There is also some evidence that in a small group of infants the presence of maternal asthma may increase the risk of asthma in the child after long term breastfeeding (*Wright* et al., 2001). However, this was not seen in another study (*Oddy* et al., 2002).

Breastfeeding seems to have a number of further long term effects on the child like promotion of brain development, decreased risk of obesity in school age (*von Kries* et al., 2000) and lower blood pressure in adolescence (*Singhal* et al., 2001). Such effects might result from some of the many signals provided via the maternal milk and illustrate the complexity of mechanisms involved.

In full-term babies there does not seem to be any convincing evidence for any early window in the immune responsiveness. Giving cow's milk proteins during the first week to a breastfed infant did not decrease or increase the risk of cow's milk allergy (*Juvonen* et al., 1996). That may be taken as evidence against an early deficiency in the capacity to develop tolerance.

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MULTIPLE CONSEQUENCES OF THE CHANGING EDUCATIONAL INPUT TO OUR IMMUNE SYSTEMS

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SUMMARY

The idea that diminishing exposure to microorganisms in the environment might play a role in the rising incidence of allergies was put forward nearly 30 years ago. Soon after the same idea was suggested for autoimmune disease. We would now add inflammatory bowel disease (IBD; Crohn's disease and ulcerative colitis) to this list, and place all three groups of disorder (allergies, autoimmunity, IBD) under a common umbrella title of "diseases of immunodysregulation", all attributable to a failure of maturation of immunoregulatory cell populations and networks. Meanwhile it has become clear that some behavioural and mood disorders are also increasing at a worrying rate in the rich, developed countries, and some of these are epidemiologically associated with the immunoregulatory disorders, and accompanied by cytokine changes that permit a tentative unifying hypothesis involving regulatory roles of interleukin 10 (IL-10) in the immune system and central nervous system.

Recent laboratory and clinical studies suggest that microorganisms can influence the "diseases of immunodysregulation" without causing overt infections. Changes in commensal flora (such as gut flora) or exposure to immunogenic but harmless saprophytes or vaccines, or alteration of our microbial exposure by antibiotics and hygiene can all exert significant effects. This information provides us with some extraordinary challenges, because although hygiene, vaccines and antibiotics are the three most useful and cost-effective achievements of medicine, the next decade will see us trying to discover how to improve vaccines so that they more closely mimic the "educational" input that the immune system requires, and how to supplement the effects of conventional vaccines with probiotics, and how to devise novel vaccines that are used, not to combat a specific infection, but rather to exploit their non-specific immunoregulatory properties.

INTRODUCTION: THE HYGIENE HYPOTHESIS AND ALLERGIC DISORDERS

In 1976 Gerrard and his colleagues, noted that North American Indians had higher levels of IgE than their white neighbours, but a much lower incidence

of allergic symptoms (*Gerrard* et al., 1976). They suggested explicitly that "atopic disease is the price paid by some members of the white community for their relative freedom from diseases due to viruses, bacteria and helminths" (*Gerrard* et al., 1976). This constitutes one of the earliest statements of the hygiene hypothesis.

Numerous subsequent epidemiological studies have confirmed that there is a steadily rising incidence of allergic symptoms in the rich developed, hygienic countries (*Strachan* et al., 1997) and several aspects of the epidemiology are compatible with the view that the relevant environmental change is the decreasing exposure to microorganisms. These include the protective effects of large family size, and of being low down in the birth order, especially if the individual has "dirty older brothers"(*Matricardi* et al., 1998; *Strachan* et al., 1997). A trend towards increases in allergic manifestations is also now being seen in urban Africans, when compared to the corresponding rural population (*Yemaneberhan* et al., 1997).

Further support came from a number of studies showing a negative correlation between allergic manifestations and evidence of response to organisms transmitted by the oro-faecal route (*Matricardi* et al., 2000), the incidence of tuberculosis (*von Mutius* et al., 2000), BCG vaccination (*Aaby* et al., 2000), and tuberculin skin test positivity (*Shirakawa* et al., 1996). While other studies failed to confirm the latter observations in other environments (*Alm* et al., 1997; *Strannegard* et al., 1998), the interpretation of tuberculin test data, and the immunological effects of exposure to mycobacteria are complex matters and the negative results are not convincing (*Rook* and *Stanford*, 1999). At that time there was widespread belief that there is a reciprocal "see-saw" downregulation of Th1 responses by Th2, and of Th2 responses by Th1. Therefore several authors expanded the view that the rising incidence of allergies might be due to a failure of our hygienic modern environments to drive sufficient Th1 activity (*Holt*, 1995; *Hopkin*, 1997; *Rook* and *Stanford*, 1998; *von Mutius*, 1998). With hindsight this was clearly never a viable hypothesis. First, it entirely ignored the possibility that Th2-inducing infections such as helminths might also protect (*Gerrard* et al., 1976). Secondly the Th1/Th2 seesaw hypothesis ignored the fact that the incidence of Th1-mediated autoimmune diseases is rising in parallel with allergic diseases, with remarkably tight correlation between the two (*Stene* and *Nafstad*, 2001).

The major themes of this article are therefore the nature of the diseases that might be increasing because of altered inputs to our immune systems, the nature of the altered input, and the prospects for using this knowledge for novel immunotherapies.

THE HYGIENE HYPOTHESIS AND AUTOIMMUNE DISEASE

With hindsight it should have been obvious that the "hygiene hypothesis" could not be explained only by changes in the Th1/Th2 cytokine balance. It was already known that microbial exposure can influence autoimmunity in animal models, whether that autoimmunity is mediated by Th2 or Th1 cells. Secondly, human Th1-mediated autoimmunity, such as type 1 diabetes (*Stene* and *Nafstad*, 2001) and multiple sclerosis (*Celius* and *Vandvik*, 2001; *Pugliatti* et al., 2001; *Sumelahti* et al., 2001) have been increasing at a rate similar to the

increase in allergies (*Stene* and *Nafstad*, 2001). Taken together these facts suggest that the hygiene hypothesis applies equally to these Th1-mediated disorders, and cannot therefore be a simple Th1/Th2 imbalance (*Rook*, 2000).

For instance, 25 years ago *Kohashi* and colleagues (1985) studied the induction of adjuvant arthritis in germ-free mice, and compared the incidence and severity of disease with that seen after re-colonisation of the gut with single species. The gut flora could either enhance or inhibit the disease depending on the bacterial species used. Similarly bacterial exposure profoundly affects rodent models of arthritis in HLA B27 rats (*Taurog* et al., 1994), pristane-induced Lupus (*Hamilton* et al., 1998), and autoimmune thyroiditis (*Penhale* and *Young*, 1988). Moreover a single dose of Freund's complete adjuvant protected non-obese diabetic (NOD) mice from a Th1-mediated autoimmune diabetes (*Sadelain* et al., 1990), and striking differences in the manifestations of diabetes in NOD mice in different animal houses have been attributed to differing microbial exposure (*Todd*, 1991).

The most striking evidence comes from the astonishingly close correlations between the incidences of allergic symptoms and type 1 diabetes whether the analysis is confined to Europe, or extended to countries outside Europe (*Stene* and *Nafstad*, 2001). Therefore Th2-mediated and Th1-mediated pathologies are increasing in parallel (*Stene* and *Nafstad*, 2001).

Does this mean that Th1/Th2 balance has no role at all? It is interesting that one study has suggested that despite the parallel increase in allergies and type 1 diabetes *within communities*, these diseases tend *not* to occur in the same individuals. Children with type 1 diabetes, and their siblings, may be partially protected from allergic disorders (*Douek* et al., 1999). This implies that there might be an underlying environmental trend that is increasing the risk of both types of disorder, but that which disorder develops is determined by the individual's genetic background and immunological experience.

THE HYGIENE HYPOTHESIS AND INFLAMMATORY BOWEL DISEASE

The picture becomes clearer when a third group of diseases is considered. There have been definite increases in inflammatory bowel disease (IBD) over the last 10 years. In Iceland, the incidence of ulcerative colitis has doubled, and Crohn's disease has increased 3-fold (*Bjornsson* et al., 1998). IBD is commoner in the North of Europe than the South, though the gap may be closing (*Shivananda* et al., 1996), and there have been similar increases in Italy over a similar period (*Trallori* et al., 1996). Any doubts about these conclusions, based on changing diagnostic criteria, for example, have been dispelled by rigorous recent studies in Scandinavia and Scotland (*Lindberg* et al., 2000; *Sawczenko* et al., 2001).

Once again there are animal models of IBD that suggest the possibility that microbial exposure can influence the incidence and severity of the disease. Interleukin-2 knockout (IL-2(-/-)) mice get colitis and autoimmunity unless germ-free. In germfree IL-2(-/-) mice the disease can be induced by antigen in Freunds complete adjuvant (*Ehrhardt* et al., 1997), and is associated with IL-12-driven Th1 without a Th2 component. The same is true of IL-10 knockout mice (*Kuhn* et al., 1993), and it is the

more recent work in which the role of IL-10 and regulatory T-cells has been defined in murine models of IBD that has consolidated the view that IBD belongs within the group of diseases that falls under the "hygiene hypothesis" umbrella, and cast light on the mechanisms involved. This is discussed in the next section.

THE HYGIENE HYPOTHESIS AND REGULATORY T-CELLS; DISEASES OF "IMMUNODYSREGULATION"

Thus we would now place all three groups of disorder (allergy, autoimmunity, and inflammatory bowel disease) under a common umbrella title of "diseases of immunodysregulation", all attributable to a failure of maturation of immunoregulatory cell populations and networks.

Regulatory cells and inflammatory bowel disease

Current evidence suggests that inflammatory bowel diseases (Crohn's disease and ulcerative colitis; IBD) are disorders in which the physiological mechanisms that inhibit immune responses to gut content (food or microbes) are failing. For instance IBD occurs in mice that lack IL-10 (gene knockout) and also in mice with severe combined immunodeficiency (SCID) that receive effector T-cells ($CD4+CD45RB^{high}$) without the appropriate regulatory cells (reviewed in (*Asseman* and *Powrie*, 1998). As in the models of autoimmunity discussed below the regulatory cells are characteristically CD25+ and their function involves CTLA4 (*Read* et al., 2000). There is good evidence that the regulatory cells that can stop the inflammatory process are associated with abundant IL-10, and they have been denoted Tr1 cells (T-regulatory 1) (*Groux* et al., 1997).

Regulatory cells and autoimmunity

Regulatory cells that can suppress inflammation mediated by autoimmunity, have also been characterised. It is not clear whether these constitute several different cell types or whether they are variants of a single regulatory cell lineage. A wide variety of organ-specific autoimmune disorders can be shown to be controlled by CD25+ T-cells that also express mRNA for IL-4, TGF-ß and IL-10 (*Seddon* and *Mason*, 2000). They are likely to be related to the IL-10-secreting Tr1 cells that can downregulate the Th1-mediated inflammation in models of inflammatory bowel disease (*Groux* et al., 1997), These cells are found in man, and for instance they can inhibit the nickel-specific Th1 responses of nickel-reactive individuals (*Cavani* et al., 2000). They may also be related to Th3 cells that secrete TGF-ß and IL-10 (*Fukaura* et al., 1996). These are readily derived from human peripheral blood (*Kitani* et al., 2000). They can inhibit both Th1-mediated (*Cavani* et al., 2000; *Fukaura* et al., 1996) and Th2-mediated autoimmunity (*Bridoux* et al., 1997). Similarly T-cells engineered to secrete TGF-ß will also downregulate both Th2-mediated and Th1-mediated responses (*Thorbecke* et al., 2000).

Regulatory T-cells and allergy

A variety of different types of cell have been implicated in the regulation of allergic responses. These include CD8-expressing cells (*MacAry* et al., 1997), and γ/δ T-cells. The latter have been implicated in the control of airway hyperresponsiveness (*Lahn* et al., 2001), and

in the development of tolerance and IL-10 production in response to low dose oral antigen (*Fujihashi* et al., 1999). It has been suggested very tentatively that γ/δ T-cells downregulate airway responsiveness to allergen challenge by controlling the 'repair' response of the airway epithelium to damage mediated by α/β T-cells (*Holt* and *Sly*, 1999). A different subset of T-cells, that may be identical to the Tr1 cells implicated in suppression of IBD, and perhaps also to Th3 cells, can inhibit the Th2-mediated response to allergen *in vivo* (*Cottrez* et al., 2000). These cells also release IL-10 which is already known to specifically decreases IgE production by IL-4-stimulated peripheral blood mononuclear cells *in vitro* (*Punnonen* et al., 1993). It can also downregulate IL-5 production by T-cells (*Zuany-Amorim* et al., 1996).

These findings do appear to relate to man, because the diameter of a house-dust mite (HDM)-induced wheal was inversely related to the quantity of mRNA for IL-10 induced by HDM *in vitro* (*Macaubas* et al., 1999). Similarly there is evidence for deficient release of IL-10 into the airways in human asthmatics (*Borish* et al., 1996).

Parasites, regulatory T-cells and allergy

Further support has come from the recent claim that decreased atopy in children infected with *Schistosoma haematobium* correlates with increased release of IL-10 from peripheral blood mononuclear cells in response to worm antigens *in vitro* (*van den Biggelaar* et al., 2000). This interpretation helps to explain the work of Gerrard and of Lynch outlined earlier (*Gerrard* et al., 1976; *Lynch* et al., 1998), and is acceptable if we assume a biphasic relationship between parasite load and the effect on atopy. This proviso is necessary because in studies of European children, those who were *Ascaris*-IgE seropositive had 10-fold higher levels of total IgE, higher prevalence rates of allergen-specific IgE seropositivity and higher prevalence of allergic rhinitis and asthma (*Dold* et al., 1998). Similarly in a separate study the means of total serum IgE and blood eosinophils were significantly higher in *Toxocara*-seropositive than in the seronegative group, and allergic asthma was associated with *Toxocara* seroprevalence (*Buijs* et al., 1997). Thus low level infection with Th2-inducing parasites clearly exerts a non-specific adjuvant effect on the Th2 response to allergens. It might however be true that chronic exposure to very high parasite loads induces sufficient anti-inflammatory IL-10 to non-specifically downregulate inflammation due to other causes. Thus the observations in children with Schistosomiasis, although greeted with much enthusiasm, pose a number of puzzles that are highlighted by the studies on *Toxocara* (*Buijs* et al., 1997) and *Ascaris* (*Dold* et al., 1998). It is extremely difficult to explain the increase in the incidence of allergic disorders in the developed countries in recent decades on the basis of changing exposure to parasites. Moreover in Estonia, where the prevalence of allergy is low, parasites are very infrequent, so the relevant changes in microbial exposure must involve other types of organism (*Julge* et al., 1997).

Symptomless atopy, or atopy leading to allergic disorders?

A further difficulty is that the Schistosomiasis study looked at total and specific IgE and at the prevalence of positive skin prick reactions to HDM. It did not study allergic *symptoms* (*van den Biggelaar* et al., 2000). In a separate study in Ethiopia the rural population had significantly *higher* levels of skin prick test sensitivity to HDM than the urban population, but a lower

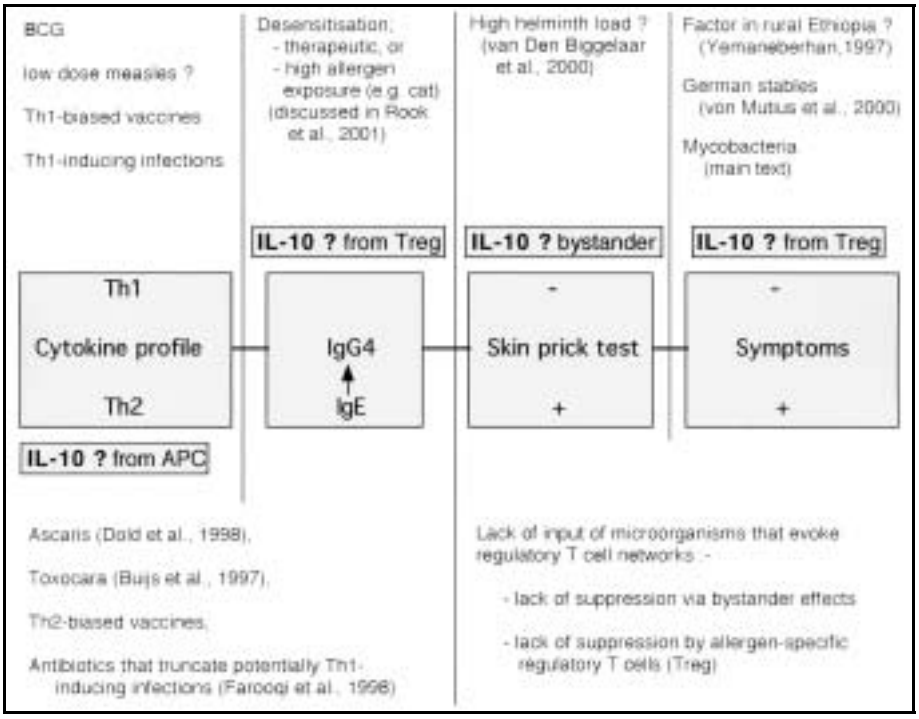

Figure 1: Regulatory cells may act at multiple levels to inhibit the inflammatory process. This figure focuses on the effects of IL-10. If antigen-presenting cells release IL-10 during the initiation of the response there may be bias towards Th2. In contrast, at later stages in the pathway, IL-10 from regulatory T-cells can switch from IgE to IgG4, downregulate skin prick test positivity, or attenuate allergic symptoms in the airways. Different subsets of regulatory cells might be involved at each stage. Moreover the regulation can be a non-specific bystander effect of anti-inflammatory cytokines, or due to *allergen-specific* regulatory cells. Similar figures could be devised for autoimmunity and inflammatory bowel disease.

prevalence of wheeze and asthma (*Yemaneberhan* et al., 1997). Figure 1 therefore points out that downregulation of allergic manifestations can occur at several different levels, and via non-specific and allergen-specific pathways.

THE HYGIENE HYPOTHESIS AND PSYCHIATRIC DISORDERS

At this point we wish to introduce an hypothesis of such potential importance that although still tentative, it deserves to be thought about. It has become clear that some behavioural and mood disorders are also increasing at a worrying rate in the rich, developed countries, and some of these are epidemiologically associated with the immunoregulatory disorders already discussed. These psychiatric problems are also accompanied by cytokine changes that parallel those seen in allergic disorders, and that can account for behavioural changes. There are two major types of evidence to support this hypothesis.

1) Associations with inflammatory disorders that are themselves increasing

The incidence of autism is increasing at the same rate as allergies etc. (*Kaye* et

al., 2001). Moreover about 50% of autistic children have gastrointestinal symptoms, and there is evidence for a mild mucosal inflammation resembling mild IBD (*Furlano* et al., 2001). Chronic fatigue syndrome is also associated with allergies (*Borish* et al., 1998). Attention deficit hyperactivity disorder (ADHD) is somewhat controversially associated with allergic manifestations (*Egger*, 1997; *Roth* et al., 1991), and many cases probably represent an infantile form of depression. There also appears to be a more general association between allergies and depression. College students with a history of clinical depression were more likely to be allergic, and allergic subjects were more likely to have mood worsening after influenza (*Bell* et al., 1991). This association seems not to be merely attributable to the stress of the allergic symptoms, because first and second degree relatives of allergic individuals have higher levels of psychiatric disorder and vice versa. Relatives of asthmatic adolescents have a higher incidence of affective disorder, antisocial personality disorder and substance abuse (*Wamboldt* et al., 1996). This association was then confirmed in a much larger twin study of depression and allergy (*Wamboldt* et al., 2000).

2) Cytokines and mood disorders

It has been noted that major depression is accompanied by indications of systemic inflammation; leukocytosis, increased acute phase proteins, increased production of IL-1ß, IL-6, IFNγ, IL-2 (reviewed in *Maes* et al., 1999), and there is some evidence that antidepressant drugs (including SSRI's and TCA's, HCA's and lithium), can reverse these cytokine changes. The recent vogue for treating hepatitis and certain cancers with IL-2 and Interferon alpha (IFN-α) has highlighted the role of cytokines in mood. In many subjects IL-2 and IFN-α rapidly induce severe clinical depression (*Capuron* et al., 2000, 2001; *Dantzer* et al., 1999; *Musselman* et al., 2001). In others there is mania after withdrawal. Interestingly IFN-α production is high in individuals with allergy or chronic fatigue syndrome while IL-10 production is low (*Borish* et al., 1998). This may be important because IL-10 is not only able to suppress the inflammatory disorders discussed above, but also able to suppress the sickness behaviour caused by signals passing to the brain from inflammatory sites. Moreover IL-10 can do this even when injected directly into the brain (*Bluthé* et al., 1999; *Nava* et al., 1997). Thus potentially these disorders may be influenced by the same defective control of inflammation, and changing cytokine balance with IL-10 deficiency, as the overtly inflammatory disorders discussed earlier.

THE UNIFYING HYPOTHESIS AND ITS CONSEQUENCES

Thus various regulatory cell types secreting IL-10, if deficient in inhabitants of the clean developed countries, can provide an unifying hypothesis to explain simultaneously the increase in allergy, autoimmunity and IBD (*Rook*, 2000; *Rook* et al., 2000; *Yazdanbakhsh* et al., 2001), and possibly changing patterns of psychiatric disorder. Indeed IL-10-secretitng cells such as certain NKT-cells can also be involved in the induction of regulatory T-cells (*Sonoda* et al., 2001), as can IL-10 secretion by antigen-presenting cells. We have recently been surprised by the huge range of background cytokine expression even in normal people. Quantitative RT-PCR using mRNA from unstimulated peripheral blood mononuclear cells from normal tuberculin-positive donors,

revealed a 10,000x range of ratios of IL-4 mRNA to IFN-γ mRNA (*Seah* and *Rook*, 2000; *Seah* et al., 2000). We are not aware of a strictly quantitative analysis of IL-10 mRNA expression in unstimulated blood cells.

One consequence of the realisation that the balance of pro-inflammatory cytokines (whether Th1 or Th2) to regulatory cytokines may be the crucial factor, is a resolution of the bizarre compartmentalisation of immunology that led workers in the field of Th1-mediated autoimmunity to seek treatments that would deviate the Th1 response towards Th2, while workers in the field of allergy were trying to deviate the Th2 response towards Th1. It now appears probable that neither approach was either safe or desirable, and that to treat both types of disorder we should be trying to evoke regulatory T-cells.

For instance, in the experimental allergic encephalitis (EAE) model and in the induction of diabetes in non-obese diabetic (NOD) mice, both of which are primarily Th1-mediated, a superimposed Th2 response can make the situation worse (*Lafaille* et al., 1997; *Pakala* et al., 1997), as it does in models of infection (reviewed in (*Rook* et al., 2000). Moreover if the immune response is incorrectly regulated, switching the incorrectly regulated Th1 response to Th2 may merely result in an incorrectly regulated Th2 response that causes a different but equally dangerous disease (*Baxter* et al., 1994; *Genain* et al., 1996). This appears to be what happened in a treatment trial in human multiple sclerosis, where the disease changed in nature without improvement from the patients' point of view, and autoimmune thyroid disease also appeared, superimposed upon the modified MS (*Coles* et al., 1999a; *Coles* et al., 1999b). These issues were discussed in detail elsewhere (*Rook* et al., 2000). The solution to this dilemma is to distinguish between Th2 effector cells, which are potentially dangerous when superimposed upon a Th1 response and Th2-like regulatory cells secreting IL-10 and/or TGF-β, which are beneficial.

The same argument holds for attempts to treat allergy by deviating the response to Th1. Allergen-specific Th1 clones can entirely fail to downregulate a Th2 response (*Cottrez* et al., 2000) or airway hyper-reactivity (*Hansen* et al., 1999). Indeed they can contribute additional immunopathology despite their ability to downregulate aspects of the Th2 response such as eosinophil infiltration (*Hansen* et al., 1999). Similarly in a recent human clinical trial, administration of IL-12 to asthmatics caused some evidence of a switch towards Th1, and some fall in eosinophil count, but failed to decrease the late asthmatic response to allergen exposure (*Bryan* et al., 2000). Moreover a monoclonal antibody to IL-5, a key Th2 cytokine, greatly reduced eosinophil levels in blood and bronchoalveolar lavage, but also failed to affect the late asthmatic response to allergen exposure (*Leckie* et al., 2000).

An alternative approach is the induction of the physiological regulatory cells that can control both excessive Th1 and Th2 activity.

HOW DO MICROORGANISMS TRIGGER REGULATORY T-CELLS?

There is no definitive answer to this question yet. Figure 2 is a crude cartoon indicating that the precursors of effector cells, (which are inevitably weakly anti-self because of positive selection in the thymus), and regulatory cells that block

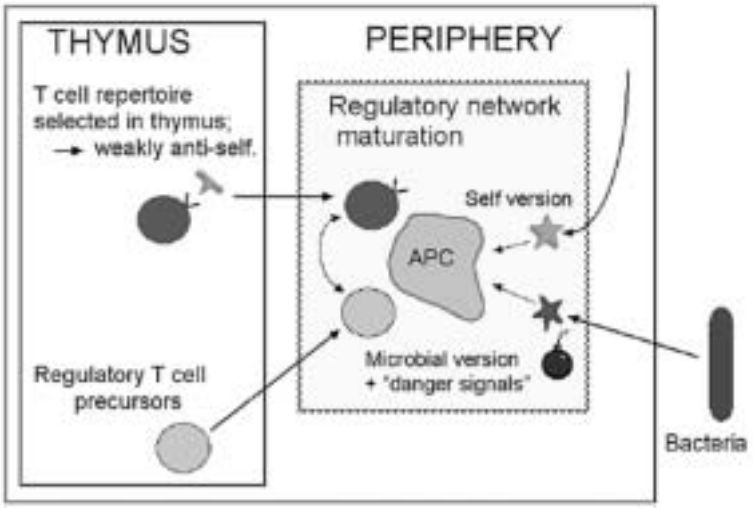

Figure 2: The induction of regulatory T-cells, and the requirement for bacterial or other microbial products is not understood in detail. Regulatory T-cell precursors need to encounter the antigen in the periphery, and the simultaneous presence of microbial homologues, presented in the context of microbe-derived "danger signals", may be essential for the correct setting up of regulatory networks and self/non-self discrimination.

autoimmunity, may be generated together in the thymus (*Seddon* and *Mason*, 2000). Then for full function the regulatory cells need to encounter the self antigens in the periphery (*Seddon* and *Mason*, 2000). It may be at this point that cross-reactive microbial epitopes, presented in the context of microbial "danger signals" (*Matzinger*, 1994), exert a crucial influence on the appropriate development of regulatory networks. Much tolerance to allergens probably develops via mucosal surfaces, and indeed Th3 cells are preferentially induced following oral administration of antigen. It is interesting that oral tolerance is difficult to evoke in germ-free mice. This ability is restored by bowel flora or bacterial products (*Sudo* et al., 1997; *Wannemuehler* et al., 1982). Effectively therefore, certain microbial components can act as "regulatory cell adjuvants". One hypothesis to explain the development of regulatory responses involves the Notch-Notch ligand system. These molecules take part in cell lineage decisions in many organs, and may be involved in the effector/regulator decision in the immune system. High-dose peptide delivered intranasally to mice induces transient expression of Delta 1 (a Notch ligand) on inhibitory CD4+ T-cells. Ligation of the Notch1 receptor on neighbouring T-cells by Delta1+ regulatory T-cells inhibits clonal expansion of effector cells and leads to suppression (*Hoyne* et al., 1999).

THE MICROORGANISMS INVOLVED

If deficient exposure to certain microorganisms that act as regulatory T-cell adjuvants is behind the steady increase in diseases of immunodysregulation in the developed countries, it becomes important to identify the organ-

isms involved. As far as recent trends in the developed countries are concerned the helminths and other parasites can probably be disregarded, as discussed earlier. In fact it is extremely unlikely that any single species or genus holds the key, but identification of any genera with the appropriate properties is clearly useful since it can lead to therapeutic trials.

Gram-negative bacteria

Recently some attention has been focused on Gram-negative bacteria, because allergic manifestations are less common in children living in an environment contaminated with high levels of endotoxin (lipopolysaccharide; LPS) (*Gereda* et al., 2000). This was confirmed by studies of farming families in Germany, where high levels of LPS are found in the homes and bedding of a population with a low incidence of allergies (*von Mutius* et al., 2000). LPS is a readily assayed and very robust component of Gram-negative bacteria, but this work does not in any way prove that these bacteria are the important ones. They might be, but we must remember that the LPS is an indicator of a lifestyle that will allow contamination with all types of organism, even if there is no way to screen easily for the others. It is however interesting that a polymorphism of CD14, which is involved in recognition of LPS by macrophages, is associated with some aspects of atopy (*Baldini* et al., 1999), but if the Notch-Notch ligand story is correct, LPS is an unlikely candidate as inducer of regulatory cells since its known interactions with Toll-like receptors will drive effector rather than regulatory cell proliferation.

Mycobacteria

The possible importance of the mycobacteria is suggested by several epidemiological approaches already discussed (*Aaby* et al., 2000; *Shirakawa* et al., 1996; *von Mutius* et al., 2000). Mycobacteria are not part of the normal commensal flora of man, but they are immensely common in mud and untreated water. There are more than 80 saprophytic species, and untreated water in developing countries can contain as many as 10^9 (approximately 1mg) per litre. Thus exposure to mycobacteria depends on lifestyle to an extent that is not true for any species that is part of the commensal gut flora, and so can help to explain the difference between developing and rich countries, and the urban-rural differences seen in both environments. The inhabitants of inner cities in the USA encounter mycobacteria very little, whereas inhabitants of developing countries usually have delayed hypersensitivity reactions to multiple environmental species as well as to tuberculosis. In fact they must have been very common in the environment throughout mammalian and human evolution, and it is possible that exposure to them is as much an evolutionary necessity as is exposure to a background level of LPS. It is important to note that children with defective receptors for IFN-γ, or IL-12 die from infections with saprophytic environmental mycobacteria (*de Jong* et al., 1998; *Newport* et al., 1996), which implies that in normal children, who frequently have such species associated with their tonsils (*Stewart* et al., 1970), the immune system is constantly and actively involved in their destruction. Conversely, the mycobacteria, when present in the environment, must be constantly modulating the immune system.

In mouse models BCG or *M. vaccae* can limit Th2 responses, even when given after the start of immunisation (*Erb* et al., 1998; *Wang* and *Rook*, 1998). This effect includes downregulation of airway hyper-reactivity (*Zuany-Amorim* et al., 2001). The

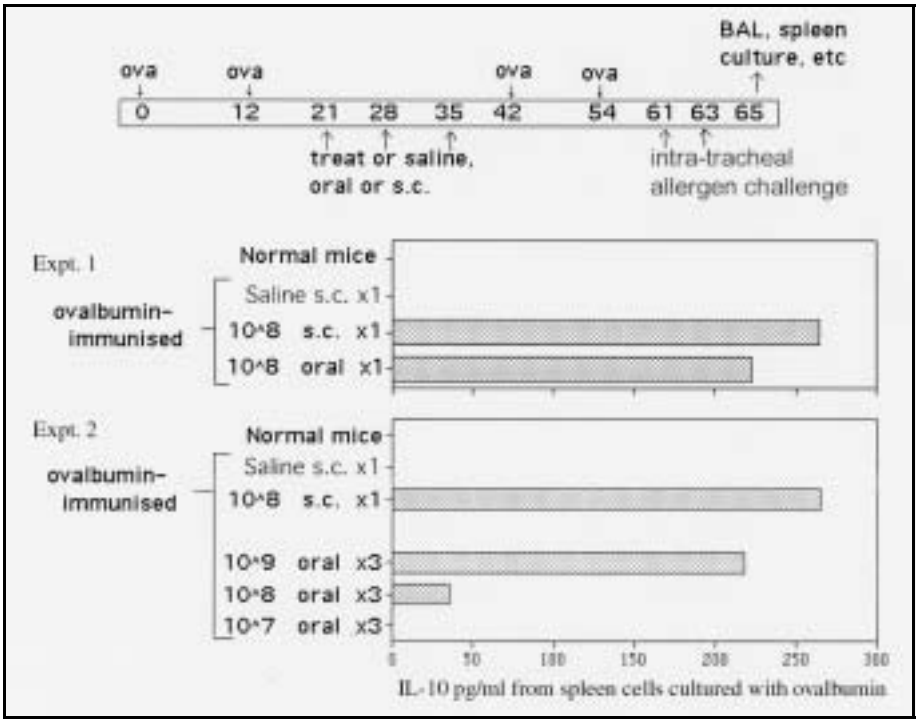

Figure 3: Spleen cells from Balb/c mice rendered allergic to ovalbumin did not release IL-10 in response to ovalbumin *in vitro*. However 0.1mg killed *M. vaccae* given s.c. at the times indicated, resulted in the presence in the spleen of a robust IL-10 response. Manifestations of the allergic response to intratracheal challenge were also supressed, correlating with the presence of allergen-*specific* regulatory cells demonstrable in a cell transfer system. Orally administered *M. vaccae* (by gavage) was similarly active.

downregulation is mediated by IL-10-secreting allergen-specific CD4+ T-cells that can be transferred into allergic recipients (discussed in *Rook* et al., 2001) and *Zuany-Amorim* et al., submitted for publication). In view of the probable involvement of the gut in tolerance induction, it is interesting that mycobacteria, whether alive or killed, are subject to very rapid and efficient uptake via M cells (*Fujimura*, 1986; *Lugton*, 1999; *Momotani* et al., 1988). It has emerged recently that tiny doses of SRL172 or SRP299 (derivatives of heat-killed *M. vaccae* NCTC11659) given by the oral route are active in the Balb/c model of allergy to ovalbumin. A full account of this work will be published elsewhere (Rosa Brunet, L., Hunt, J., and Rook, G.A.W., in preparation). Figure 3 shows, for example, that one dose of SRL172 given after the first 2 of 4 immunisations, caused the appearance in the spleen of cells that released IL-10 in response to ovalbumin *in vitro*. Again, allergen-specific regulatory cells are induced.

Clinical studies with mycobacterial derivatives in allergic disorders

Derivatives of *M. vaccae* administered by the intradermal route have been tested in several clinical studies in human asthma (*Camporota* et al., 2000; *Hopkin* et al., 1998; *Shirtcliffe* et al., 2001) and eczema (*Arkwright* and *David*, 2001). The results are encour-

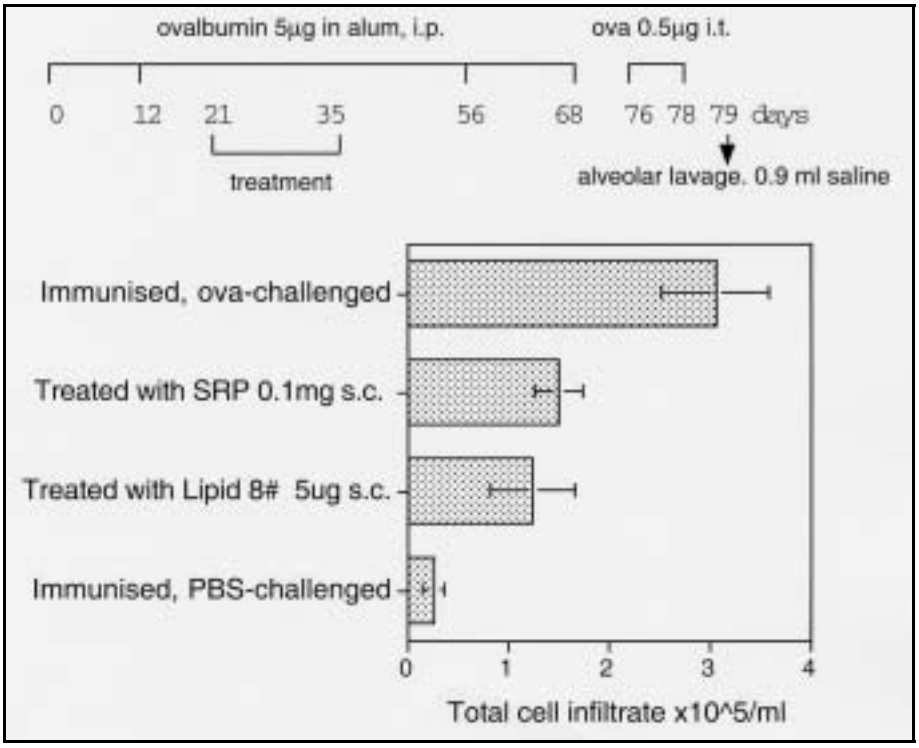

Figure 4: Using a protocol similar to that shown in Figure 3, two s.c. injections of lipid fraction 8 from *M. vaccae* NCTC11659 caused suppression of cellular infiltration into the lungs following intratracheal (i.t.) challenge with ovalbumin. This fraction can also lead to the presence of IL-10 secreting cells, and to suppression of eosinophilia, without any switch to Th1 (not shown). We speculate that some mycobacterial lipids might be essential to the immune system, and act as regulatory T-cell adjuvants.

aging, and a Phase II study is now in progress. Interestingly a delipidated preparation appeared to be inactive (*Shirtcliffe* et al., 2001), and recent work suggests that certain lipid fractions may be important active components of the organism (Figure 4).

Clinical studies with mycobacterial derivatives in autoimmune disease

M. vaccae derivatives have also shown promise in several clinical studies in psoriasis (*Balagon* et al., 2000; *Lehrer* et al., 1998), and BCG vaccine was recently tried in patients with multiple sclerosis (MS) (*Ristori* et al., 1999). Since BCG is predominantly a Th1-inducing vaccine, and MS is a predominantly Th1-mediated disease, this trial represented a striking conceptual advance in which the physicians were thinking in terms of regulatory T-cells rather than Th1/Th2 balance (*Rook* et al., 2000). The results of this small preliminary study were most encouraging (*Ristori* et al., 1999).

Lactobacilli and the hygiene hypothesis

Lactobacilli were a common part of the diet of primitive man, but now occur mostly as a minor additive in some foods. Levels of gut *Lactobacilli* are lower in populations with high rates of allergy (*Sepp* et al., 1997). Therefore

this genus is also generating interest, and since it colonises the human gut there are exciting possibilities, and several small clinical studies have taken place. These are covered by other chapters within this volume and will not be considered further here.

CONCLUDING REMARKS

As we live in increasingly hygienic and infection-free environments, so vaccination schedules become an increasingly large proportion of the educational input to our immune systems. If the immune system has evolved in the anticipation of a particular type and sequence of inputs, the change represented by modern vaccination schedules may be inappropriate. Vaccines will inevitably modify the priming of regulatory T-cells. First, they provide exposure to organisms in a sequence that is different from the evolutionarily experienced/programmed sequence. Peter Aaby has evidence that the *sequence of vaccination* has effects on subsequent overall health (personal communication). Secondly, the vaccines used prime an unbalanced response, mostly Th2-orientated, particularly in the USA where BCG is not used and hepatitis B vaccination at birth is routine. It is not clear that priming antibody responses drives adequate maturation of regulatory T-cells. Thirdly, most current vaccines *replace* the educational input previously provided by recovery from the infection, with a quite different type of immunological response (for instance antibody that blocks infection rather than a cell-mediated response that cures it). These changes are taking place against a background of antibiotic use, already shown to affect susceptibility to allergies (*Farooqi* and *Hopkin*, 1998), that will also alter bowel flora and the education of the immune system.

Against this background there are now several studies indicating that vaccinations can affect the overall survival of children from diseases not directly related to the target infection for which the vaccine was intended. Thus measles vaccine and BCG were beneficial for overall survival, whereas Diphtheria-Pertussis-Tetanus (DPT) opposed this beneficial effect (*Kristensen* et al., 2000). Thus while many groups, including ours, are seeking to use vaccines as immunomodulators to correct inappropriate priming of immunoregulation in the developed countries, others are already showing that vaccines do indeed exert non-specific effects on health, even against the greater background of exposure to infection in a developing country (*Kristensen* et al., 2000). It is to be hoped that novel vaccine design and novel vaccination schedules with greater emphasis on the activation of regulatory cells, can work together with probiotics (or indeed with orally administered vaccines) to retain the huge benefits of vaccination, while simultaneously reducing the diseases of immunodysregulation.

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POTENTIAL BENEFIT FROM INACTIVATED WHOLE CELL VACCINES

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INTRODUCTION

The potential for the development of new and better vaccines against infection is greater today than at any time in the past two centuries since Jenner's initial vaccinations against smallpox. This opportunity has been fuelled by the great strides made in immunology and biotechnology. One potentially useful approach to achieve immune protection through immunisation at mucosal surfaces is the use of inactivated whole cell vaccines. As will be described below, new technologies are making this approach a very promising means to alleviate human suffering.

Infectious diseases continue to be the leading cause of morbidity and mortality world-wide. With the increasing occurrence of antibiotic resistance to many infectious agents, new vaccination strategies for mucosal immunisation may provide efficacious preventive and therapeutic treatments. Mucosal membranes are the most frequent portals of entry for pathogenic organisms. These facts have prompted studies aimed at developing vaccination protocols to increase protection of mucosal surfaces. Systemic immunisations are of limited effectiveness in the induction of mucosal protection (*Bakke* et al., 2001); however, ingestion or inhalation of antigens results in a generalised mucosal immune response. Mucosal immunity will not only protect the surfaces against colonisation, but also has the potential to reduce existing colonisation by pathogens inhabiting mucosal surfaces. Further, mucosal immunisation can also increase systemic immunity (*Bakke* et al., 2001; *Stiles* et al., 2001) which could make the mucosal route appropriate for needle-less delivery of many current and future vaccines.

Inactivated micro-organisms offer potential advantages as vaccines for mucosal immunisation. Physically, they are naturally occurring microparticles and possess multiple antigens that should enhance interactions between the antigens they carry and mucosal lymphoid tissues. Presentation of multiple antigens may be particularly important for pathogens for which protective antigens are not known, or are not readily available in purified or recombinant forms. Also, whole cell vaccines can often be developed relatively quickly and produced and administered economically. A killed whole cell cholera vaccine made in Vietnam, for example, consists of four cell types, 2.5×10^{10} cells per type, and costs just 10 cents per dose (*Trach* et al., 1997). Inactivated whole cell vaccines are generally safe for mucosal administration and, in clinically useful combinations, may be safer than similar combinations of attenuated micro-organisms.

Several considerations are paramount for successful development of inactivated whole cell vaccines. One, the bacteria grown in vitro must express important antigens associated with pro-

tection. Second, the antigens so expressed must be retained upon inactivation. Here, both the process of inactivation and the method of preservation are important. Other considerations include immunomodulary techniques and delivery formulations, which will facilitate intact delivery and maximise the appropriate host immune response to antigens presented to the mucosal surface. In short, the key to successful mucosal vaccination is to induce the appropriate mediated responses to provide strong protective immunity without associated illness.

MECHANISMS OF IMMUNITY ASSOCIATED WITH ORAL VACCINATION

A mucosal immune system exists whereby antigens encounter specialised epithelial cells and then the underlying lymphoid cell aggregates (*Craig* and *Cebra*, 1971). In the gastrointestinal tract, these lymphoid aggregates are the Peyer's patches. The predominant pathway of lymphocyte effector cells within the intestinal immune system is from the gut-associated lymphoid system to other mucosal sites (*Rudzik* et al., 1975). Other pathways do, however, exist (*McGhee* et al., 1992). Lymphoid tissue throughout the mucosal system exists either as well defined and clearly organised tissue such as the lingual, palatine and pharyngeal tonsils, and the Peyer's patches along the intestine, or it exists as diffuse collections of lymphocytes, plasma cells, and phagocytes such as in the lung and the lamina propria of the intestinal wall. In the intestine, antigen entering the Peyer's patches does so in pinocytotic vesicles across specialised epithelial cells (M cells). Within the follicle, antigen is processed by antigen presenting cells to stimulate antigen sensitive T and B lymphocytes. These subsequently drain into lymph, migrate via the mesenteric lymph nodes and the thoracic duct lymphatics, and pass from the blood into the mucosal lamina propria where they differentiate into mature effector T cells and predominantly IgA producing plasma cells (*Craig* and *Cebra*, 1971).

The common mucosal immune system can be differentiated into two areas. Initial responses are induced at the site where the antigen is first encountered by the lymphoid cells (inductive site). The second and larger area of induced immunity occurs at distant mucosal sites where the lymphocyte effector cells migrate and thus activate IgA production and cell-mediated immunity for local immune protection. The distant mucosal site commonly has a predominance of T cells of the CD4+ helper type. It is enriched in B cells and plasma cells, usually of an IgA isotype, and is covered by epithelial cells producing the polymeric immunoglobulin receptor secretory component that transports polymeric IgA into external secretions (e.g., saliva, mucus) (*McGhee* et al., 1992). Exposure to most environmental antigens is usually via the respiratory or gastrointestinal tracts, which therefore act as the first lines of defence. Thus, the common mucosal immune system provides the network for the distribution of specific humoral and cellular immune responses from inductive sites, particularly in the gastrointestinal tract, to peripheral mucosal sites. The generation of protective mucosal immune responses by oral presentation of antigen is therefore a feasible vaccine approach.

At least two criteria determine the efficacy of antigens in the gastrointestinal tract for the induction of mucosal immune responses: firstly, the protection

of antigenic components from the gastric acid and digestive components and, secondly, access to the mucosal immune apparatus which preferentially interacts with particulate matter. The first of these criteria can now be achieved by use of protective enteric formulations or co-administration of buffer. The second requires consideration of particle size, shape, and sufficient antigen delivery and immunomodulation at the mucosal surface. Traditionally, live organisms were thought to give a more effective mucosal immune response compared with whole killed organisms. An appropriately formulated inactivated whole cell vaccine, however, which safely presents an array of protective antigens should be able to induce a mucosal immune response that is equivalent to that obtained with live vaccine delivery systems. Evidence, for this possibility is available for at least one organism (*Cardenas* et al., 1994). *Salmonella dublin* strains expressing the *Escherichia coli* heat-labile enterotoxin B subunit were inactivated with several different methods and used to orally immunise mice. Immunoglobulin G and IgA responses to B subunit on the inactivated cells were equivalent to the immune response following infection with live organisms expressing the same labile toxin B-subunit. More recently, orally administered formaldehyde-killed recombinant bacteria expressing a mimic of the shiga toxin receptor were found to protect mice from a normally fatal challenge with shiga-toxigenic *E. coli* (*Paton* et al., 2001). These data suggest that inactivated whole cells may be suitable as vectors to deliver foreign antigens that have been cloned into them.

The efficacy of a mucosal vaccine delivery system may be dependent on the type of immune response induced. It has been shown that oral immunisation with attenuated *Salmonella typhi* strains carrying foreign genes elicits a strong systemic cell-mediated immunity that is of a Th1-type, but not Th2-type (*Sztein* et al., 1994). Oral delivery of a *Salmonella* vector having a recombinant tetanus toxin C induces a dominant Th1-type response, whereas tetanus toxin C co-administered orally with cholera toxin (CT) induced a predominantly Th2-type response in CD4+ T cells (*Xuamamo* et al., 1994; *Jackson* et al., 1996). This suggests that the immune response to the recombinant antigens expressed in *Salmonella* strains may be directed by the *Salmonella* carrier. Interestingly, both vaccine delivery systems elicited high levels of mucosal secretory IgA. It has been suggested that following oral immunisation with recombinant *Salmonella*, antigen-specific Th1 cells producing IFN-gamma and IL-2, Th2-derived IL-10, and IL-6 from macrophages, all provide important signals for the development of mucosal IgA in the absence of a dominant Th2 response (*Vancott* et al., 1996).

EXPERIENCE WITH KILLED WHOLE CELLS FOR ORAL IMMUNISATION

Vaccines for Enteric Pathogens

Campylobacter Vaccine Development

During the past decade *Campylobacter jejuni* has become the focus of growing attention due to its increasing frequency of isolation (*Nachamkin* et al., 1992; *Taylor* and *Blaser*, 1991; *Stern* and *Kazmi*, 1989). No vaccine is currently available to protect against this pathogen. American adult volunteers challenged with *C. jejuni* developed serum and intestinal antibodies and were

protected from subsequent illness, but not against infection, following challenge (*Black*, 1988). This observation is consistent with reports documenting prolonged excretion or symptoms, or both, in patients with IgA deficiency (*Johnson* et al., 1984; *Melamed* et al., 1983). An in vitro mucus assay was used to study the role of sIgA in preventing attachment of *C. jejuni* to INT 407 cells. Mucus from rabbits previously colonised with *C. jejuni* completely blocked adherence to the underlying cells (*McSweegan* et al., 1987). Anti-*Campylobacter* sIgA was readily detected in these mucus samples and was responsible for eliminating bacterial adherence. This was shown by loss of inhibition after mucus absorption with *Campylobacter* cells.

Since natural infection confers IgA-associated immunity against *Campylobacter*, both living and non-living vaccine approaches against *Campylobacter* have been considered as means to safely confer immunity. The use of inactivated *Campylobacter* whole cells (CWC) offers a practical approach to vaccination against the pathogen and, at present, this has been the most studied approach to development of a vaccine against this pathogen.

A formalin-inactivated whole cell vaccine for *C. jejuni* has been extensively studied in humans and animals. Mice have been orally immunised with a 3-dose primary series of particles of CWC (48-hour intervals) at doses of 10^5, 10^7, or 10^9 cells (*Baqar* et al., 1995). The vaccine was given to mice with or without the mucosal adjuvans consisting of the heat-labile enterotoxin of *Escherichia coli* (LT) (*Walker* and *Clements*, 1993). Baqar's studies showed that the *Campylobacter*- specific intestinal IgA response was dependent on the use of LT, whereas serum immunoglobulin responses were not. Upon subsequent oral challenge, colonisation resistance was found to be induced over a broad range of vaccine doses when LT was included. However, only the highest dose (10^9) of CWC alone gave comparable levels of protection against colonisation. Both the adjuvanted and unadjuvanted formulations of CWC given at the intermediate dose (10^7) provided equivalent protection against systemic spread of challenge organisms.

The CWC vaccine is currently being evaluated in a series of clinical trials by a team from Antex Biologics Inc and the Naval Medical Research Center. Studies completed so far indicate that the vaccine is safe and immunogenic (D. Tribble, personal communication). Future answers concerning the optimal dosing and the contribution of adjuvans to the vaccine are needed.

Shigella Vaccine Development

Shigellae are enteric pathogens that invade and spread between the epithelial cells of the colonic mucosa, thereby causing inflammation and ulceration, resulting in diarrhoea or dysentery. Vaccine development for this disease has focused on oral administration of invasive attenuated strains. For many of these vaccine candidates it has been difficult to balance protective efficacy with reactogenicity. To date, no licensed vaccine for *Shigella* spp. exists. Although not previously thought to be effective (*Hale*, 1995), it has recently been shown that heat-killed *S. flexneri* 2a could induce protective immunity in guinea pigs if co-administered with mutant labile toxin of enterotoxigenic *Escherichia coli* (*Hartman*, 1999).

Hartman's studies (*Hartman*, 1999) used a 2-dose (days 0 and 14) oral regimen containing 10^{10} killed bacteria per dose. No protection was seen with this regimen unless the adjuvans LT_{R192G} [an attenuated LT (*Dickenson* and *Clements*, 1995)] was included. It

remains to be seen whether an increased dosing regimen could overcome the need for the adjuvans. In support of this idea, Hartman also found that the 2-dose regimen (5 x 10^7 CFU) did produce protection without the adjuvans if given nasally. Further support for the concept of increased vaccine dosing comes from work with an attenuated oral vaccine for typhoid fever, Ty21a. Although not a killed cell preparation, the typhoid vaccine studies showed that a fourth dose was significantly more protective than a three dose regimen which was much better than a 2-dose regimen (*Levine* et al., 1989).

At present no clinical data are available regarding inactivated whole cell vaccines for *Shigella* spp. The fact that human challenge models for this pathogen do exist should facilitate eventual clinical studies.

Cholera Vaccine Development

Inactivated cholera vaccines now under development rely on oral delivery of the mucosal immunogen. The best studied of these vaccines is a preparation composed of a mixture of the non-toxic B sub-unit of cholera toxin and killed whole *V. cholerae* cells (WC-BS). There were no adverse events among volunteers in a clinical safety trial of the WC-BS cholera vaccine administered orally at 5 x 10^{10} bacterial cells per dose (*Svennerholm* et al., 1984). A random, double-blind placebo-controlled field trial, involving 63,000 individuals in rural Bangladesh, established the safety, immunogenicity and efficacy of the WC-BS vaccine. Two and three doses of the WC-BS vaccine conferred 85% protection against cholera for the first six months in all age groups tested, and 51% overall protection after three years (*Clemens* et al., 1990). No adverse events attributable to the vaccine were reported. More recently, a new formulation of the WC-BS cholera vaccine containing a recombinantly produced cholera toxin B sub-unit was also found to be non-reactogenic (*Sanchez* et al., 1993) and gave high levels of protective immunity (protective immunity = 86%) against symptomatic cholera in Peruvian military recruits (*Sanchez* at al., 1994). These vaccines provide proof of principal that orally administered mucosal vaccines can be a safe and effective means to protect against disease.

ETEC Vaccine Development

An approach similar to that used with the cholera vaccine has been used to develop a vaccine against enterotoxigenic *E. coli* (ETEC). The vaccine consists of a combination of 5 strains (2 x 10^{10} particles of each strain) that expresses the most important colonisation factor antigens (CFA) on their surface and also contain the main O antigens (*Holmgren* and *Svennerholm*, 1990). The bacteria are grown under conditions to maximise the expression of CFAs, then inactivated with a mild formalin treatment. The CFAs are resistant to degradation in gastric juice when presented in this whole cell complex, rather than as free macromolecules (*WHO*, 1990). The B sub-unit of cholera toxin, which cross-reacts with the heat labile toxin of ETEC, is included in the vaccine to provide antitoxin immunity. This vaccine, when given to Egyptian school children as two doses two weeks apart, is safe and immunogenic. The vaccine also induced a prompt mucosal immune response to multiple CFA antigens (*Savarino* et al., 1997). Further trials to demonstrate protection are needed.

Helicobacter pylori Vaccine Development

Helicobacter pylori infects nearly half of the world's population, resulting in chronic active gastritis which persists throughout life unless the organism is

eradicated (*Drumm*, 1993; *Fiocca* et al., 1987). Most infected individuals remain asymptomatic, but some develop peptic ulcer disease as well as run an increased risk of gastric cancer (*Graham* et al., 1992; *Rauws* and *Tytgut*, 1990; *Correa* et al., 1990; *Forman* et al., 1991). Treatment of *Helicobacter* infections is difficult and the use of vaccines for treatment and prevention of *H. pylori* infections is being actively explored.

Helicobacter seems to differ from other enteric pathogens in that colonisation persists in spite of a strong host immune response (*Crabtree*, 1993). Conflicting data are available regarding the importance of different components of the immune response in clearing this pathogen. One consistent observation, however, is that an immune response induced mucosally by *Helicobacter* antigens alone is not sufficient to affect colonisation. Protective responses are seen only if mucosal adjuvans such as cholera toxin (*Lee* and *Chen*, 1994) or LT are used with antigens from *Helicobacter*.

A formalin-inactivated whole cell preparation of *H. pylori* (HWC) has been tested as a vaccine candidate. A Phase I clinical trial with the HWC + LT_{R192G} vaccine was conducted to establish its safety and immunogenicity in humans (*Kotloff* et al., 2001). An initial dose response study was conducted among 23 volunteers to determine whether increasing inocula of HWC, co-administered with 25 µg of LT_{R192G}, were well tolerated, and to evaluate whether increasing HWC inocula enhanced the immune response. It was anticipated that the optimal dose would contain 2.5 x 10^{10} HWC + 25 µg LT_{R192G}. Groups of 3-10 *H. pylori* infected and *H. pylori* uninfected subjects were assigned in an unblinded fashion to receive three oral doses of vaccine, on days 0, 14, and 28, at an inoculum of either 2.5 x 10^{6}, 2.5 x 10^{8}, or 2.5 x 10^{10} HWC plus 25 µg LT_{R192G}. Safety was established at each dose level before a new group of volunteers received a higher inoculum of vaccine. For the purpose of characterising the dose-response, the eight *H. pylori* infected subjects who received 2.5 x 10^{10} HWC plus 25 µg LT_{R192G} as part of the Randomised Safety and Immunogenicity Study included in this trial were also included in this analysis.

A randomised study was conducted among *H. pylori*-infected subjects to investigate in a preliminary fashion the safety and immunogenicity of the oral HWC vaccine administered with and without the adjuvans. Twenty *H. pylori*-infected subjects were randomly assigned, in a double blind, placebo controlled fashion, to receive, on days 0, 14, and 28, either 2.5 x 10^{10} HWC plus placebo-adjuvans, placebo-vaccine plus 25 µg LT_{R192G}, placebo-vaccine plus placebo-adjuvans, or 2.5 x 10^{10} HWC plus 25 µg LT_{R192G}.

Of the 41 subjects who participated in the trial, six experienced diarrhoea (three who had baseline *H. pylori* infection); one subject received placebo-vaccine plus LT_{R192G} and the remaining five received 2.5 x 10^{10} HWC plus LT_{R192G}. Thus, diarrhoea was seen only among subjects who received LT_{R192G} (with or without vaccine), and only following the highest (2.5 x 10^{10}) HWC dose. Diarrhoea followed the first inoculation in all but one subject. The episodes lasted for 1-3 days, during which time these subjects passed a total of 3 to 17 loose stools.

Immunisation elicited rises in geometric mean serum and mucosal anti-HWC antibodies only among subjects who received the highest (2.5 x 10^{10} HWC) vaccine dose. Whereas post-vaccination increases in geometric mean peak serum IgA and IgG titres were marginal ($p=0.06$), and only seen among *H. pylori*-infected subjects, the

faecal and salivary IgA responses were statistically significant and occurred in both *H. pylori*-infected and uninfected volunteers. Anti-HWC ASC responses were meagre (none exceeded 10 cells per 10^6 PBMC), and so were not subjected to statistical analysis.

Immunisation with 2.5 x 10^{10} HWC plus LT_{R192G} resulted in significant ($p<0.05$) increases among *H. pylori*-uninfected volunteers in mean group IFN-gamma production to the *H. pylori* sonicate at 2 µg/ml; significant rises were observed in 7 of the 10 volunteers studied. No significant increases in mean interferon gamma production were observed when PBMC were incubated with either recombinant catalase or BSA. In contrast, significant increases in mean interferon gamma production to the *H. pylori* sonicate were not observed following immunisation of *H. pylori*-infected volunteers.

Vaccines for Non-Enteric Pathogens

Neisseria gonorrhoeae Vaccine Development

A systemic-oral vaccination approach has been used to vaccinate mice against *Neisseria gonorrhoeae* (*Arko* et al., 1997). In this approach, systemic administration of a gonococcal synthetic peptide was followed by oral administration of killed gonococcal cells made deficient in protein III, which interferes with protective immune responses. These studies showed that vaginal clearance of gonococci from mice was faster in the vaccinated group than in control animals.

Development of Vaccines for Respiratory Pathogens

Oral administration of formalin-killed whole cell *Pseudomonas,* non-typeable *Haemophilus influenzae* and *Streptococcus pneumoniae* has been shown to protect rats against acute respiratory infection (*Wallace* et al., 1989; *Yoshimura* et al., 1991; *Cripps* et al., 1994). Similarly, intestinal Peyer's patch immunisation with formalin-killed whole cell *Branhamella (Moraxella) catarrhalis* provides protection against acute respiratory infection in mice when intestinal immunisation is accompanied by an immunisation boost delivered to the lungs (Alan Cripps, personal communication). In human studies, adult patients were protected against acute bronchitis after receiving a series of immunisations with 10^{11} killed non-typeable *Haemophilus influenzae* in enteric-coated tablets (*Lehman* et al., 1991). In an earlier study (*Clancy* et al., 1985) in which patients were given this vaccine, a ten-fold reduction in the incidence of infection was noted. There was no clear correlation, however, between clinical protection in these studies and either carriage of *H. influenzae,* or the level of antibacterial antibody in saliva. Subsequent studies in a mouse model of acute respiratory infection (*Wallace* et al., 1991; 1995) have supported the hypothesis that the protective mechanisms induced in the lung include a combination of opsonic antibody and T-cell upregulation of polymorphonuclear cell recruitment and activation.

Oral immunisation of guinea pigs with formalin-killed *Streptococcus pneumoniae* in enteric capsules, following systemic sensitisation with the bacterial preparation, enhanced IgA mediated mucosal immunity (*Yoshimura* et al., 1991). This immunity was associated with reduction of histological changes in the middle ear mucosa compared to control animals. More recently intranasal immunisation with killed unencapsulated whole cells with cholera toxin as an adjuvans was used in rats to prevent colonisation and invasive disease by capsulated pneumococci (*Malley* et al., 2001). In this way, multitypic protection against unrelated pneumo-

coccal serotypes could be obtained associated with the production of systemic antibodies.

The possibility of acts of bioterrorism has led to intense interest in a vaccine against *Bacillus anthracis.* The non-capsulated attenuated Sterne strain of *B. anthracis* has been used to construct genetically detoxified derivatives, thus making a safer non-toxic vaccine candidate (*Mock* et al., 2001). A cellular vaccine may be useful because the cell-free protective antigen (PA) vaccine is less effective in animal models than live strains. This suggests that other components and/or various immune response mechanisms are required for optimal protection. Addition of formaldehyde-inactivated spores of the non toxic Sterne strain protected 100% of mice and guinea pigs against challenge with virulent *B. anthracis* strains under conditions in which PA alone is ineffective.

FUTURE CONSIDERATIONS

Human and animal data indicate that the inactivated whole cell vaccines are safe when administered orally. This characteristic may be made even more important if whole cell vaccines of various pathogens are eventually formulated in a multi-pathogen vaccine or administered simultaneously. The work with the CWC vaccine could lead to a vaccine against major enteric pathogens. Previous experience with cholera and ETEC suggest that this type of vaccine should be effective.

The CWC vaccine could be administered as part of an enteric diseases vaccine. The ETEC component of a combined enteric vaccine should consist of strains to include the major adhesin antigens. Colonisation factor antigen I (CFA/I), CFA/II and CFA/IV are individually expressed by a majority of ETEC isolates from most geographic regions (*Holmgren* and *Svennerholm*, 1990). CFA/I is antigenically homogeneous, whereas CFA/II is composed of coli surface antigen (CS) 3 alone or in combination with CS1 or CS2, and CFA/IV is composed of CS6 alone or in combination with CS4 or CS5. The most advanced ETEC vaccine is a group of inactivated whole cells representing the major CFAs (*Holmgren* and *Svennerholm*, 1990; *Savarino* et al., 1999). It is possible that other organisms, such as *Shigella,* can serve as a platform for passenger antigens, which could include the colonisation factor antigens of ETEC. For example, *Noriega* et al. (1996) used an attenuated *S. flexneri* 2a as a live vector for ETEC antigens CFA/I and CS3. This preparation induced immune responses to the ETEC passenger antigens in mice and guinea pigs. Whether this could also be accomplished with a non-living whole cell preparation of this organism remains to be seen. The *Shigella* component of an enteric vaccine would not only contain *S. flexneri* 2a, but also *S. flexneri* 3a, and *S. flexneri* 6 which would cover most clinically significant strains of this Shigella species (*Noriega* et al., 1999), *S. sonnei,* and *S. dysenteriae*.

Oral immunisation with inactivated whole cells shows promise as a vaccination strategy. Some pathogens, however, have mechanisms, which challenge the success of immunisation. Evidence presented above suggests that *H. pylori* is one of these pathogens. While reduction in colonisation is fairly easily achieved, culture data in animals suggests complete eradication is rarely achieved (*Lee* et al., 1999; *Sutton* et al., 2000). This finding illustrates the need to develop better ways to present anti-

gens and modulate the immune system. Indeed, improved clearance of *Helicobacter* from rodent models has been described in recent papers using a variety of immunisation strategies (*Sutton* et al., 2000; *Guy* et al., 1999; *Eaton* and *Mefford*, 2001). Even reduction of colonisation may be sufficient to relieve symptomatic aspects of infection. *H. pylori* colonisation may protect against diarrhoeagenic gastrointestinal infections (*Rothenbacher*, et al., 2000), indicating that in some circumstances, infection may not be detrimental to the host.

Several associated technologies may be useful with whole cell vaccines. The role of the adjuvans LT_{R192G} in orally administered vaccines remains to be determined. It can certainly serve as an antigen to protect against certain enteric toxins. The adjuvans seems to also enhance the local IgA response in mice (*Rollwagen* et al., 1997; *Baqar* et al., 1995), but this is yet to be shown in humans. Protection in mice could be obtained without adjuvans if the dose of CWC was sufficient (*Baqar* et al., 1995). In contrast, no protection was seen in *Helicobacter* - challenged mice unless the adjuvans was present. Future human trials with inactivated whole cell vaccines should consider the immune responses to a multiple dose, 10^{10} cells per dose, regimen which would include groups with and without a mucosal adjuvans. In this way the IFN-gamma and faecal IgA responses in the presence of adjuvans could be monitored and assessed.

The observation that inclusion of LT-based adjuvans induce a Th1 response (*Bowman* and *Clements*, 2001) may not only be important for protection against the specific pathogen. By inducing immune responses closer to those seen with live organisms, the adjuvans could affect the Th1-Th2 balance that develops after birth. A current view is that Th2-mediated immunity exists in the foetus, but gradually changes to a Th1-mediated immunity (*Rook* and *Stanford*, 1998). This shift is thought to be due to childhood infections, which, through this induction process, could provide significant protection from atopy. It may be prudent to use appropriate adjuvans to ensure that vaccines do not merely protect from infections, but actually replace them as immunologic stimuli (*Walker* and *Zuany-Amorim*, 2001).

In addition to mucosal adjuvans, the method for inactivation could be important in optimising responses to whole cell vaccines. Antibody responses obtained with *Salmonella dublin* varied markedly depending on whether the cells were inactivated with heat, formalin, acetone or ethanol (*Cardenas* et al., 1994). Another approach to inactivating cells is to use expression of cloned PhiX174 gene E in Gram-negative bacteria to cause lysis of the bacteria by formation of an E-specific transmembrane tunnel structure through the cell envelope complex (*Eko*, et al., 1994). Bacterial ghosts prepared with this approach do not suffer from possible denaturation of relevant immunologic determinants obtained with other inactivation procedures.

Another associated technology for use of whole cell vaccines involves their delivery to the mucosal immune system. Frequently buffers have been used to protect antigen from gastric acidity (*Sack* et al., 1997; *Clemens* et al., 1986). These have been particularly important for protein antigens. A less-studied approach is the use of enteric-coated microparticles to induce antibodies against orally administered antigens. Heat-killed *E. coli* were encapsulated in microcapsules coated with a pH-sensitive enteric coating (*Flanagan* et al., 1996). The whole killed bacteria encapsulated by an enteric coating and administered orally to mice effectively

induced an antibody response to its LPS.

Although encouraging data are accumulating, the foregoing illustrates that there is much work to be done to fully realise the potential benefits of orally administered inactivated whole cell vaccines. The safety factor, multi-antigen local and systemic immunity obtained and relative ease of vaccine production and delivery associated with oral immunisation with inactivated whole cell vaccines certainly makes it worthwhile to meet these challenges.

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IMMUNOREGULATORY ACTIONS OF LIVE AND KILLED ENTEROCOCCI AND *E. COLI* PROBIOTICS

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SUMMARY

During the past two decades an aggressive marketing campaign for so-called „probiotics“ resulted in an increased consumer awareness for these „health-promoting bacteria“. Among them, certain lactic acid bacteria of human origin dominate. Effective market strategies in the past, associated with the consumption of these bacteria for attractive possibilities to improve well being and health, even for such medically relevant and serious disease entities like atopic allergy or inflammatory bowel disease. However, to effectively market these health-claims, probiotic bacteria need to have scientific credibility. The latter can only be achieved by well-controlled clinical studies together with pre-clinical well-designed experimental work using refined cell culture models preferably with human leukocytes. However, clinical data supporting the above claimed health benefits remained scant. So far, the best-documented clinical application of probiotics is the treatment of acute diarrhoea in adults and infants, as shown recently by well controlled small-scale studies.

Whereas the successful treatment of acute diarrhoea presumably needs the oral administration of viable bacteria, whose numbers could markedly differ between different fermented milk products, dependent on the manufacturing process and strain-specific properties, distinct immunomodulatory effects can be mediated by inactivated germs too. Hence, the central question to be answered by this presentation was, whether live or dead probiotic bacterial strains, in particular *E. coli* derived probiotics, *Enterococcus faecalis* and a mixture of both strains (Pro-Symbioflor®), may exert similar immunomodulatory effects, tentatively speculated to be useful for immune system related disorders.

To address this question may be the more important in view of the rising tendency of the food industry to use several state-of- the-art biotechnological tools to design „better“ probiotic strains aimed to achieve „optimal“ gut colonisation, better adhesion properties and metabolic profiles, anticancer properties, bacteriocin production and much more. Introducing such genetically modified bacteria into the human gastrointestinal tract could have a profound and still yet unknown impact on the physiology and complex interactions of the normal gut flora, so that probiotic bacteria with a suitable immunomodulatory profile, comparable to selected live strains, might be a more safe alternative, particular under conditions of long-term use.

The ability of mammalian host cells to distinguish harmful virulent pathogens from dead ones, point to the existence of so-called pathogen-associated molecular patterns (PAMS), recognised for example by the Toll-like receptor family of proteins. Other host defence strategies, represented by CD14 antigen, the acute phase proteins, the scavenger receptors, mannose binding lectin and much more emerged to be indispensable in promoting phagocytosis without promoting inflammation with subsequent more effective killing of Gram-positive as well as Gram-negative bacteria. Accordingly the presentation summarises recent published data with regard to those receptors involved in the recognition of Gram-positive and Gram-negative bacteria, which may be of importance in the recognition of *E. coli* and *Enterococcus faecalis*.

The results revealed similarities as well as differences in view of the molecular recognition of dead versus live bacteria and whole versus purified cell wall components illustrating, that the physical composition of their cell wall components have an impact for the subsequent activation of immune cells. Also, recent laboratory work providing evidence for powerful immunomodulatory actions of *Enterococcus faecalis* and *E. coli* were presented. Both strains, being the constituents of a „medical" probiotic preparation named Pro-Symbioflor®, possess a profound cytokine-modulating capacity suggesting that Th1-cells were the main target population. These distinct immunomodulatory properties of Pro-Symbioflor® may be of great importance for therapeutic interventions in Th2 dominated diseases.

PATHOGEN–ASSOCIATED MOLECULAR PATTERNS (PAMPs): KEY STRUCTURES NOT ONLY FOR ANTIMICROBIAL DEFENCE BUT ALSO FOR THE NON-PHLOGISTIC REMOVAL OF APOPTOTIC OR INJURED CELLS

For the survival of most multi-cellular organisms, the recognition of infecting microbes followed by the induction of an effective immune response is essential. Additionally, it is equally important, that the immune response is not induced upon the recognition of self antigens or non-infectious non-self antigens which seem to be of great importance, when injured or apoptotic cells must be removed without causing harm to the host. Thus, the central question is: How does the immune system decide which antigens to respond to and which to ignore? From an evolutionary point of view, microbes, such as bacteria, impose a general thread to the host organism, so that proper and effective recognition of microbial antigens is the first step to ensure the survival of mammalian cells.

But what are the responsible structures to interact with the immune system, as microbes normally possess a huge variety of potential harmful antigenic structures? These microbial structures should be expressed by as much as possible potentially harmful pathogens, enabling the host to broadly recognise and kill as much as possible pathogens with sufficient specificity. The problem comes to a solution as it has been suggested by *Medzhitov* et al. in 1997, that host cells infecting microbes are recognised by a limited number of highly conserved chemical

Table 1: Overview on PAMPs with their proposed ligands (for explanations see text)

PAMPs	Ligand(s)/functions
Soluble and membrane-bound CD14 (sCD14,mCD14) (*Sher* et al., 1991)	Interaction with LBP-bound monomers of LPS, sLPS > rLPS > diphosphoryl LipidA >Monophosphoryl Lipid A liporabinomannans of mycobacteria, soluble peptidoglycans, polymers of rhamnose and glucose of streptococci and whole bacterial cell walls of streptococci and staphylococci
LPS binding Protein (LBP) (*Fenton* and *Golenbock* , 1998)	Lipid A part of endotoxin
Scavenger receptor subtypes (SRs) (*Rigotti* et al., 1997)	SR-A and B subtypes interact with polyanionic ligands, such as structurally modified lipoproteins (oxidised LDLs, HDLs, etc.)
Mannose Binding Lectin (MBL) (*Stahl* and *Ezekowitz* , 1998)	A member of the collectin family, calcium-dependent binding of multiple lectin domains of a wide spectrum of oligosaccharides of several bacterial species
Serum amyloid P (SAP) (*Coker* et al., 2000)	A member of the pentraxin family of proteins, calcium dependent binding to FcG receptors like an opsonin
C-reactive protein (CRP) (*Fenton* and *Golenbock*, 1998; *Gregory* , 2000)	A member of the pentraxin family reacting with Phosphorylcholine domains in bacterial cell walls
Phosphatidyl Serine Receptor (PS-R) (*Gregory* , 2000)	Expressed as scavenger Receptor on Macrophages for non-phlogistic removal of apoptotic cells, cytokine inducible
Thrombospondin (TSP) (*Gregory* , 2000)	A matrix component enhanced on apoptotic cells, ligands reported to be CD36 or the Vitronectin receptor

structures produced only by microorganisms and not by multi-cellular hosts. Consequently, these structures are referred to as pathogen-associated molecular patterns (PAMPs) and comprise cell wall components of Gram-negative as well as Gram-positive bacteria as well. Their recognition by immune cells is followed by the induction of a more or less intense inflammatory response enabling the host to respond as fast as possible to the invading pathogens.

The concept of PAMPs, introduced by *Medzhitov* et al. (1997), was proposed to be mostly an element of the „primitive“ immune system. PAMPs can define ligands in the bacterial cell wall common for both Gram-positive and Gram-negative bacteria (such as peptidoglycans or lipoproteins/lipopeptides) or different ones, such as lipoteichoic acids (LTAs) or lipopolysaccharide (LPS). Of note, it turned out in the past years, that recognition of PAMPs operates quite efficiently in higher vertebrates as well. The different PAMPs together with their respective receptors (pattern recognition receptors, PRRs) compose therefore powerful recognition entities aimed to interact with manifold chemical entities highly conserved in microbes. Among PRRs are the serum amyloid P (SAP) (*Coker* et al., 2000), the newly described phosphatidylserine receptor (PS-R) (*Gregory*, 2000), the family of scavenger receptors (SRs) able to interact with modified lipoproteins (SR subtypes A and B) (*Rigotti*, 1997; *Zingg* et al., 2000; *Williams* et al., 1999; *Platt* and *Gordon*, 1998), the mannose binding lectin (MBL) (*Stahl* and *Ezekowitz*,

1998; *Turner*, 1998), the selectins (*Malhotra* and *Bird*, 1997), the C-reactive protein (CRP) and last not least the CD14 antigen (*Fenton* and *Golenbock*, 1998), as is summarised in Table 1.

The latter glycoprotein is indispensable for the recognition of the Lipid A complex within the complex of lipid-binding-protein/LPS, together with a recently described new family of PRPs, the so called Toll-like receptor family (TLRs). Notably a new function of CD14 was recently described as a scavenger receptor for the clearance of apoptotic cells (*Gregory*, 2000). In particular, this newly described function of mCD14 could be of utmost importance for the organism to minimise or prevent overshooting inflammation associated with the removal of apoptotic and injured cells implicating that the receptor/ligand recognition process must generate signals for downregulation of inflammatory mediators. The report by *Gregory* et al. (2000) is therefore worthwhile to mention because the previously described functions of CD14 as a main LPS sensor generally were associated with „danger" for the immune system but turned out now to be involved in the limitation of inflammatory processes.

TOLL LIKE PROTEINS: RECEPTORS TO DISCRIMINATE BETWEEN GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA

The first evidence for the involvement of Toll proteins in signalling against antibacterial defence came from analyses of *Drosophila* mutants, carrying loss-of-functions mutations in the various components of the Toll-pathway (*Lemaitre* et al., 1995; *Belvin* and *Anderson*, 1996). These mutants were unable to recognise fungal infections and produced among several other antibacterial peptides drosomycin, a major antifungal peptide. In *Drosophila*, the prototypic gene named Toll encodes a plasma membrane receptor, known to be involved in dorsal-ventral polarisation of the embryo. On the basis of using expressed sequence tags with homology to Toll, several members of the human Toll superfamily were recently cloned during the past years from human cDNA libraries including TLR4, originally designed human Toll (hToll) (*Medzhitov* et al., 1997; *Medzhitov*, 2000). Today's knowledge on members of the human Toll like receptor family and their putative ligands, the PAMPs of different bacterial species, are summarised in Figure 1.

Accordingly about 10 members of the TLR family are known at present. They represent type 1 transmembrane proteins, characterised extracellularily by so-called leucine rich repeats (LRRs) of different length. According to Figure 1, TLRs recognise PAMPs that often represent molecular signatures of a particular pathogen class: LPS is the main signature of Gram-negative bacterial cell walls and a huge amount of experimental data, obtained from gene knockout mice for different Toll proteins and transfection experiments with Toll receptors into cell lines, summarised by *Janeway* and *Medzhitov* (1999), *Beutler* (2000), *Anderson* (2000), *Heldwein* et al. (2001), *Brightbill* et al. (1999), and *Akira* et al. (2001), point to the prominent role of TLR4 as an important mammalian LPS sensor and receptor.

For example, a knockout mutation of TLR2 in mice has no effect on LPS signal transduction, as these animals were as competent in their response to LPS as their wildtype littermates. By contrast, a knockout in the TLR4 gene produced a phenotype completely unresponsive to

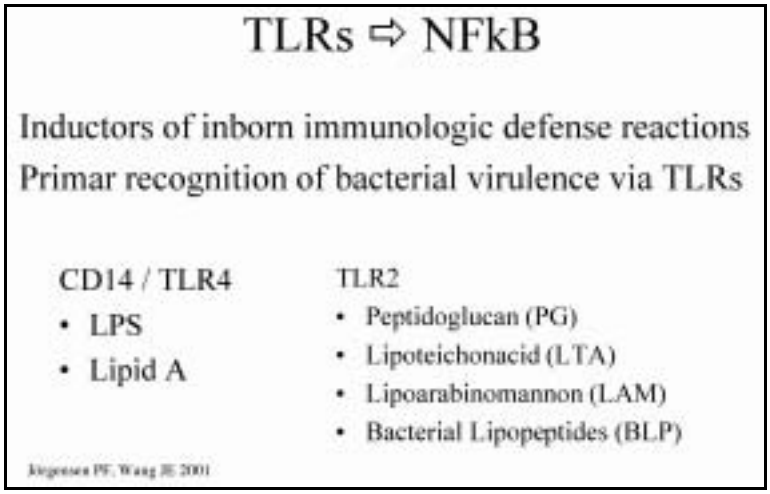

Figure 1: Subtypes of Toll-like receptors involved in the recognition of Gram-negative and Gram-positive bacterial cell walls

LPS stimulation. Furthermore, TLR4 mutations are also associated with hyporesponsiveness in humans. Most importantly, overexpression of TLR4 in embryonic human 293 kidney cells did not automatically conferred responsiveness to LPS suggesting that other additional molecule(s) are required for LPS signalling through TLR4. This molecule was subsequently identified as the secreted protein MD-2 (*Viriyakosol* et al., 2001). Transfection studies revealed that neither MD-2 nor TLR4 alone were able to confer responsiveness to LPS, but co-transfection did. MD-2 is physically associated with the extracellular domain of TLR4 on the cell surface (*Viriyakosol* et al., 2001).

According to Figure 1, the TLR2 recognises *lipoproteins* and *glycolipids* (reviewed by *Akira* et al., 2001; *Anderson* , 2000; and *Heldwein* et al., 2001), so that TLR2 is associated with the recognition of both Gram-negative and Gram-positive bacterial species. Moreover, other ligands included yeast cell walls, mycobacterial-derived lipoarabinomannans, whole mycobacteria, whole Gram-positive bacteria and Peptidoglycan. The TLR5 member was described to interact with flagellin, a 55 kD protein monomer obtained from bacterial flagellae. Flagellin is like the other bacterial cell wall components also a potent inducer of the inflammatory response accompanied by phagocytosis of bacteria, as it rapidly activates the NF-kappaB pathway (*Frendéus* et al., 2001; *Hedlund* et al., 2001) although it seemed to be partially independent of CD14. And last not least the TLR9 has been described to interact mainly with bacterial DNA, containing unmethylated CpG oligonucleotides (*Wagner*, 2001). Recently published data deal with CpG oligonucleotides as one of the most potent inducers of B-cell proliferation or dendritic cell maturation and activation, favouring the use of CpGs as potent adjuvans for vaccines. As TLR9 knockout mice were completely unresponsive to CpGs it seems likely that the TLR9 is essential in the signalling cascade of CpGs.

CD14 IN RECOGNITION OF PAMPs BY IMMUNOCOMPETENT CELLS INDUCED INHIBITORY AS WELL AS STIMULATORY SIGNALS

There is no doubt, that the major constituent of the outer membrane of Gram-negative bacteria plays a crucial role in mediating host response to Gram-negative bacterial infections by stimulating the release of inflammatory mediators, including cytokines from various target cells, such as endothelial cells, macrophages or polymorphonuclear cells. Beside that LPS activation of these mediators is thought to be responsible for the clinical manifestations of septic shock, these pathophysiological events may also play a role in chronic inflammatory disorders. Three cloned families of molecules on the surface of leukocytes are known to bind the Lipid A moiety responsible for the endotoxic activities of the LPS molecule.

These include the CD14, the macrophage scavenger receptors (SR-A family) and the β_2 or CD11/CD18 leukocyte integrins (*Fenton* and *Golenbock*, 1998). LPS binding to CD14 on the surface of immunocompetent cells is enhanced by serum factors, including the acute phase proteins and the LPS binding protein (LBP). LBP is known to catalyse the transfer of LPS monomers within the LPS/LBP-complex to the soluble or the membrane-bound form of CD14 (sCD14, mCD14). This serves to increase the sensitivity of cells towards LPS. These interactions of LBP with the Lipid A moiety of the LPS complex, intensely mediate and control bio-availability and the transport of LPS from biological fluids to responsive cell types and vice versa, opening sometimes under critical ill conditions, the life saving possibility for the organism to control neutralisation and stimulatory properties of this important molecule as best as possible. Details of these molecular interactions have been described extensively elsewhere (*Landmann* et al., 2000; *Malhotra* and *Bird*, 1997; *Mathison* et al., 1992; *Su* et al., 1995; *Tapping* et al., 1998; *Tobias* et al., 1999).

However, since the discovery of CD14 as a central LPS-receptor on mammalian cell types, a lot of subsequent work revealed two additional very remarkable unexpected findings: the first is that CD14 could interact not only with LPS of Gram-negative bacteria but with cell wall components of Gram-positive bacteria too, such as LTAs, lipoproteins, lipo-arabinomannans from *mycobacterium tuberculosis*, manuronic acid polymers from *Pseudomonas species*, soluble peptidoglycans from *S. aureus*, rhamnose-glucose polymers from *Streptococcus mutants* and insoluble cell wall components from several Gram-positive bacterial species (*Gupta* et al., 1996; 1999; *Heumann* et al., 1994).

These experimental findings open the possibility for the immune system to respond either in a more refined, but on the other hand, also in a broader manner to Gram-positive and Gram-negative bacteria as well. In the case of mixed bacterial infections, several different pathogens may invade the organism at the same time and the immune system has to decide how to handle them in the best way to avoid harm to the host. Nevertheless, signalling pathways induced by these molecular interactions may not be necessarily uniform but may include divergent as well as convergent signal transduction pathways, depending not only on the cell type, but also on the activation state of the cell in their natural environment.

In view of the particular composition of Pro-Symbioflor®, representing a

mixture of heat-inactivated Gram-positive and Gram-negative bacteria, together with their soluble and particulate cell wall components, synergistic actions of muramyldipeptides, Lipoteichoic acids (LTAs) together with LPS were shown by several publications, highlighting the dose-dependent effects of these bacterial stimulants concerning cytokine synthesis (*Rabehi* et al., 2001; *Liu* et al., 2001; *Sellati* et al., 1998; *Cauwels* et al., 1997; *Cleveland* et al., 1996; *Yang* et al., 2001).

In this context, the expression of certain cytokine genes, such as Interleukin-12 was reported to be synergistically activated by sequentially acting bacterial stimulants, for example LTAs and LPS, whereas the expression of other cytokine genes remained unaffected (*Cleveland* et al., 1996). Of note, the recent report by *Sugawara* et al. (1999) implicated clearly, that structural different LTAs from different bacterial species possess the capability to deliver to immune cells stimulating (agonistic) as well as inhibitory (antagonistic) signals, which may be of great importance, considering explanations for the immunomodulatory properties of such probiotic bacterial preparations composed of mixed bacterial strains such as Pro-Symbioflor® (described below).

The authors found, that depending on the level of expression of the CD14 antigen of different cell types (human monocytes, human gingival fibroblasts), LTAs were able to antagonise the effects of purified LPS and synthetic Lipid A, particularly, when high concentrations of LTAs were used to elicit cytokine induction (*Sugawara* et al., 1999).

The relevance of lipoproteins, being constituents of both Gram-negative and Gram-positive bacteria as important cytokine modulating PAMPs, was stressed by the report of *Giambartolomei* et al. (1999), showing the induction of pro- as well as anti-inflammatory cytokines (IL-6, IL-10) at the same time by the same pathogenic agent. The spirochete *Borrelia burgdorferi*, whose cell wall definitively lacks lipopolysaccharide but nevertheless proved to be a very strong immunomodulating agent in the cell wall of the heat-killed pathogen. Of utmost importance was the finding by the authors, that it was not the protein moiety itself found to play a role in cytokine induction, but the acylation of the peptide in form of tripalmitoyl-cysteine residues.

Of note, unlipidated outer surface proteins of *Borrelia burgdorferi* were unable to induce cytokines as IL-10 or IL-6 from human monocytes. It is reasonable to assume from these interesting experimental findings, that although LPS constitutes a biologically highly important immunomodulatory bacterial cell wall component, the acylation pattern of lipoproteins in Gram-negative and Gram-positive bacteria should be considered as strong immunomodulatory bacterial components too, which could promote the release by monocytes of a very differential cytokine profile.

Yet, considering the sometimes contrasting results with different bacterial species with regard to the activation of immune cells, it could be assumed, that these were highly influenced by the test system used, the leukocyte population under investigation, the sensitivity of immunocompetent cells, reflected by the density of receptors expressed, which correlates with the maturation state, the presence of serum in the culture and finally, the physicochemical composition of the bacterial cell wall-derived stimulants. All is being equally important for the net outcome of an immunological response. To underline these interesting findings in the literature, concerning the new important immunoregulatory role of the CD14 molecule as an important PAMP for interac-

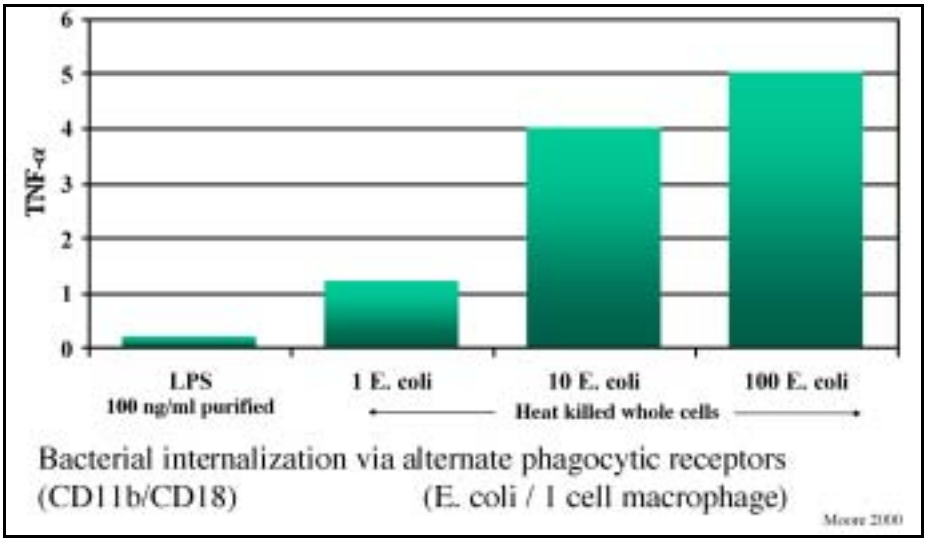

Figure 2: Stimulatory capacity of whole bacterial cells in CD14 knock out mice. It can be seen, that despite the failure of CD14 expression, whole bacterial cells of *E. coli* were able to induce TNF-α production in mouse macrophages derived from these animals, whereas even high concentrations of purified LPS remained ineffective, demonstrating that additional recognition structure in CD14 knock out animals operate together to sense bacteria.

tion with Gram-positive as well as Gram-negative bacteria, the Figures 2, 3, and 4 summarised some of the above mentioned data.

In Figure 2, the response of macrophages from CD14 knockout mice are shown with respect of the recognition of whole cells of *E. coli* compared to purified LPS (*Moore* et al., 2000). It can be seen that purified LPS were unable to induce TNF-α, chosen as a read out. However, despite the absence of CD14 on the cell membrane, murine macrophages produced dose-dependently remarkable amounts of TNF-α upon interaction with heat killed whole *E. coli* cells. This may be a clear hint for other molecules than CD14, to be involved in the recognition of whole bacteria, in comparison to purified LPS. The authors have suggested that the β_2 integrin CD11b/CD18 might have compensated for the loss of function of CD14. Moreover it is worthwhile to mention, that CD11/CD18 integrins do not have such a high affinity than the CD14 molecule for LPS in its monomeric form. Instead β_2 integrins preferentially were reported to interact with larger aggregates, including whole bacteria. The participation of β_2 Integrins in LPS recognition by CD14 knockout animals, was confirmed by the authors (*Moore* et al., 2000). Using inhibition experiments with neutrophil inhibitory factor, which blocked the integrin receptor, thereby diminishing the recognition of whole bacteria. About half of this CD14-independent response could be inhibited by integrin blockade abrogating TNF-α production.

In Figure 3, the influence of different bacterial cell wall components on the level of expression of the CD14 molecule was shown. Interaction of human monocytes with purified bacterial cell wall components derived from Gram-positive bacteria resulted in a marked upregulation of CD14 expression. In contrast, stimulation by LPS of human monocytes downregulate CD14, underlining the complex role of the CD14 an-

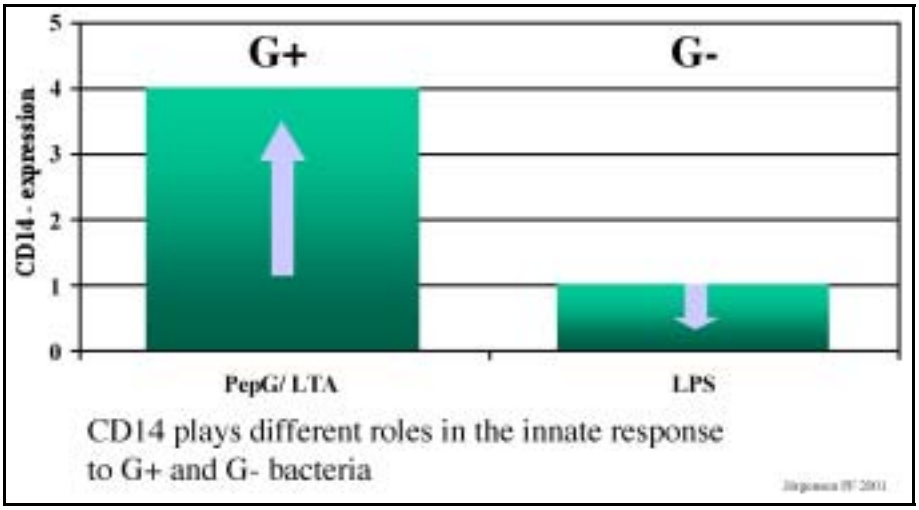

Figure 3: The important immunomodulatory roles of Gram-positive bacterial cell walls in comparison to Gram-negative bacterial cell walls in view of the level of CD14 expression monocytes It can be seen, that upon interaction of human monocytes with purified bacterial cell wall components derived from Gram-positive bacteria CD14 expression was highly increased. In contrast, stimulation by LPS of human monocytes down-regulate CD14, indicating the complex role of the PAMP CD14 in the recognition of different bacterial species.

tigen as an important PAMP operating in recognition of different bacterial species which could result in the generation of different signalling pathways. In Figure 4, the above mentioned synergistic effects of some distinct bacterial cell wall components on cytokine induction were summarised according to *Jörgensen* et al. (2001).

The importance of the experimental design for investigating bacterial immune cell interactions was outlined by *Cauwels* et al. (1997), reporting a dose-dependent cytokine production *ex vivo* with heparinised blood by LPS from *E. coli*, heat-inactivated whole pneumococci or purified cell walls which were all potent inducers of TNF-α, IL-1 and IL-6. The authors could further demonstrate in this system, that the whole blood assay is 1,000 fold more sensitive than the use of a human monocytic cell line THP-1 cells as it responded to as little as 1 ng LPS.

The cytokine response to 5 ng LPS corresponded according to *Cauwels* et al. (1997) to $5x10^5$ *E. coli* and proved to be similar to 1 µg cell wall (= 10^7) bacteria and 10^5 intact heat killed bacteria.

This means that isolated LPS was about 200 times more potent than the purified cell wall of pneumococci for induction of comparable levels of TNF-α, IL-1ß or IL-6, but roughly equipotent to whole pneumococci.

These experimental data underline not only the importance of the chemophysical composition of the bacterial cell wall components to interact with immune cells, but also the use of an appropriate sensitive test system to evaluate the binding specifities of different bacterial cell wall components from Gram-negative and Gram-positive bacteria. Moreover the authors suggested from their results the co-existence of CD14 dependent and CD14 independent stimulation pathways particularly by Gram-positive bacteria.

In addition, anti-CD14 antibodies could inhibit the response of whole

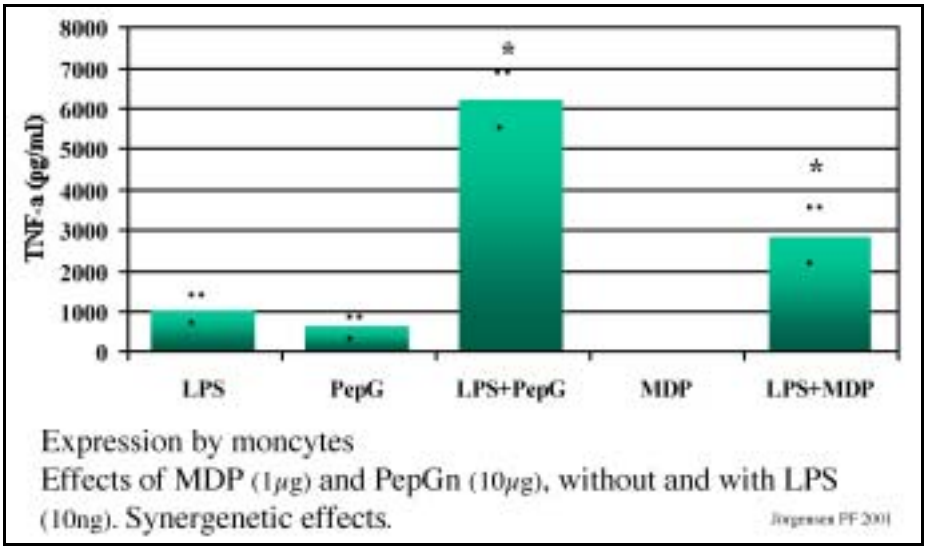

Figure 4: Synergistic effects of different bacterial cell wall compounds of either Gram-positive or Gram-negative bacteria on cytokine production (according to *Jörgensen* et al., 2001). Whereas purified MDP or peptidoglycans in the whole blood assay were only weakly effective for TNF-α production (shown in pg/ml), the simultaneous combination of both stimuli dramatically enhanced TNF-α production 3-fold to 6-fold.

blood cells to purified LPS, but not to stimulation with whole bacterial cells, which could not be abrogated by anti-CD14, indicating, that different mechanisms operating in whole blood for the recognition of whole bacteria compared to isolated cell wall components. This is shown in Figure 5.

DO DIFFERENCES EXIST BETWEEN LIVE VERSUS DEAD BACTERIA WITH REGARD TO THE CYTOKINE PROFILE THEY INDUCED?

The next question to be addressed in the context of the probiotic bacterial preparations manufactured by the SymbioPharm Herborn is to clarify, whether there will be differences between dead and live bacteria in terms of cytokine production.

The situation turned out to be equally complex as with the different PAMPs of Gram-negative and Gram-positive bacteria. Several conflicting reports support the conclusion, that, with regard to active immunisation procedures, living bacteria may be more potent than dead ones (*Cooper* et al., 1997; *Sher* et al., 1991; *Chambers* et al., 1997; *Zhan* and *Cheers*, 1998; *Cheers* and *Zhan*, 1996; *Sander* et al., 1995).

For example, *Cambers* et al. (1997) reported that protection of mice with virulent *Mycobacterium tuberculosis* was strongly associated with the inoculation of live but not dead BCG. Although live and dead bacteria induced comparable cellular responses during the first week after vaccination determined by immunohistochemical analyses of the draining lymph nodes, the typical migration of live parasites into local lymph nodes, which resulted in subsequent recruitment on mononuclear cells, was only seen with live bacteria.

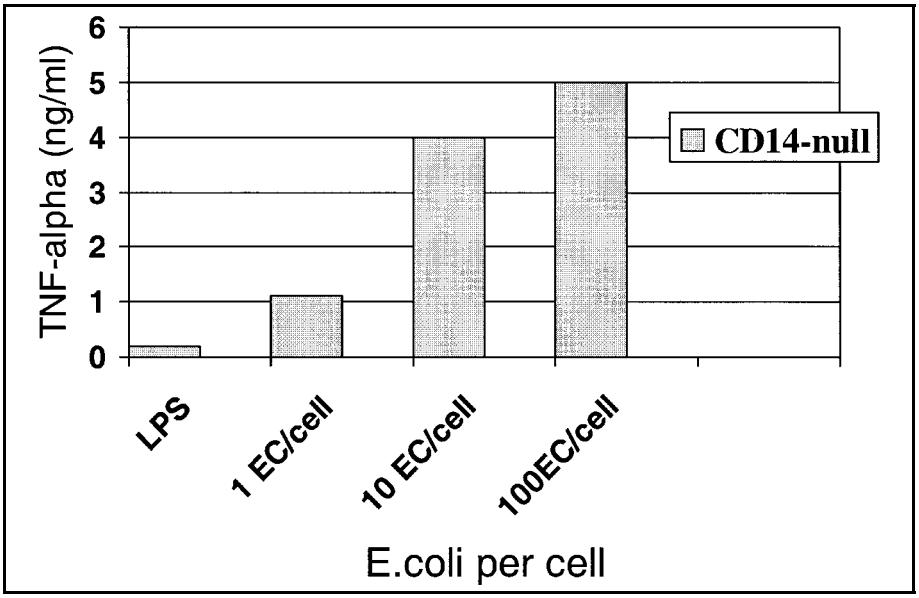

Figure 5: Different effects of anti-CD14 antibody aimed to inhibit signal transduction by bacterial cell wall components dependent of the test system and the physico-chemical composition of the bacterial components used. Whereas anti-CD14 inhibited the response of whole human blood leukocytes against purified LPS, no inhibition was observed with whole pneumococcal cell walls.

It is generally assumed, that the elimination of intracellular bacteria like mycobacteria, brucellae, listeriae or some salmonellae species required a strong Th1 biased immune response associated with a prominent release of IL-12 and interferon-gamma *Cheers* et al. (1996) as killed vaccines tend to be ineffective because they induced a strong Th2 driven response. A protective immune response, elicited by vaccination seemed to be strictly dependent on the generation of interleukin-12, which is a very important cytokine released after vaccination with attenuated but not dead BCG. However under in vitro conditions, IL-12 was reported to be released by live and dead bacteria (*Cheers* et al., 1996) so that the situation in view of IL-12 remains complex, because dead bacteria or their cell wall products could be either effective (*Mahon* et al., 1996) or not (*Zhan* and *Cheers*, 1995; 1998).

Further, not only species differences can play a prominent role in these observations, but also the structure of the bacterial cells too (*Mahon* et al., 1996; *Sander* et al., 1995): Using a murine respiratory infection model, the authors demonstrated, that infection with *Bordetella pertussis* (Gram-negative rod) or immunisation with a whole cell pertussis vaccine (e.g. the intact cell membrane with the natural configuration of lipopeptides, LPS and proteins) induced a strong antigen-specific Th1 dominated response. In contrast, immunisation with an a-cellular vaccine consisting of *B. pertussis* components or filamentous haemagglutinins generated a Th2 response, which was associated with delayed bacterial clearance from the lungs of the animals. However, addition of Interleukin-12 to the a-celluar vaccine strongly increased the Th1 response.

This may be a good example, how vaccine structure could influences the

Table 2: Characterisation of binding specifities of the AutoColiVaccine (ACV) compared to a commercial LPS preparation, a synthetic lipopeptide (PAM_3CSK_4), and a natural LPS (*S. friedenau*) using CHO-cells as test system

	CHO wildtype		CHO/CD14		CHO/CD14/TLR2	
TLR4	Natively expressed		Natively expressed		Natively expressed	
MD-2	Natively expressed		Natively expressed		Natively expressed	
CD14	-		Rec. Expressed		Rec. Expressed	
TLR2	-		-		Rec. Expressed	
Synthetic lipopeptide (PAM_3CSK_4)	-		-		>100 ng	
IL-1β control	↑		↑		↑	
AutoColiVaccine	-	-	+	>100 ng	+	>1000 ng
S. friedenau LPS	+	>100 ng	+	>1 ng	+	>10 ng
Commercial LPS (055:B5, *E. coli*)	+	>10 ng	+	>1 ng	+	>10 ng

Due to the failure to express TLR2, wild type CHO cells did not react with synthetic lipoproteins and only to high concentrations of LPS. When CD14 was additionally expressed, cells became responsive to the ACV at high concentration and, as could be expected the sensitivity to LPS increased due to CD14. Notably, CHO cell sensitivity to the ACV seemed to be increased in CD14 positive cells but this did not change after TLR2 expression. The result, that commercial LPS showed a rise in sensitivity after TLR2 expression could indicate for the presence of lipoproteins in the LPS, as the activity of *S. friedenau* LPS was comparable with that of commercial LPS regarding the activation of the CHO cells. Thus, the failure of the ACV to activate wild Type CHO cells might indicate a partially independent TLR4/MD-2 activation pathway triggered by the ACV.

outcome of an immune response. In the human system *Sander* et al. (1995) evaluated cytokine production of human leukocytes at the single cell level after stimulation with live attenuated *Mycobacteria bovis* BCG. It was shown, that although major cytokines like IL-12 and Interferon-γ were sequentially produced, later on a Th2 polarised lymphocyte response occurred, reflected by the appearance of IL-4, IL-5 and IL-10 intracellularly. This strongly indicates that in humans a mixed T-helper cell profile operating together for the successful elimination of *M. bovis*.

RECOGNITION OF A *E. COLI* PREPARATION IS PARTIALLY INDEPENDENT FROM THE CLASSICAL LPS RECEPTOR IN MAMMALS TLR-4/MD-2

Another important bacterial derived immunomodulator manufactured at Herborn is the AutoColiVaccine (ACV). This bacterial preparation derived from the stool flora of patients, resembled in its chemical composition in many parts the structure of Lipid A, but has different acylation patterns and a low endotoxic activity. Nevertheless it can be assumed, that commercially obtained LPS compared to the ACV will use possibly known receptors involved in the LPS-signalling pathway, such as the TLR-4-MD2/CD14 complex.

Together with an experimental group at Borstel it was investigated, which type of the known classical LPS receptors might be recognised by the ACV

Table 3: Characterisation of binding specifities of the AutoColiVaccine (ACV) compared to a commercial LPS preparation, a synthetic lipopeptide (PAM_3CSK_4) and a natural LPS (*S. friedenau*) using HEK293-fibroblasts as test system

	HEK293 wildtype		HEK293/TLR2		HEK 293/ TLR4/MD2		HEK 293/ CD14/TLR4/MD-2	
TLR4	-		-		Rec. Expressed		Rec. Expressed	
MD-2	-		-		Rec. Expressed		Rec. Expressed	
CD14	-		-		-		Rec. Expressed	
TLR2	-		Rec. Expressed		-		-	
Synthetic lipopeptide (PAM_3CSK_4)	-		>1000 ng		-		-	
TNF-_ control	↑		↑		↑		↑	
AutoColiVaccine	+	>1000 ng	+	>100 ng	+	>1000 ng	+	>10 ng
S. friedenau LPS	-	-	-	-	+	>100 ng	+	0,1 ng
Commercial LPS (055:B5, *E. coli*)	-	-	+	>100 ng	+	>10 ng	+	0,1 ng

Despite wild type HEK 293 fibroblasts did not express any of the classical LPS recognition structures, the ACV was able to stimulate the cells for increased IL-8 production. Most importantly, the expression of TLR2 by HEK 293 cells increased the sensitivity from 1000 to 100 ng and this was further enhanced after expression of CD14 (1000 to 10 ng). It is therefore reasonable to assume that the Lipid A analogue ACV contains additional molecules with specificity for TLR2 and CD14 but with a lower binding specificity for TLR4/MD2 compared to commercial LPS. This assumption may be also confirmed by the observation that HEK 293 wild type cells and HEK 293 TLR4/MD2 cells showed nearly the same reactivity against ACV with 1000 ng being necessary for cellular activation.

using binding studies with transfected cell lines. The latter represent a useful molecular tool to solve this question, because they harbour the cloned human TLR proteins together with their necessary adapter protein MD-2. Kirschning et al. (1998) and Yang et al. (1998) recently described the test system. The authors used the transfection of the human 293 embryonic kidney fibroblast cell line (HEK 293), with cloned human TLR-proteins and their necessary co-factors. As a parameter of HEK 293 activation upon challenge with the bacterial preparations, the release of IL-8 was determined after stimulation either with a commercial LPS preparation (*E. coli* 055:B5), a natural LPS from *S. friedenau* or the Lipid A analogue named ACV.

A second test system included Chinese hamster ovary cells (CHO-cells) co-transfected with a reporter gene to investigate the activation potential of LPS in comparison with ACV with respect to the expression of the CD25 surface marker. In this CHO cell line, the expression of CD25 was induced by a minimal structure of the NF-kB promotor from the selectin gene as described previously (*Yang* et al., 1998). The Tables 2 and 3 summarised the characteristics of the cell lines used together with the results.

The results from Tables 2 and 3 revealed some unexpected findings, which support evidence, that the receptors involved in the recognition of the ACV preparation seem to be partially independent from the classical TLR-4/MD-2 receptor system for the following reasons: In wild type (WT) CHO-cells containing the full repertoire of TLR-4/MD-2 the AutoColiVaccine

did not induce a marked CD25 expression compared to commercially obtained LPS.

As WT CHO cells did not express the TLRs, they failed to react with the synthetic lipopeptide used as a control (PAM_3CSK_4), but were fully reactive to TNF-α. Additionally, despite HEK 293 WT cells were devoid of all classical LPS receptors, they responded to high concentrations of the ACV whereas the positive control LPS could not activate HEK 293 fibroblasts. And what may be of utmost importance is, that the introduction of the TLR-2 in CHO cells rendered them more responsive to the ACV.

The same was seen with the HEK 293 fibroblasts: Whereas WT HEK 293 cells could react to high concentrations of the ACV, but not to natural *S. friedenau* LPS or commercial LPS, the co-transfection of HEK 293 cells with TLR2 and CD14 seem to increase the sensitivity of the cells to ACV.

It may be concluded, that the ACV contained a compound with TLR2 specificity and these results do fit very well the above cited publications of *Giambartolomei* et al. (1999) and *Kreutz* et al. (1997) demonstrating the importance of the lipoproteins/lipopeptides in cytokine induction with the pattern of acylation of a synthetic PAM_3CSK_4 being more relevant for the capacity of a lipopeptide to modify cytokine synthesis.

Accordingly, the results by *Bainbridge* et al. (2001) implicated that although LPS (and ACV as an *E. coli* derived bacterial preparation) seem to be highly conserved in its core structure among different bacterial species, some subtypes of LPS even do not bind to the classical TLR4 receptor. Instead for example, the LPS from *Porphyromonas gingivalis* was reported to interact with TLR2 in stable transfected CHO cells. Furthermore *Porphyromonas gingivalis* LPS was able to submit stimulating signals to monocytes, while at the same time inhibiting endothelial cell activation by interfering with the p38MAP kinase pathway (*Bainbridge* et al., 2001).

THE PROBIOTIC PREPARATION PRO-SYMBIOFLOR®, COMPOSED OF HEAT-INACTIVATED *E. COLI* AND *ENTEROCOCCUS FAECALIS* INDUCED A CYTOKINE PROFILE CHARACTERISTIC FOR Th1 T-HELPER CELLS

The experimental results shown in Figures 6 to 9 contrasts some recent reports by *Hessle* et al., (2000) and *Haller* et al. (1999). Not only were differences between live and dead bacteria reported with respect to cytokine production, but growth-related differences and strain specific differences (*Hessle* et al., 2000) were also shown. For example *Hessle* et al. (2000) examined the synthesis of IL-10 and IL-12 by human blood monocytes after challenge with UV killed bacteria of 7 Gram-negative strains and 7 Gram-positive strains (commensals and pathogens). According to these experiments Gram-positive bacteria induced a predominant IL-12 production while Gram-negative bacteria enhanced preferentially the synthesis of IL-10. The lower capacity of Gram-negative bacteria to induce IL-12 was independent of IL-10 itself, as blocking IL-10 with antibodies did not resulted in an enhancement of IL-12 (*Haller* et al., 1999; *Hessle* et al., 2000).

In view of these findings, the series of experiments presented in Figures 6 to 9 argued more for a qualitative difference of some bacterial preparations rather than a species-origin determined

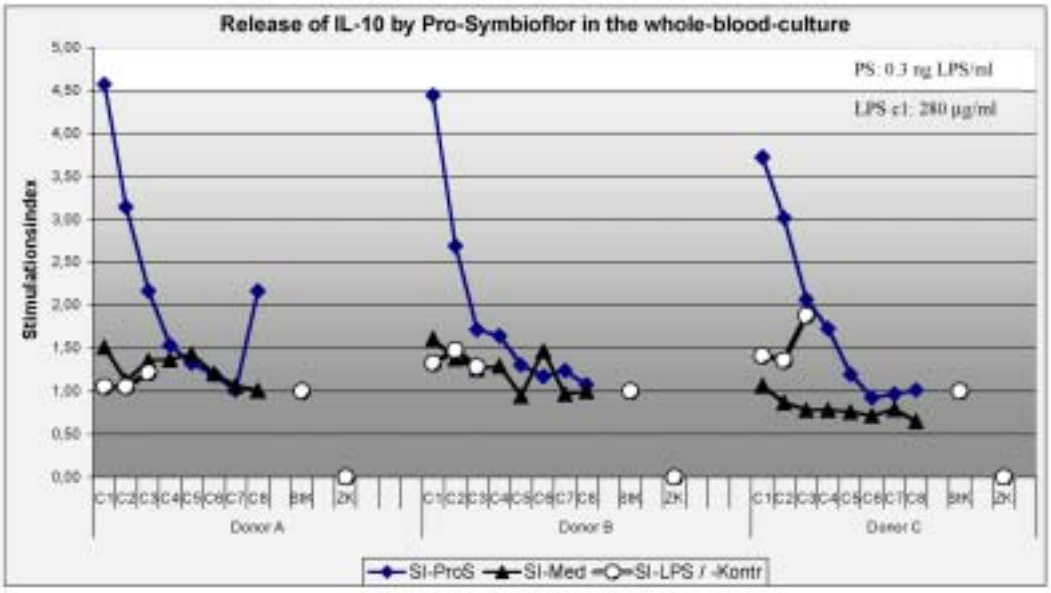

Figure 6: The effect of Pro-Symbioflor® on the LPS induced production of IL-10. Whole blood cell cultures were stimulated with sub-optimal concentration of opsonised zymosan together with eight different concentrations (C1–C8) of Pro-Symbioflor®. Medium-treated cells served to evaluate the background cytokine production and the positive control included LPS (*E. coli* 055:B5, Sigma). IL-10 was determined after 24h of incubation with ELISA. It can be seen that Pro-Symbioflor® dose-dependently stimulated the synthesis of IL-10 to a great extent, which was evident with leukocytes from all three donors tested.

difference. One probiotic preparation manufactured by SymbioPharm composed of live *Enterococcus faecalis* bacteria (Symbioflor 1®) was recently reported in a double-blind, randomised clinical study to significantly reduce number and severity of relapses in patients suffering from chronic recurrent bronchitis. An important medical disease entity thought to be associated with recurrent infections of the respiratory tract.

The latter may be a consequence of a suppressed immune system either due to inherited or to an adapted capacity of the immune system, to mount an appropriate antibacterial defence to effectively kill bacteria *Habermann* et al. (2001). To address the question, whether this clinically relevant effect could have been correlated with an immunomodulating effect of *Enterococcus faecalis* or *E. coli*, another probiotic bacterial preparation by SymbioPharm was investigated for immunomodulatory properties called Pro-Symbiofor®, using the whole blood cell culture system with blood from three healthy donors. The bacterial strains in Pro-Symbioflor® were of human origin and non-pathogenic, manufactured as a heat-inactivated preparation of cells and bacterial cell wall components with different concentrations of LPS and LTAs. The LPS content of Pro-Symbioflor® is in the range of about 0,3 ng/ml.

In these experiments, whole blood cultures were treated with eight different concentrations of Pro-Symbioflor® and sub-optimal concentrations of co-stimuli, able to activate specifically the synthesis of monokines (opsonises zymosan, IL-6, IL-10, IL-12) and lymphokines (anti CD3 plus anti-CD28, IL-4, IL-5 and IFN-γ) was used. The background control included unstimulated

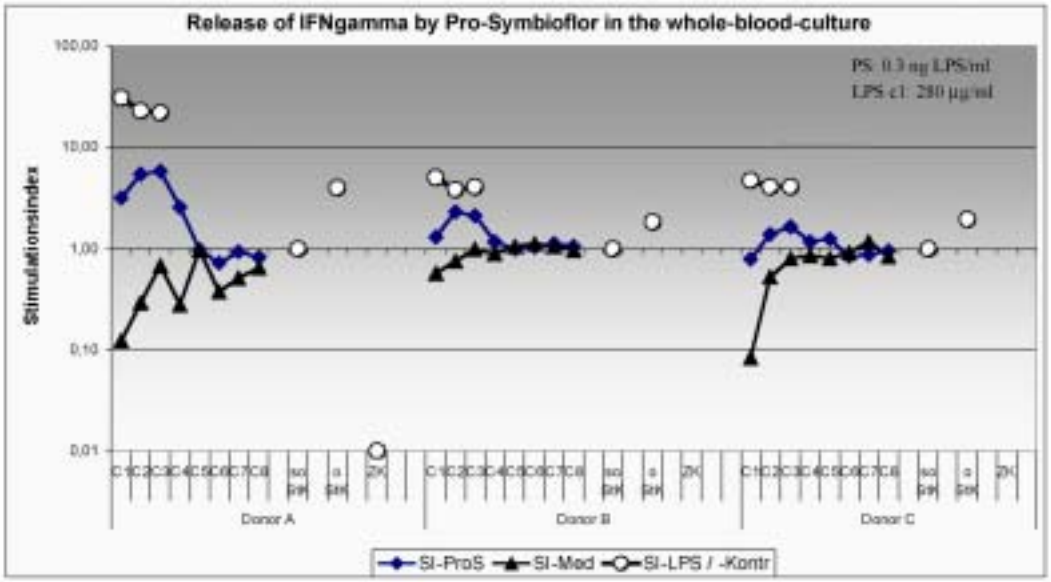

Figure 7: The effect of Pro-Symbioflor® on the LPS induced production of IFN-γ. As Co-stimulus a combination of anti-CD28 plus anti-CD3 antibodies was used. Pro-Symbioflor® dose-dependently induced great amounts of IFN-γ with leukocytes from all three donors tested.

cells (ZK), and the stimulus control included *E. coli* serotype 055:B5 obtained from Sigma. The results of these experiments were illustrated in Figures 6 to 9.

The important anti-inflammatory cytokine IL-10 was dose-dependently increased in whole blood cultures of all three donors (Figure 6). The synthesis of IL-12 (Figure 7) showed more inter-individual variations and seemed to be increased within the concentration ranges C2 to C4. With whole blood cells from two donors, IL-12 was inhibited at the highest concentration of Pro-Symbioflor®. The important pro-inflammatory and immunoregulatory cytokine Interferon-γ proved to be consistently stimulated with whole blood cultures from all three donors (Figure 8) and the cytokines IL-5 (Figure 9) and IL-4 (data not shown) were profoundly suppressed over a wide concentration range. So far, although these are first results on immunomodulatory properties of Pro-Symbioflor®, these results were so consistent that they should justify the conclusion to suggest a profound influence of Pro-Symbioflor® on the activation of Th1-helper cells.

Thus, in the context of the previously described experimental findings reported by *Hessle* et al. (2000) or *Haller* et al. (1999), these results obviously do not encounter the immunopharmacological profile of Pro-Symbioflor® being a mixed heat-killed preparation of *Enterococcus faecalis* and *E. coli*. This „medical" probiotic was able to stimulate both cytokines at a high extent.

A reasonable explanation for these apparent differences may be the use of different test systems, the former involved separated cells, where the data reported herein were generated with the whole blood cell cultures. The latter resembles the *in vivo* conditions as close as possible, because the bacteria interact with immune cells in their natural environment allowing the full spectrum of receptor-ligand interactions as close as possible to the situation *in vivo*.

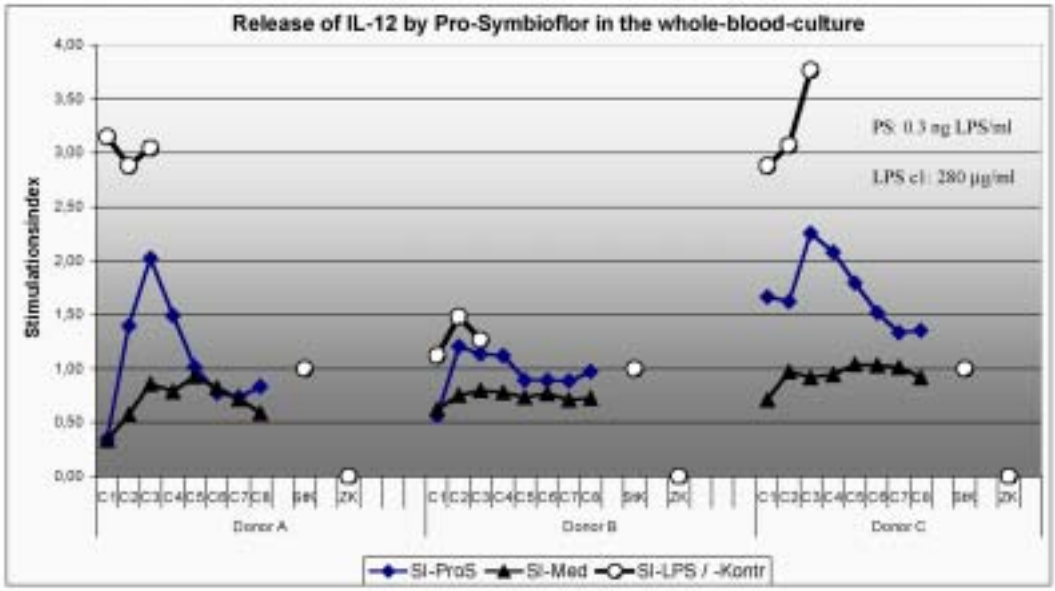

Figure 8: Pro-Symbioflor® profoundly affects the co-stimulated release of IL-12 (opsonised zymosan as co-activator, measurement after 24 h with ELISA) in whole blood cell cultures from three different donors. Although IL-12 production varied between donors in response to different concentrations of Pro-Symbioflor®, this important cytokine involved in the enhancement of cell-mediated immunity was profoundly modulated.

PRO-SYMBIOFLOR® FOR THE TREATMENT OF TYPE 1 ALLERGIC DISEASES?

Assumed, that in atopic allergy a predominant Th2 response dominate, it is tempting to speculate, that a bacterial preparation with such a remarkable immunomodulatory activity profile like Pro-Symbioflor® may be an ideal candidate for therapy of type 1 allergic diseases, characterised by an overproduction of IgE. Generally, bifidobacteria and lactobacilli as classical representatives of probiotic bacterial strains, were investigated extensively in the past in numerous experimental studies for their immunomodulatory properties and in some small controlled clinical trials for their „anti-allergic“ and anti-infective potential (reviewed by *Vaughan* et al., 1999; *Gill* and *Rutherfurs*, 2001; *German* et al., 1999; *de Roos* and *Katan*, 2000; *Gismondo* et al., 1999; *Sanders*, 2000; *McNaught* and *MacFie*, 2001; *Cross* and *Gill*, 2001; and *Cross* et al., 2001).

As an example, the list of probiotic bacteria-mediated effects include antimicrobial activity, colonisation resistance, antigen-non-specific immune system activation by cytokine induction and stimulation of phagocytosis of peripheral blood leukocytes, stimulation of secretory IgA production, anti-mutagenic effects, anti-genotoxic effects and influence on enzyme activity (*Sanders*, 2000). Unfortunately most of these health claims were until now not sufficiently supported by randomised, double-blind large controlled clinical studies.

The best documented clinical application for the use of probiotic bacteria seem to be preventing or shortening episodes of acute diarrhoea and gastro-

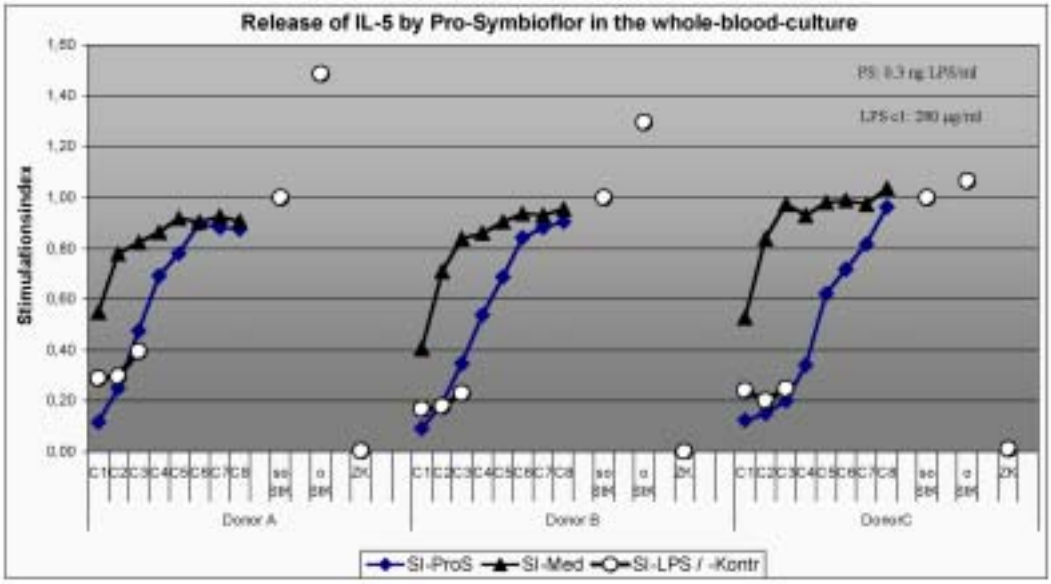

Figure 9: The effect of Pro-Symbioflor® on the co-stimulated production of IL-5. As Co-stimulus a combination of anti CD28 plus anti CD3 antibodies was used. Pro-Symbioflor® dose-dependently inhibited the release of IL-5 with leukocytes from all three donors tested over a wide concentration range. Data for IL-4 looked quite similar and were not presented here.

enteritis in infants and adults (*McNaught* et al., 2001; *Elmer* et al. 2001). Serious concerns may raise in view of the rising tendency of the food industry to genetically „design" food-related bacteria, specifically lactic acid bacteria, with new metabolic and functional properties not originally found in the parent strain (*Kuipers* et al., 2000; *Saarela* et al., 2000; *van der Werf* et al., 2001; *Dunne* et al., 2001) aimed to improve functional properties of the strains.

Presumably one reason for these extensive research activities may be the perhaps questionable assumption, that many of the claimed health promoting effects of probiotic bacteria could only be achieved with sufficient high numbers of bacteria (about 10^9 to 10^{10} per day), which should colonise the human gastrointestinal tract. Despite, that the survival of probiotic bacteria during passage through the human gut, when administered in fermented milk products, has been investigated intensely in recent years the survival rates have been estimated only to be about 20% - 40% for selected strains (*Bezkorovainy* et al., 2001).

Thus, although it is generally proposed that a probiotic bacteria mediated effect can be better achieved if the germs would adhere to the intestinal mucosa, reality has shown, that most of the bacteria passed into faeces without having attached to the mucosa or without having multiplied. Consequently, to get a continuous health effect by an exogenously introduced probiotic strain, the bacteria were recommended to be ingested by an individual continually.

However little is known, what defined molecular and functional properties an „ideal" probiotic bacterial strain must have to exert the above mentioned health effects. Simply, because under in vivo conditions the interactions of the introduced probiotic strain with the individual indigenous microflora of each

individual, might be extremely complex and influenced by several additional factors: Among them are the nutrition behaviour of an individual, the actual composition of the microbiota, external factors like stress and use of antibiotics or other pharmaceuticals. Therefore the important question arise, whether dead probiotic bacterial strains, if endowed with a suitable immunopharmacological profile, might be more safe for use in human in the context of immunomodulation, instead of ingesting large numbers of genetically modified strains, whose long term behaviour *in vivo* is presently largely unknown.

With regard to the immunopathogenesis of atopic allergy, there is compelling evidence from a vast amount of hundreds of published papers during the past 10 years, that established type 1 allergy is dominated by a preferential activation of Th2 cells, responsible for the initiation of atopic dermatitis or allergic asthma (*Rothe* and *Grant-Kels*, 1996; *Kapp*, 1995; *Vercelli*, 1995; *Masman* and *Sad*, 1996; *Romagnani*, 1997; *Leung*, 2000; 2001; *O'Garra* and *Murphy*, 1996). However, despite this intense research, the aetiology of e.g. atopic dermatitis, characterised as a chronic, highly purulent inflammatory skin disease with elevated levels of total and antigen-specific IgE and tissue eosinophilia, is largely unknown. Moreover, the cytokine profiles measured in biopsy specimens of atopic donors expressed high variability depending on the disease state (*Hamid* et al., 1994; 1996; *Thepen* et al., 1996; *Langeveld-Wildschut* et al., 2000; *de Vries* et al., 1997).

Together with other underlying factors, such as severe skin colonisation with super-antigenic exotoxin-producing *Staphylococcus aureus* (*Bunikowski* et al., 2000) or a chemokine triggered non-specific homing of specific lymphocytes into inflamed skin lesions (*Nickel* et al., 2000), contrasting results pointing to an IL-4 independent rise of IgE (*van der Pouw-Kraan* et al., 1994; *Virtanen* et al., 1995).

IL-4 is considered to be the main cytokine involved in the switch of antibody production by B-lymphocytes from IgG to IgE. All these reports strongly suggest that the immunopathogenesis of atopic eczema is not simply a mere shift towards a CD4-Th2 dominated immune response. This was recently confirmed by observations of a participation of CD8-positive T-cells in allergic responses (*Nakazawa* et al., 1997). Nevertheless, immunotherapeutic strategies used in the past in form of active desensitisation regimens using increasing doses of allergens implied, that an induction of the change in cytokine profiles which correlated with symptom improvement (*Hamid* et al., 1997; *Movérare* et al., 2000; *Secrist* et al., 1993) may be a reasonable strategy for the treatment of IgE mediated hypersensitivities.

A major breakthrough in the understanding of the etiopathogenesis of type 1 allergy which showed a dramatic rise during the past years, affecting approximately 30% of children during childhood (*Endres* et al., 2000), has come from the elucidation of the role of the gastrointestinal flora. The early manifestation of the disease soon after birth took place in a time span of about 6 months post partum, which decides whether atopic allergy will develop or not (*Bjorksten* et al., 2001).

A first clue came from the important observations of *Björksten* et al. (1999) about differing colonisations patterns of allergic Estonian and Swedish 2-year-old children. In the following years *He* et al. (2001) and *Ouwehand* et al. (1999) confirmed differences in the adhesion properties of the Gram-positive gut flora between healthy and allergic infants. Faecal *Bifidobacteriaceae* from

healthy infants expressed markedly higher adhesive properties than those of allergic infants. In addition, *Böttcher* et al. (2000) were able to demonstrate microflora-associated characteristics in faeces from allergic and non-allergic infants, by means of the measurement of the faecal concentrations of eight different short chain fatty acids.

They showed that allergic infants have lower levels of propionic, i-butyric, butyric, i-valeric and valeric acid and higher levels of i-caprioc acid. The latter short chain fatty acid has been associated with the presence of *Clostridium difficile*. Subsequently several experimental studies pointed to a possibly hopeful concept considering probiotic bacteria as a useful treatment of severe type 1 allergies such as atopic eczema (*Isolauri* et al., 1990; 2000; *Isolauri*, 1997; *Majamaa* and *Isolauri*, 1996; 1997; *Majamaa* et al., 1996; *Pessi* et al., 1998).

This intense research trend continued during in the following years and resulted in the appearance of first small clinical trials for the treatment of allergic infants by the oral application of Gram-positive probiotics (*Isolauri* et al., 2001; *Kalliomäki* et al., 2001; *Pelto* et al., 1998; *Pessi* et al., 2000; *Salminen* et al., 1995; 1996; *Simmering* and *Blaut*, 2001). In the light of these encouraging findings and the intriguing immunopathological profile of atopic diseases, it seems reasonable to assume, that a probiotic bacterial preparation such as Pro-Symbioflor® might help to improve symptoms associated with atopic eczema.

This the more, as Pro-Symbioflor® acts not by blocking only a distinct cytokine but instead activates those cytokine peptides responsible for the polarisation of T-helper lymphocytes into the Th1 direction while down-regulating those responsible for Th2 lymphocyte activation. This represents a real immunomodulatory capacity of Pro-Symbioflor® raising the question of the therapeutic value of such a „medical" probiotic for the treatment of type 1 allergy which will be the subject of further intense research in the field of probiotics in form of clinical trials.

To summarise the immunomodulatory properties of probiotic bacteria such as *E. coli* and *Enterococcus faecalis* and a mixture of them turned out to be highly complex, which may be the result of the initiation of different activatory and inhibitory pathways in immune cells. The outcome of such an interaction may not only be determined by the pathogenic class e.g. Gram-positive or Gram-negative bacteria, but much more by the sequential and spatial interactions of the PAMPs with their respective ligands, which is further modulated by the environmental cytokine milieu and the activation state of a given target cell population in a tissue.

These molecular interactions may induce, among other at present unknown effects of probiotics under in vivo situations, the elaboration of different cytokine profiles subsequently contributing to prominent changes in the activation of the innate as well as the adaptive immune system.

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CHARACTERISATION OF NANOSTRUCTURES OF AUTOVACCINES OBTAINED FROM *E. COLI*

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SUMMARY

Dilute aqueous dispersions of autovaccines have been characterised by transmission electron microscopy, scanning electron microscopy, static and dynamic light scattering and conductivity measurements for volume fractions between $1.0 \times 10^{-4} \leq \emptyset \leq 3.5 \times 10^{-4}$. The static structure factor of the autovaccines molecules depends on the number density of the particles, on temperature, salt concentration, and the nature of counterions, e.g. Ca^{2+}, Mg^{2+}, and 50 -100 mM NaCl. We found that the variation of the particle number density of Lipid A significantly influences the height of the first peak of S(Q) revealing, that the experimentally determined structure factor at low particle concentration shows considerably more structure than expected. The magnitude of the structure factor S(Q) depends strongly on Ca^{2+} ions, rather less on Mg^{2+} ions, but also on the order of the addition of the anions to the autovaccine suspension. We observed small changes in the radii of gyration of the autovaccine suspensions in the presence of Ca^{2+} (11.3 nm) or Mg^{2+} (12.6 nm). The experimental structure factors S(Q) for the various particle densities were analysed in terms of an effective particle charge Z^* and a corresponding screening parameter k^* which were obtained from the Poisson-Boltzmann-Cell (PBC) model. The light scattering data are also in accord with a model, that a small fraction of the ionisable surface sites of the autovaccine which contains phosphate groups, are only partly dissociated (~15%) according to titration and conductivity measurements. It can be concluded that the light scattering data are well represented by a PBC-model of ordering of autovaccines at low volume fractions where colloidal crystals are absent. It was discovered that a given amount of Ca^{2+} (1.0 – 5.0 nM) influences the structure much more than does Na^+ at a considerably higher concentration (0.010 M). This behaviour may explain an ion-exchange mechanism that takes place with Ca^{2+} but at a much lower concentration than for Na^+. Furthermore, the addition of Ca^{2+} at 1.0 – 5.0 nM in the presence of 0.01 M NaCl to the colloidal autovaccine assembly resulted in only small exchange of Na^+ from the sites previously occupied by Ca^{2+}. Comparing the different structure factors S(Q) for Autovaccine-Na^+ and -Ca^{2+}, it

can be concluded that the structure factor does not depend simply on the ionic strength of the solution. The liquid-like interactions at moderate ionic strength, in the presence of Na^+ or Ca^{2+} can be treated as a fluid or polymer electrolyte with a certain degree of ordering in solution, which accounts for S(Q) and the secondary minimum at sufficiently large separation.

Dilute aqueous solutions of autovaccines ($3.5 \times 10^{-3} < Ø < 1.2 \times 10^{-2}$) form stable and regular shaped, colloidal crystals of sizes between 1 to 2 μm. The morphological shapes of the obtained crystals are cubic or rectangular, and by increasing the particle number density of Lipid A above 1.5×10^{-2} they cluster into ordered, long ropes of diameter of 2.8 nm separated by a rope of 4.6 nm having only short range order. The individual cubic crystals are very thin of about 30 nm, and fragile to exposure to the electron beam because of melting. From electron diffraction measurements, various diffraction pattern were obtained, which could be referred to a cubic lattice with a lattice constant of a = 36.14 ± 0.2 nm. In the diffraction patterns obtained from the crystalline specimens of the autovaccines the {110} (d_{110} = 25.50 ± 0.5 nm) reflection is visible and strong, and the diffraction pattern can successfully indexed in a body centred cubic lattice (bcc), with the most likely space group $I\,m\bar{3}m$. In addition, a face centred cubic lattice (fcc) was also found with a = 57.21 ± 0.5 nm occurring at high volume fractions of autovaccines ($0.75 \times 10^{-2} < Ø < 1.2 \times 10^{-2}$) having the possible space group $F\,d\,\bar{3}\,m$ (Q^{227}).

INTRODUCTION

Recently it has been demonstrated that enteric bacteria, e.g. commensurable forms of *E. coli*, are affected through acute or chronic diseases, i.e. bacterial infections in response to foreign and resistant invaders, viral infections and environmental conditions, rheumatism and allergic reactions against mite, different pollens, hair from animals as well as food. Allergic reactions, e.g. dust mite house allergens, storage mites (*lepidoglyphus destructor*) also play significant roles on the formation of distinct pattern of lipopolysaccharides (LPS) of Gram-negative bacteria. The transformation of LPS or Lipid A from commensurable *Enterobacteriaceae* to modified Lipid A molecules includes i.e. magnesium-limited growth and conditions encountered during mammalian infection as reported recently for cystic fibrosis patients after infection with the opportunistic Gram-negative bacteria *Pseudomonas aeruginosa* (*Ernst* et al., 1999).

LPS or Gram-negative endotoxins form the surface of Gram-negative bacteria the major component of the outer leaflet of the outer membrane (*Raetz*, 1990). The major component of LPS is *lipid*, where Lipid A is linked to a core of oligopolysaccharides. Lipid A consists of a β-1,6-linked D-glucosamine (2-amino-2-deoxy-D-glucose) disaccharide carrying six saturated fatty acid residues and one or two negatively charged phosphates at the reducing and non-reducing end of the glucosamine (Figure 1). The basic structure is a β-1' linked D-glucosamine disaccharide which is phosphorylated in position *1* and *4*. Four molecules of R-(-)-3-hy-

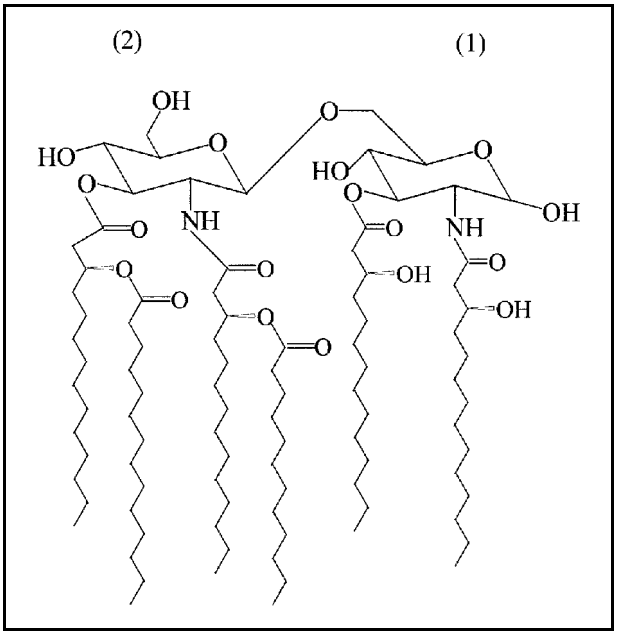

Figure 1: Chemical structure of autovaccines. Chemically, autovaccines consists of a 1,4-di-phosphorylated ß-1,6-linked D-glucosamine disaccharide with four residues of amide and esterified (R)-3-hydroxy fatty acids and carrying two hydroxylated acyl groups. The de-phosphorylated Lipid A is devoid of the inorganic phosphate groups at the reducing and non-reducing end of the disaccharide (see text).

droxytetradecnoic acid are linked to the two glucosamine residues as a backbone. Moreover, each glucosamine (glucosamine *1* & *2*) is substituted by one ester and one amine linked to hydroxy fatty acids. The 3-hydroxy group of the ester linked 3-hydroxytetradecanoic acid of glucosamine *2* is always esterified by tetradecanoic acid, whereas that of glucosamine-N-*1* is not. The amide-bonded 3-hydroxytetradecanoic acid at glucosamine is only substituted with 3-O-aclyted by decanoic acid or tetradecanoic acid while that of glucosamine-N-*1* is only substituted in some cases, mostly by hexanoic acid. The autovaccines so far studied have almost the same chemical pattern, however, some of them do not carry the phosphates, or only one phosphate, and have also been isolated as a mixture of 80% (w/w) of unphosphorylated and 20% (w/w) diphosphorylated Lipid A depending on the history of the disease of the patients (*Paradies* et al., 2000, *Thies* et al., 2001).

The outer membrane is absent in Gram-positive bacteria, hence the structure if the outer leaflet is unique for Gram-negative bacteria. Roughly 3.5 million LPS molecules cover the surface of Gram-negative bacteria, however, the nature of the molecular assembly of LPS in bacteria is of considerable interest in understanding the physical and biological properties of LPS, hence also of Lipid A, because its release in the course of severe infection is the cause of septic shock in patients with endotoxaemia. According to a survey of the Center of Disease Control (Morbidity and Mortality Weekly Report, 1997), this problem caused more than 21,000 mortalities in 1996 in the USA alone.

Moreover, there is considerable interest in understanding of the chemical, biochemical and physical properties of LPS, pyrophosphorylated Lipid A and the phosphorylated forms of free Lipid A, because its release e.g. of free pyrophosphorylated Lipid A in the course of bacterial infections is the cause of septic shock in humans (*Glauser* et al., 1991). Recently a molecular simulation study of LPS has been reported (*Kotra* et al., 1999) visualising the LPS molecule by means of atomic force microscopy (AFM) and molecular simulation, revealing the highest resolution images of any bacterium to date of 50-Å lateral and 5-Å vertical resolution. However, the physical structure of Lipid A (monophosphate & diphosphate) is also of considerable interest, i.e. with potential application in vaccine therapy, compounds known to bind to LPS, which covers the surface of Gram-negative bacteria, chain length dependent agglutination of oligosaccharide clustering by multivalent anion binding, or to find endotoxin inhibitors, which requires the generation of compounds that will prevent cells from overreacting in response to foreign bacterial LPS (*Raetz*, 1996). A complete different application and role in nature is its use as templates for the production of nano-sized materials (*Hinze* et al., 2000; *Clancy* and *Paradies*, 1998), in biomineralisation (*Archibald* and *Mann*, 1993), and drug delivery systems in the presence of double chained cationic surfactants (*Clancy* et al., 1994; *Thies* et al., 1997). In addition, it is contributing to our knowledge of lipid polymorphism (*Mariani* et al., 1988), and the various cubic phases of Lipid A containing systems (*Luzzati* et al., 1992). Therefore, the physical-chemical solution behaviour and its structure in solution of free Lipid A (monophosphate and diphosphate) is of considerable interest in many aspects, particularly prone to their specific transformation in the course of bacterial and viral infections as well as their defence against foreign invaders.

FORMATION OF NANOSTRUCTURES OF AUTOVACCINES IN AQUEOUS SOLUTIONS

The supermolecular structures of LPS have been studied by neutron scattering, small-angle X-ray scattering and electronmicroscopy (*Hayter* et al., 1987; *Seydel* et al., 1987, *Din* et al., 1993, *Brandenburg* et al., 1996; *Seydel* et al., 1993), free Lipid A has been investigated by *Seydel* et al. (1989). None of these studies observed or attributed their experimental findings with the formation of discrete structures in solution, e.g. colloidal crystallisation nor considered to apply many-body effects in solution of Lipid A in terms of colloidal crystals or liquid-like ordering, respectively, although the high potential of these materials to aggregate and the iridescence of these dispersions have been known for a long time. Scattering & diffraction methods (light), e.g. light scattering and small-angle neutron scattering (SANS) are particularly suitable for determinations of sizes, forms and interactions of particles such as the autovaccines in aqueous solutions at different ionic strengths, temperature or in the presence of specific cations e.g. Ca^{2+}, Mg^{2+}, K^{+} and Na^{+}. Autovaccines due to their preparation are colloidal suspensions of dispersed free Lipid A analogue (*Paradies* et al., 2001a) showing highly ordered structures of sizes of ~650 μm in aqueous solutions containing 0.154 M NaCl. This colloidal system exists in a non-equilibrium state, but can be driven into such a state

by application of extremely week forces, by low shear or at very low ionic strength applying tangential ultrafiltration (*Faunce* et al., 2001). Treating the autovaccines as colloidal equilibrium structures and assuming to a first approximation an isotropic distribution of spherical particles, short-range order in this particle distribution can be described by the pair distribution function, g(r), which is proportional to the probability density of finding a pair of particles separated by a distance r. This quantity can also be expressed as an autocorrelation function of the number density fluctuation normalised by the square of the average number density, n, so that g(r) reaches unity at large separations for independent particles. The pair correlation functions are accessible through scattering experiments where the structure factor, S(Q), is measured:

$$S(Q) = 1 + n\int[g(r) - \mathbf{1}]e^{-i\boldsymbol{Q}.\mathbf{r}}\, d^3r \qquad (1)$$

From the normalised measured scattering intensity $I_p(Q) = K\phi a^6 P(Q)$. S(Q), the structure factor, S(Q), can be evaluated as a function of Ø, where Ø is the particle volume fraction and *a* is the radius of this particle. Here, K is a constant depending on the optical properties of the particle and of the solvent. The quantity $Q = |\mathbf{Q}|$ is related to the scattering angle θ by $Q = (4\pi/\lambda) \sin(\theta/2)$, where λ is the wavelength in the suspension. The form factor P(Q), which contains information on the hydrodynamic shape and internal structure of the particle, is the square of the normalised scattering amplitude, B(Q). S(Q), thus contains information about the correlation between particles j and k centred at R_j and R_k, respectively. Equation (1) can be rewritten as:

$$S(Q) = 1 + \frac{1}{N}\sum_{j\neq k}^{N}\sum_{j\neq k}^{N}\left\langle e^{i\mathbf{Q}\cdot(\mathbf{R}_j - \mathbf{R}_k)}\right\rangle \qquad (2)$$

where ⟨ ⟩ denotes the ensemble average and *N* is the total number of particles. For a system of monodisperse particles which interact through strongly repulsive forces, S(Q) reveals a characteristic shape, e.g. at low Q the structure factor is small due to the low value of the osmotic compressibility Υ_T because $S(0) = (1/\beta)\,\Upsilon_T$. For $Q \sim 2\pi\, N^{1/3}$ a characteristic first peak appears, the position of which corresponds to the mean interparticle separation, where g(r) shows a maximum. Moreover, at higher Q, there are less pronounced higher order maxima in S(Q), which decay progressively to unity.

The structure factor, S(Q) was analysed in terms of available liquid state theories (*Hansen* and *McDonald*, 1988; *Oosawa*, 1971; *Roij*, 2000), and an effective pair potential of mean force has to be chosen (*Verwey* and *Overbeck*, 1948; *Hayter* and *Penfold*, 1987), as briefly outlined in this section. For a colloidal system, the potential

$$U(r) = \pi\varepsilon_0\varepsilon_r(2a)^2\psi_0^2\exp[-k(r-2a)]/r, \quad r>2a \qquad (3)$$

is widely used, where r is the center-to-center distance between two particles, ψ_0 is the surface potential, ε_r the dielectric constant of the solvent medium, ε_0 the permittivity of free space, 2*a* the diameter of the particles and *k* is the Debye-Hückel screening parameter. U(r) holds only for low ionic strength

conditions and low particle surface charge density or low surface potential ψ_0. In the dilute colloidal regime of interest here, the interparticle distances are sufficiently large for the interparticle interactions to be given by Equation (3) with an effective or rescaled particle charge Z^* and a rescaled Debye screening parameter k^*. Within this limit, the effective surface potential ψ^*, is related to the effective charge Z* by

$$\psi^* = Z^* /[\pi\varepsilon_0\varepsilon_r(2a)(2 + k^*(2a)] \qquad (4)$$

Since we are interested in the structure of the macro-ion only, an effective one-component description without the degrees of freedom of the small ions is more appropriate. *Medina-Noyola* and *McQuarrie* (1980) showed that within the limit of vanishing macro-ion concentration and for electrolyte ions of vanishing size the mean spherical approximation, yields a screened Coulomb or Yukawa potential of the form:

$$U(r) = \frac{Z^2 e_0^2}{4\pi\varepsilon_0\varepsilon_r}\left(\frac{e^{ka}}{1+ka}\right)^2 \frac{e^{-kr}}{r} \qquad (5)$$

where a is the radius of the particle, e_0 is the charge of the electron and k is the reciprocal Debye screening length, where $k = (\beta e_0^2 \sum_i \rho_i / \varepsilon_0\varepsilon_r)^{1/2}$ and $\beta = 1/k_BT$ is the reciprocal of the thermal energy; the sum here is taken over the univalent counterions and an electrolyte with the bulk number density ρ_i. The solvent enters the interaction potential through its dielectric constant ε_r, only, whereas the macro-ions have an effective charge of Ze_0. For low salt concentrations and higher volume fraction ($0.002 < Ø < 0.02$), Belloni (1993) modified Equation (5), which actually is equivalent to the far-field limit ($r \to \infty$) of the Derjaguin-Landau-Verwey-Overbeck (DLVO) potential (*Verwey* and *Overbeck*, 1948; *Hayter* and *Penfold*, 1987), by applying the mean spherical approximation. The factor $\left(\frac{e^{ka}}{1+ka}\right)^2$ is replaced by a function which depends on ka, Z, and N. Note: In de-ionised suspensions of particles, the radii of which are large compared to the Bjerrum length $l_B = \beta^2 e_0^2 /(\varepsilon_0\varepsilon_r)$, the relative deviation of the above-mentioned factor is not greater than 0.001. For moderate polydisperse systems, the interaction potential between particles having radii of a_μ and a_ν and effective charges Z_μ and Z_ν, Equation (5) can be written as:

$$U_{\mu\nu}(r) = \frac{Z_\mu Z_\nu e_0^2}{4\pi\varepsilon_r\varepsilon_0}\frac{e^{ka_\mu}}{1+ka_\mu}\frac{e^{ka_\nu}}{1+ka_\nu}\frac{e^{-kr}}{r} \qquad (6)$$

For the calculation of the effective charge Z*, or the effective surface potential ψ^* we used the Poisson-Boltzmann Cell model according to *Alexander* et al. (1984) applying a charge renormalisation (*Thies* et al., 2001).

PARTICLE CHARACTERISATION AND POLYDISPERSITY

From the transmission electron micrographs (TEM) the quantities measured in light scattering, e.g. $\overline{R_h}$ and $\overline{R_G}$ are calculated from the moments of the size distribution. For the radius of gyration, the expression $\overline{R_G} = [3\,\overline{R^8} / (5\overline{R^6})]^{1/2}$ was used and for the hydrodynamic radius $\overline{R_h} = \overline{R^6} / \overline{R^5}$ was employed. Assuming that particle interactions can be treated according to Equations (2-6) for volume fractions $1.0 \times 10^{-4} \leq \Phi \leq 3.5 \times 10^{-4}$ and salinities of 10 mM and 100 mM NaCl, $I_p(Q)$ was calculated and its Gaussian distribution, $\rho(x)$, was determined, and the results compared to the experimental values obtained from static light scattering and TEM. The best fit was for an average radius of gyration of $\overline{R_G} = 9.85 \pm 0.50$ nm from the light scattering measurements. The values of $\overline{R_h}$ from TEM were consistently lower by 8.0-9.5%, and 10.0-11.5% lower than the values determined by quasi-elastic light scattering (Table 1, $\overline{R_h} = 13.3 \pm 0.50$ nm). It is not unusual for particle sizes determined from TEM to be smaller than the ones obtained by light scattering, small-angle X-ray scattering or neutron scattering methods because the shrinkage of the hydrated particles caused by the exposure to the electron beam as well as errors in the calibration of the electron micrographs. However, we can not exclude the pos-

Table 1: Summary of the characterisation of the autovaccine particles for volume fractions $1.0 \times 10^{-4} \leq \Phi \leq 3.50 \times 10^{-4}$ at 25°C. The first column lists the number average particle radius $\bar{R}$, the polydispersity ρ_{TEM}, ρ_{LS} and ρ_{QELS}, the radius of gyration $\overline{R_G}$, and the hydrodynamic radius $\overline{R_H}$. The results obtained for 5.0 nM Ca^{2+}, 5.0 μM Mg^{2+} and 50 mM Na^{+} are included. The second column summarises the quantities calculated from the size distribution corrected for polydispersity with respect to the independently measured quantities, and the average weight molecular weight, $\overline{M_w}$, the ratio of weighted average molecular weight to number averaged molecular weight $\overline{M_n}$, $\overline{M_w} / \overline{M_n}$*.

	Quantity (uncorrected)	Size distribution independently (corrected) determined	Size distribution measured
R/ nm	11.5 ± 0.30	12.64 ± 0.30	……
ρ_{TEM}/ %	8.0 ± 0.60	8.70 ± 0.60	……
R_H/nm (QELS)	13.3 ± 0.70	12.95 ± 0.50	13.40 ± 0.55
R_H/nm, (QELS), Ca^{2+}	14.5 ± 0.50	14.48 ± 0.50	14.50 ± 0.50
R_H/nm, (QELS), Mg^{2+}	15.5 ± 0.80	15.40 ± 0.60	15.65 ± 0.55
R_H/nm, (QELS), Na^{+}	14.2 ± 0.30	14.30 ± 0.50	14.35 ± 0.40
R_G/nm (LS)	10.5 ± 0.30	9.80 ± 0.30	9.85 ± 0.55
R_G/nm (LS), Ca^{2+}	11.3 ± 0.30	11.00 ± 0.40	11.10 ± 0.50
R_G/nm (LS), Mg^{2+}	12.6 ± 0.45	12.00 ± 0.55	12.00 ± 0.50
R_G/nm (LS), Na^{+}	10.9 ± 0.50	10.50 ± 0.60	10.80 ± 0.63
ρ_{LS}/%	5.0 ± 0.70	5.00 ± 0.70	……
ρ_{QELS}/%	5.7 ± 0.75	5.75 ± 0.75	……
$\overline{M_w} \cdot 10^6$/gmol^{-1}	3.75 ± 0.86	3.69 ± 0.75	3.60 ± 0.40
$\overline{M_w} / \overline{M_n}$	1.032	1.032	1.025

* $\overline{M_n}$ was obtained from osmotic measurements (Knauer Membrane Osmometer, 15,000 MW membrane; *Paradies*, unpublished).

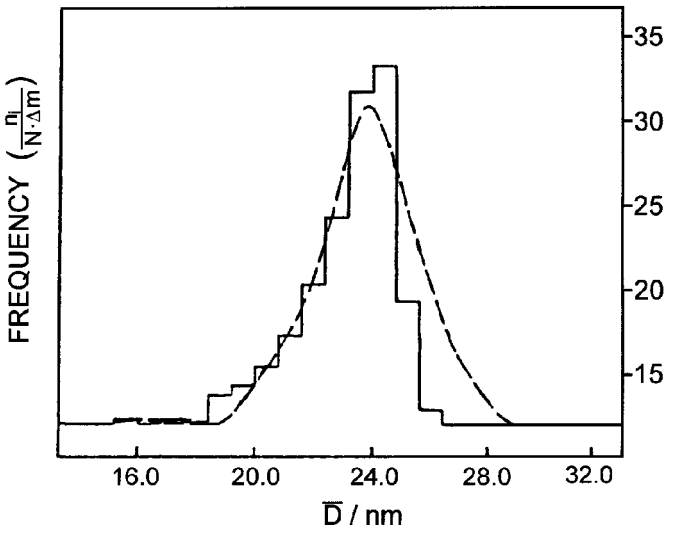

Figure 2: Size distribution of Lipid A aggregates obtained by transmission electron microscopy. Full line histogram, original data: dashed line is the smoothed distribution calculated from the histogram with the average radius of $\bar{R}$ = 12.64 nm and polydispersity ρ = 8.7%.

sibility that the differences between the radii of TEM and static light scattering could be attributed to the polydispersity of the system. Figure 2 shows the size distribution of the autovaccines, whereas Figure 3 illustrates autovaccine particles at a volume fraction Ø = 2.5 x 10^{-4} revealing spherical particles of average diameters of d ~25 nm at low salt (0.005 M). Figure 4 shows SEM electron micrographs of colloidal crystals of autovaccines obtained at volume fractions of Ø = 3.5 x 10^{-4} and 4.0 x 10^{-4}, respectively. Using the radius obtained from the TEM and a size distribution with the normalised variance $\overline{\rho_{TEM}}$ = 7.1 ± 0.8%, an estimate of the hydrodynamic radius was calculated to $\overline{R_h}$ = 12.50 nm from the radius of gyration which shifted the size distribution by 8.5%. Reasonable agreement can be found between $\overline{R_h}$ and $\overline{R_G}$, the values obtained from TEM and the light scattering experiments, respectively. This results in an average of $\overline{R_h}$ = 12.64 ± 0.2.5 nm and a polydispersity of $\overline{\rho_{TEM}}$ = 8.7 ± 0.6%. According to *Pusey* (1980) the particle polydispersity, ρ_P, can be expressed as

$$\rho_P = \sigma / a \qquad (7)$$

and set to ρ_P < 5% as a criterion for a system which can be treated as a one-component system. Applying this criterion for polydispersity, we calculate, for the TEM results, a value of 6.4% for ρ_P, which is consistent with the value found from light scattering measurements of 9.5%. As has been pointed out by *Hayter* and *Penfold* (1991) it appears that a higher degree of polydispersity can in fact be accepted for charged particle systems.

Furthermore, it should be noted that the TEM electron micrographs may give a false impression that a Gaussian distribution is appropriate where in fact the distribution could have been a log normal, where a tail exists extending to smaller particle sizes even though we could not detect any significantly smaller particles. If this were the case, the particle size distribution parameter could still be higher than 8.7 ± 0.6%. Applying a log normal distribution of

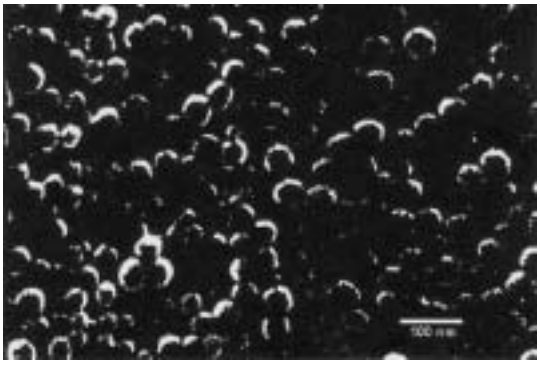

Figure 3: SEM electron micrograph of Lipid A aggregates at a volume fraction of Ø = 2.5×10^{-4}. The size bar is 100 nm.

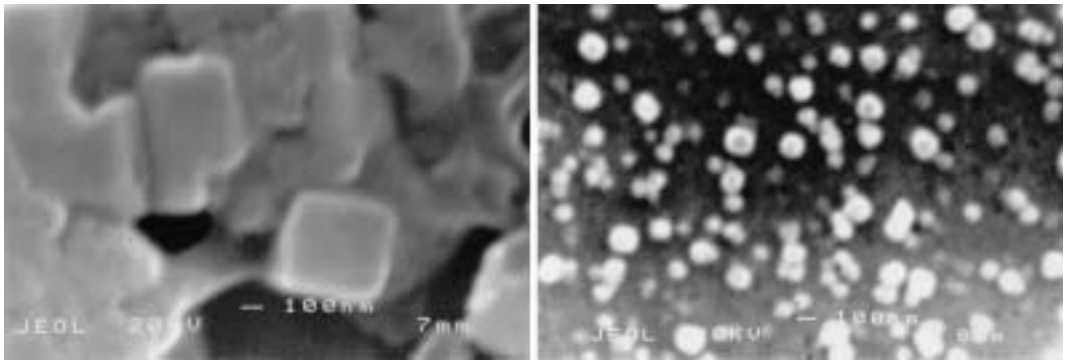

Figure 4: SEM electron micrographs of colloidal crystals of Lipid A obtained at a volume fraction Ø = 4.0×10^{-4} (left), and at a volume fraction Ø = 3.5×10^{-4} (right), showing large colloidal crystals. The size bar is 100 nm.

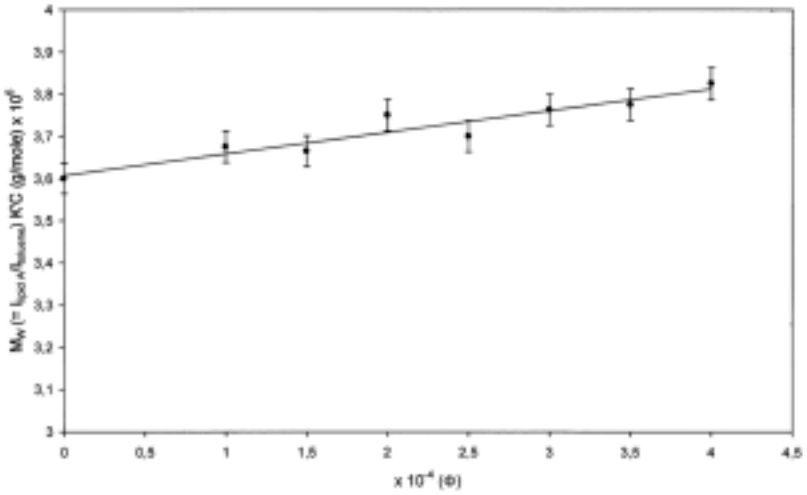

Figure 5: A plot of $I_P / K' c \cdot I_{toluene}$ vs. volume fraction Ø yielding a molecular weight of $M_w = (3.6 \pm 0.4) \times 10^6$ g/mol in the presence of 1 mM NaCl and Q = 2.5×10^{-2} nm^{-1}.

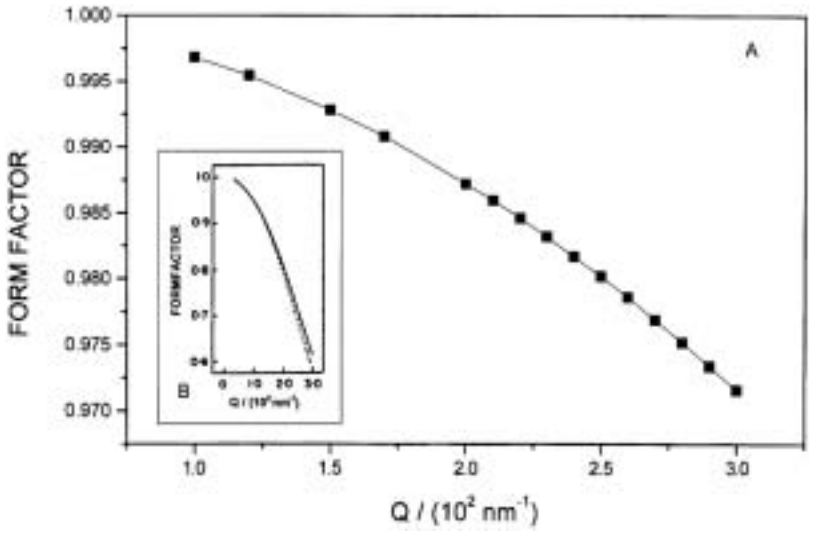

Figure 6: (A) Experimental particle form factor of Lipid A (◆····◆) and best fit calculation applying the Guinier approximation with $\overline{R_G}$ = 10.1 nm.
(B) Guinier approximation for a polydisperse form factor (8.7%) calculated with Mie theory as a homogeneous sphere with complex index of refraction r = 1.532 + i 0.0015. The dotted line is the prediction with ρ = 8.7% and $\overline{R}$ = 12.64 nm.

the particle size polydispersity recalculated from the M_w distribution, we found a value of the order of 8.5±0.7%.

The weighted-average molecular weight, M_w, for the volume fraction in the range $1.0 \times 10^{-4} \leq Ø \leq 3.50 \times 10^{-4}$ was determined in the presence of 1.0–100.0 mM NaCl by plotting $(I_P / K' c \cdot I_{toluene})$ vs. Ø (Figure 5), revealing a slight positive slope indicating a repulsive potential between like-charge species which is almost independent of the range of the volume fraction studied ($1.0 \times 10^{-4} \leq Ø \leq 4.0 \times 10^{-4}$), and yields after extrapolation to Ø = 0, $M_w = (3.60 \pm 0.40) \times 10^6$ g/mol, $N_{agg} = 2.2 \times 10^3$, $K_{as} = 10^{8.5}$ L/mol (Table 1). The positive slope does not preclude the possibility that excluded volume effects are not present, for this would give rise to repulsive interactions for any type of particles whether they are charged or not. However, the repulsive interactions would be less sensitive towards addition of salt.

STRUCTURE FACTORS

The structure factor S(Q) was extracted from the average intensity by normalising I(Q) to the average particle form factor P(Q). One of the main problems of this technique for polydisperse suspension compared to monodisperse ones is an increase in scattered intensity at low Q values which makes precise measurements difficult for dilute suspensions. This, of course, can lead to considerable errors in S(Q). However, as outlined above, by applying the polydispersity criterion of *Pusey* (1980) we can treat this dilute suspension to a one-component system to a first approximation for extraction of the form factor P(Q) from I(Q). Considering that we have strong interactions in the dilute

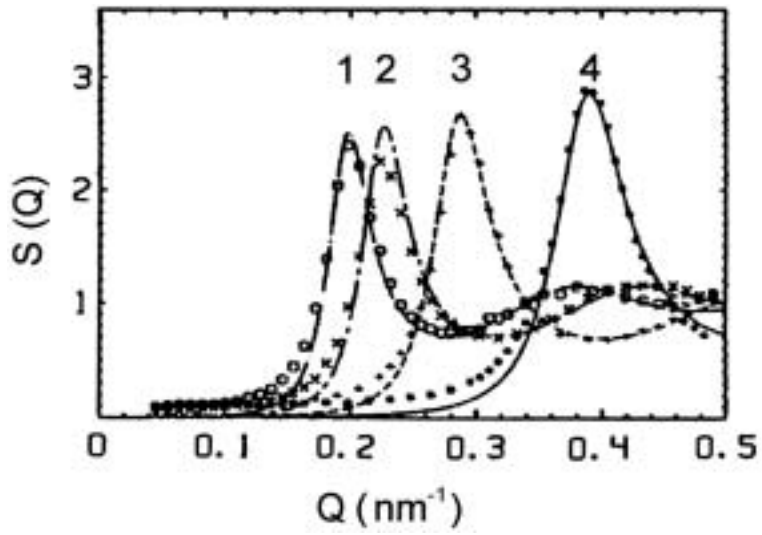

Figure 7: Experimental structure factors S(Q) as obtained from the PBC model and the RSMA for different particle number densities: 2.45 x 10^{15} particles/ml (1); 3.05 x 10^{-4} particles/ml (2); 3.9 x 10^{15} particles/ml (3) and 4. 9 x 10^{15} particles/ml (4).

system in the absence of salt as well as at low salinity e.g. 10 mM NaCl, it is safe to calculate the form factor P(Q) according to Guinier's approximation (*Guinier*, 1955), since the compressibility (χ) for a particle with a = 12.64 nm amounts to 0.0067 for a monodisperse system in the absence of salt, and 0.0063 for a polydisperse system after allowing for the determined polydispersity index. The experimentally obtained radius of gyration was used, $\overline{R_G}$ = 9.80 ± 0.55 nm (Table 1), to normalise the form factor, and then P(Q) vs. Q was calculated according to the Guinier approximation as shown in Figure 6 together with the experimental data and compared with the form factor calculated using the Mie theory assuming a homogeneous sphere having a hydrodynamic radius of $\overline{R_h}$ = 12.64 nm with a complex index of refraction r = 1.532 + i0.0015. Figure 7 shows a series of examples of measured structure factors, S(Q), with different volume fractions for the *Autovacccine* suspensions in water after applying the PBC model and the rescaled mean spherical approximation (RSMA) according to *Hansen* and *Hayter* (1982).

The effective charges and volume fractions were determined by fitting the positions and heights of the peaks in the calculated structure factors S(Q) to the experimental scattering data. The structure factor, S(Q), was calculated using the (RSMA) algorithm as a convenient tool to generate theoretical structure factors for comparison with the experimentally obtained structure factors, S(Q), particularly for these dilute suspensions assuming an average particle radius of $\overline{R_h}$ = 12.64 nm and a polydispersity of ρ_{LS} = 5.0%. In comparison, the volume fractions found ranged from 1.15 x 10^{-4} to 3.75 x 10^{-4}, and were surprisingly close to the ones calculated from dilution experiments. Within this volume fraction range, the effective charges were determined to be of the order of Z^* ~ 373-460 grouping around 400 for a bare particle charge of Z_P = 780 and for $\overline{R_h}$ = 12.64 nm. Table 2 lists the parameters for calculation of the structure factors, S(Q), of this Lipid A dispersion for a particle density of

Table 2: PBC model with effective charge Z^*_p and effective reduced, screening parameter $k^*/(2a)$ for various particle density numbers and for a fixed charge of $Z_P = 800$

Number density (particles/cm^3)	Z^*_p	$k^* \cdot (2a)$
1.5 x 10^{15}	421	0.21
2.0 x 10^{15}	400	0.23
2.5 x 10^{15}	378	0.25
3.0 x 10^{15}	360	0.28
3.2 x 10^{15}	348	0.37
3.5 x 10^{15}	300	0.47

1.25 x 10^{15}/cm^3 and particle diameter of d = 25.28 nm. Note: The effective charges obtained from a mono-disperse fit to the light scattering data after applying the (RSMA) closure for calculating the structure factor, the true effective charges can be underestimated by underestimating the colloidal structure due to polydispersity.

These effective charges and the volume fractions $\kappa a > 0.1$, and the deviation from the pre-factor in the Yukawa potential, were found to be of the order of 10^{-3}. Moreover, Figure 7 shows structure factors, S(Q), for four different particle number densities of Lipid A suspensions which are close to $\Phi \cong 3.0 \times 10^{-4}$, ranging from a particle number densities from 2.45 x 10^{15}/cm^3 to 4.9 x 10^{15}/cm^3 and again the effective charge values Z* cluster around $Z^* \cong 390$ - 410. The appearance of the structure factors, S(Q), for these various number densities and the height of the first peak do not change significantly in the presence of 10 mM to 50 mM NaCl. The height of the first peak in all the S(Q) curves is well above 2.0 as it is also the case for the structure factors, S(Q), for all volume fractions. According to the rule of *Verlet* and *Hansen* (1969), crystallisation occurs as the structure factor of ordinary liquids exceeds a value of 2.85. This effect is seen especially for autovaccine particles having a number particle density of 4.0 x 10^{15} particles/cm^3. Here, an increase in height of S(Q) and a shift towards higher Q-values even occurs at volume fractions as low as $\Phi \cong 1.50 \times 10^{-4}$. The situation does not change significantly upon increasing the ionic strength to 50 mM NaCl, or 100 mM NaCl as long as the number particle density is below ~5.0 x 10^{-4}. However, a significant ordering of the autovaccine particles at this low volume fractions of ~1.50x10^{-4} in the presence of 100 mM NaCl (25°C), or 5.0 nM Ca^{2+} and 50 mM NaCl, respectively, results in a significant increase in height of S(Q) to 3.0. Indeed, this solution does show a strong tendency to crystallise sometimes into colloidal crystals of sizes up to 1.5 – 5.0 μm. After stirring (2-3 hrs) or heating the solution (20-30 s) to 50°C, these colloidal crystals of autovaccines dissolve, which can also be monitored in a UV-VIS spectrophotometer at ~540 nm due to changes in the transmission. Furthermore, in the calculation of S(Q), the charge $Z_\mu$of component μ has been taken into account (Equation 5 and 6), because Z_μ scales with a^2_μ. The values of S(Q) were calculated for $Z_\mu \propto a_\mu$ keeping the average charge constant. No significant polydispersity was found with respect to the width and height of the first peak, once again indicating that the polydispersity does seem to be rather low.

The total anionic charges of 5940 per particle, the experimentally determined exposed surface charges per particle of

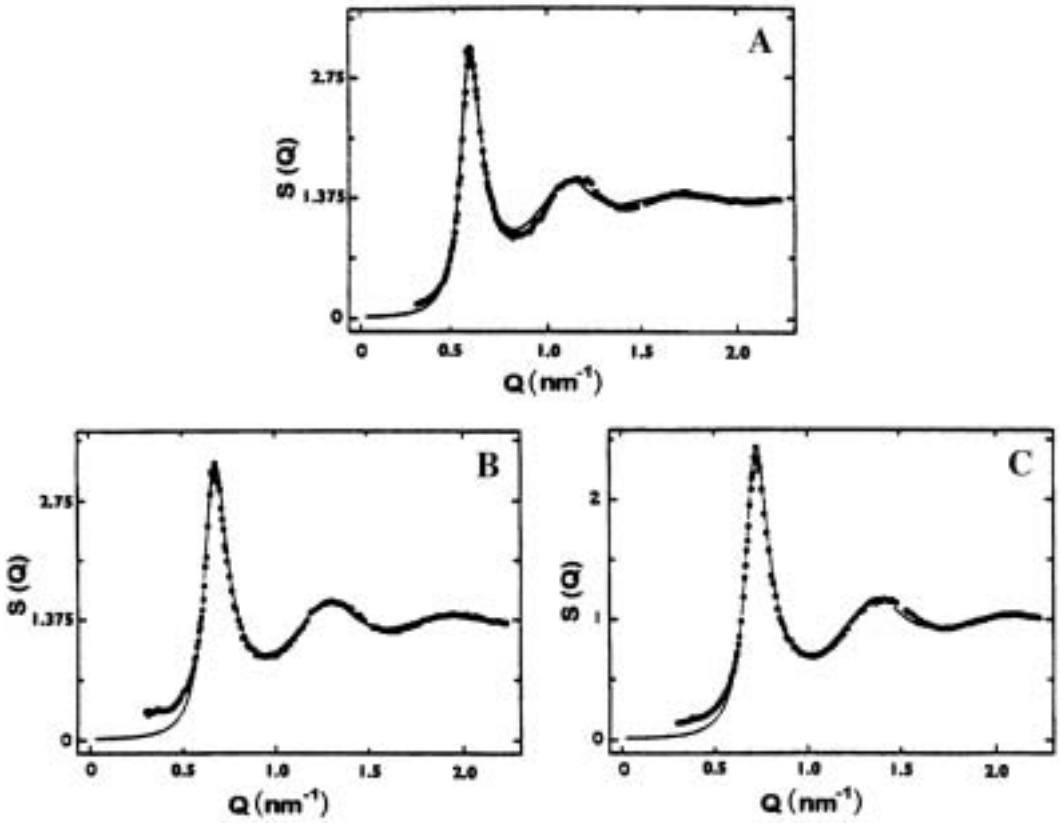

Figure 8: Experimental structure factors S(Q) as obtained from the PBC-model and the RSMSA, for a particle density of 1.60 x 10^{15}/ml in the presence of 5.0 nM Ca^{2+} (A), 3.5 nM Ca^{2+} (B), and 2.5 nM Ca^{2+} (C). Experimental structure factors (• --- •) and calculated ones (full line) according to the PBC model.

~380 including the bound protons (~60), which remain electrostatically bound to the particle, and the determined pK_a value of 7.21 of the autovaccine suspensions enables us to study the interactions of Ca^{2+} and Mg^{2+} with the negatively charged Lipid A and to study their influence on the structure factor S(Q). This interaction is among other biological actions a process of utmost biological significance due to

1) it is the biochemical mechanism by which Lipid A influences plasma levels of various interleukines (*Ernst* et al., 1999);
2) due to the increased stimulation of susceptibility to innate immune killing, and
3) the significant decrease of inflammatory response in the presence of autovaccines or Lipid A derivatives from clinical isolates and laboratory strains grown in low Mg^{2+} medium.

The autovaccine suspensions have been studied in the same volume fraction and number particle density as described before, but in the presence of 1.0 – 5.0 nM Ca^{2+}, 1.0 – 5.0 μM Mg^{2+}, and as a mixture of both at the same molarities (*Paradies* et al., 2001). Figure 8 shows the structure factor, S(Q), for a number particle density of 1.6 x 10^{15}/cm^3 in the presence of 1.0, 2.5, 3.5, and 5.0 nM Ca^{2+}, which were added as $CaCl_2$ to the Lipid A suspension. It is interesting to observe, that here the height of the first

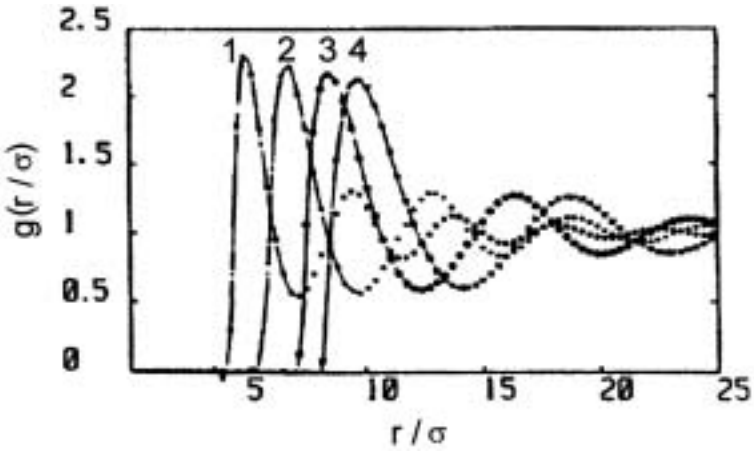

Figure 9: Radial distribution function g(r) for different particle concentrations: 2.5 x 10^{15} particles/ml (1); 3.5 x 10^{15} particles/ml (2); 4.0 x 10^{15} particles/ml (3), and 4.9 x 10^{15} particles/ml (4) obtained by Fourier transformation of the measured S(Q).

peak of S(Q) exceeds the value of 2.85 according to the Hansen-Verlet melting rule by a factor of 1.16. At low Q, the structure factor S(Q) approaches a constant value different from zero, which might be due to polydispersity of the Lipid A suspension in the presence of Ca^{2+}. It can be seen from Figure 8, that increasing the Ca^{2+} concentration above 3.5 nM gives no further increase in height of the first peak. No significant effect on the magnitude of the structure factors, S(Q), can be noted after adding 50 mM or 100 mM NaCl to this sample. However, when the same experiment is conducted in the reverse order, e.g. adding the NaCl solution (50 mM or 100 mM,) prior to the 1.0 nM – 5.0 nM Ca^{2+} solution, a significant decrease in the height of the structure factor S(Q) was observed by a factor of ~1.9. This indicates a loss of structure when compared to the structure factors S(Q) of the autovaccine suspensions in pure water or low NaCl solutions. Addition of concentrations higher than 3.5 nM up to 15.0 nM Ca^{2+} have no detectable effects on the magnitude of the structure factor, S(Q). Furthermore, determinations of the form factor as a function of scattering vector as well as the radius of gyration as determined of these suspensions of autovaccines, did not change in comparison to those determined for the free Lipid A suspensions in the absence of salt (Table 1).

Pursuing the same experiments in the same order as the ones conducted for Ca^{2+} but with Mg^{2+}, using much higher concentrations than for Ca^{2+}, of the order of 1.0 – 5.0 μM, a significant loss of structure was observed, which can be reversed with the addition of 50 mM or 100 mM NaCl, to yield almost the same value of the structure factor S(Q) as before the experiment was started in the presence of 50 mM salt. Alternatively after two hours, the same value for the structure factor S(Q) can be recovered having the same magnitude and appearance as in Figure 9. Moreover, no change was noticed at higher Mg^{2+} concentrations as long as the temperature is kept at 25°C. It would appear, that the addition of Mg^{2+} in the presence of 50 mM or 100 mM NaCl results only in a small decrease of the height of the structure factor S(Q), which does not change with time (24 hrs) at constant temperature. However, the radius of gyration for this system changed to a higher value (12.6 nm) unlike the one

for Ca^{2+} which was 11.3 nm (Table 1). However, we observed that colloidal crystallisation of the autovaccines occurred already at volume fraction of 1.7 x 10^{-4}.

Adding 5.0 nM Ca^{2+} and 5 μM Mg^{2+} to the de-ionised Lipid A suspension with a number particle density of 1.6 x $10^{12}/cm^3$, the same appearance with respect to magnitude of the structure factor S(Q) and position is observed as for Ca^{2+} alone, so what matters is the order of addition, e.g. with Ca^{2+} first then Mg^{2+}, the structure factor S(Q) for Ca^{2+}-autovaccine is unaffected by Mg^{2+} at all. The addition of salt solutions of the order of 50 mM NaCl or 100 mM NaCl to this suspension does not affect the magnitude of the structure factor S(Q) if the temperature is kept at 25°C. No changes in the radius of gyration (R_G = 12.6 nm) or in the polydispersity index were observed in the presence of the 5.0 nM Ca^{2+} and 5 μM Mg^{2+}. The dissociation constant of Ca^{2+} of K_d = 2.4 nM is much lower than for Mg^{2+} K_d = 9.7 μM, indicating that binding of Ca^{2+} is much stronger than the one for Mg^{2+}, which implies preferential binding of Ca^{2+} over Na^+ and Mg^{2+} ions. Removing the Ca^{2+} through the addition of 5 mM EDTA and extensive dialysis in the presence of 10 mM NaCl, the same structure for the autovaccines is seen. It is quite surprising that Ca^{2+} ions at such low concentrations do improve ordering of the Lipid A clusters in solution to such an extent, and that neither Mg^{2+} nor Na^+ ions are able to exchange Ca^{2+} (*Paradies* et al., 2001).

It is apparent that Ca^{2+} sites must select Ca^{2+} in preference to the much more numerous Na^+ ions present in solution, even though the two cations have precisely the same diameter. The Lipid A or the autovaccines obviously bind Ca^{2+} very tightly with K_d = 3.5 nM as opposed Mg^{2+} = 1.5 mM, and upon doing so, blocks Na^+ binding to some extent. This still raises the problem as to how Ca^2 ions pass through the Lipid A or autovaccine aggregate to their binding sites without some change in the shape of the Lipid A or autovaccine molecule. Since the form factor P(Q) and the dependence of P(Q) on Q (R_G = 10.21 ± 0.8 nm, Figure 6), or the size distribution observed by TEM or SEM of the autovaccine aggregate in the presence of 5.0 nM Ca^{2+}, and the weighted average molecular weight ($\overline{M_w}$ x 10^6 = 3.85 ± 0.9) does not change at all as observed here. It may be assumed according to the titration data that the binding sites for Ca^{2+} or Mg^{2+} are not only located on the surfaces of the dispersed Lipid A colloids. Another explanation can be offered analogous to the one for the binding sites of Ca^{2+} or Mg^{2+} like EDTA chelators, considering the favoured pK_a at low pH (5.5 – 6-5) where no phosphate groups are involved. A model can be constructed which permits eight oxygen atoms, each built with charge of –1/2 and Ca^{2+} to float freely in a confined volume with a dielectric coefficient, so that the oxygens then interact with ions that pass in and out of the volume, always acting to maintain electroneutrality within this space. This would explain the retention of the number of surface charges in the presence of the Ca^{2+} as well as the insignificant change on the surface charges of Na^+. Moreover, the almost constant surface charge of 350 ± 70 found for Na^+ in the presence of Ca^{2+}, and the small reduction of surface charge after addition of Na^+ from 350 ± 70 to 200 ± 50 in the presence of Ca^{2+}, may have its origin in the replacement of two Na^+ by one Ca^{2+}, leaving approximately 70 – 80 Ca^{2+} ions located at the surface. Titration of these Ca^{2+} ions by an ion selective electrode as well as by a Ca^{2+} specific indicator yielded values of 76 ± 10 Ca^{2+} ions, the rest of the Ca^{2+} ions are apparently located in the interior of the

Table 3: Variation of the total and of the effective charge of the autovaccines with added NaOH at constant volume fraction Ø = 3.5 x 10^{-4}. The surface parameters at low salt concentrations (5 mM NaOH) are: Total surface charge density ρ_T = - 0.12 C/m^2, effective particle radius α = 12.8 nm, Stern capacitance, C_S = 0.48 F/m^2, pK_a of the phosphate group is 7.20, and α is the degree of dissociation.

[NaOH]/M	pH0	pHd	Z	Z*	Φ0/mV	α
10^{-7}	1.51	5.70	900	170	- 230.4	5.40 x 10^{-4}
10^{-6}	1.66	6.55	950	200	- 250.3	5.10 x 10^{-4}
10^{-5}	3.46	8.51	1010	210	- 263.5	2.78 x 10^{-2}
5.0 x 10^{-5}	4.66	9.31	1200	215	- 290.3	1.50 x 10^{-1}

cluster, and possibly chelated within the region of the chiral (R)-3-hydroxyoctanoic acid residues of the colloidal suspension of Lipid A.

The low affinity of Mg^{2+} compared to Ca^{2+} can be related to the ability of Mg^{2+} to bind water unusually tightly. Also it is noteworthy that the Ca^{2+} associated strongly with this autovaccine can be found analytically in MALDI-TOF-mass-spectroscop, by Ion Spray-mass-spectroscopy, or by combined Capillary electrophoresis and MALDI-TOF in the same total amount after extensive dialysis or electrodialysis against water, and by calculating the difference in the average number of charges of the calcium bound Lipid A (250, 265 for the autovaccines) prior to addition of sodium chloride or vice versa. The strong influence of Ca^{2+} ions on the solution structure, S(Q), may have also reflect the observation, that the autolysis of the autolysine-susceptible cell walls of *Staphylococcus aureus* during growth is dependent on low Ca^{2+} but in the presence of high salt (*Ochiai*, 1999; 2000).

SURFACE CHARGE

Preliminary data on particle surface charges were determined by comparing $S_{eff}(Q)$ obtained from light scattering for diluted systems having volume fractions of 1.5 x 10^{-4} ≤ Ø ≤ 2.0 x 10^{-4}, from values obtained by fitting to the PBC model. The fitting procedure yielded an average surface particle charge of 400 ± 80 in the presence of 50 mM NaCl, close to that deduced from conductivity measurements. No significant differences were noticed between the low salinity (1.0 mM, 410 ± 70) and high salinity (100 mM, 395 ± 70) solutions (*Thies* et al., 2001). From the results obtained when NaOH was added to the autovaccine suspensions during titration and conductivity measurements, and by applying the PBC model, predictions were made of the effective charges at intermediate salt concentrations (Table 3).

Basically, free protons are excluded from the surface of the colloidal Lipid A particle, so when using the Stern model, $[H^+]_0$ has to be replaced by the proton concentration at the outer Helmholtz plane according to the equation:

$$[H^+]_d = [H^+]_b \cdot e^{\Psi} \quad (8)$$

This expression includes the dissociation of the phosphate, where the concentration is given as volume concentration and the subscripts of d, b and s mean the concentration in the diffuse layer, the bulk solution and in the Stern layer, respectively, as defined by Equation (9):

$$K_{R\text{-}O\text{-}P(=O)(OH)_2} \cdot e^{\beta\sigma/C_S} = [R\text{-}O\text{-}P(=O)(OH)O^-] \cdot [H^+]_d / [R\text{-}O\text{-}P(=O)(OH)_2] \quad (9)$$

where σ is the total charge density and C_S the Stern capacitance. Table 3 lists the variation of the effective charges as the suspension of the autovaccines is titrated with NaOH at a constant volume fraction of $\Phi = 2.0 \times 10^{-4}$. The effective charges were obtained from the PBC model. The corresponding effective charge, $Z^* = 215$ at the highest NaOH concentration used (5.0×10^{-5} M) is significantly lower than the saturation value of ~400 predicted by the BPC model. Using the criteria by *Alexander* et al. (1984), it has been found, that if $Z^* = 215$ is taken as the structural particle charge for the linearised Poisson-Boltzmann equation predicts a value of 170 at 50 mM salt, and at 200 for 100 mM salt. This would imply, if all sites were charged, a surface charge of 400 sites/particle for this suspension. However, the bare charges of this suspension increase with NaOH concentration at constant volume fraction primarily due to the resulting increase of the pH. The observed discrepancy between the number of total charges (~400) and the structural colloidal charge of ~170 – 200 detail, one has to increase the volume fractions, use small-angle light scattering techniques, and small-angle X-ray scattering. However, the use of scattering techniques is also limited since the autovaccine particles form colloidal crystals at higher volume fraction.

It is thus clear that indicates that only half the fraction of the available sites is ionised. So, with the inclusion of the charge regulation, the resulting effective charges are always lower than their saturation values in the absence of charge regulation. To investigate the surface charges of this colloidal aggregate in more further detailed study of the dynamics of the ordered structure of Lipid A or the autovaccines in the presence of both Ca^{2+} and Mg^{2+} ions is most desirable, particularly to help us to understand changes in the physical surface charges, their salt-, pH- and temperature dependence, as well as their dependence on higher number densities of solute. Moreover, there is strong evidence that this ordered structure of the autovaccines in aqueous solution is stable over several months when the Lipid A concentration is maintained within the relevant volume fraction range.

Having established the spherical size of the autovaccines with diameters of d = 24.5 nm and the onset of crystallisation at volume fractions of $\Phi = 3.5 \times 10^{-4}$, we should expect close packed arrays of spheres of free Lipid A or autovaccines. These systems, termed colloidal crystals, are composed of either polymer like polystyrene sulphonates, viruses such as *Tipula iridescent virus* (TIV) suspensions (*Klug* et al., 1959) or free Lipid A and autovaccines, which have been shown for the first time under strictly controlled conditions to form these crystalline structures (*Thies* et al., 2001; *Paradies* et al., 2000; *Faunce* and *Paradies*, 2001; *Paradies* et al., 2000, *Faunce* et al., 2001). Like the natural gem stone opal they diffract light as a result of the submicrometer diameters of these colloids (*Hiltner* and *Krieger*, 1969; *Luck* et al., 1963**;** *Hachisu* et al., 1973; *Asher* et al., 1998), where the colloidal crystals of free Lipid A or autovaccines spontaneously form under ambient conditions as reported before.

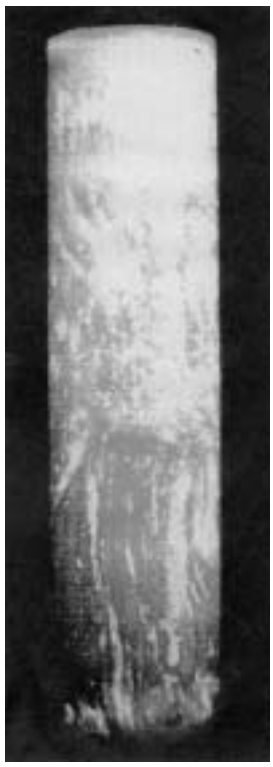

Figure 10: Image plate photograph of colloidal crystals of autovaccines at ionic strength of 0.0005 M (25°C) after coating with C_{16}-fluorescein thiocynate, showing Bragg reflections in dimethylsulphate/water, 70/30 (v/v).

FORMATION OF COLLOIDAL CRYSTALS OF THE AUTOVACCINES

Above a volume fraction of Ø = 3.5 x 10^{-4} at low salt (I=0.0001 M), or in the presence of 0.150 M NaCl, however at Ø =1.5 x 10^{-4}, colloidal crystals of the autovaccines are appearing (Figure 3), whereas Figure 10 shows colloidal crystals of autovaccines, which have been doped with fluorescein thiocyanate (C_{16}) and illuminated with laser light of λ = 621.1 nm, revealing intense Bragg reflections of pseudo-hexagonal closed packed (hcp) colloidal crystals of autovaccines. These colloidal crystals, sometimes of sizes of 2 and 5 μm but rather thin (50 nm in thickness) form only if a narrow size distribution of the particles is preserved. When these colloids settle into surface features, quite a few structures can be generated as it has been observed in TEM and electron diffraction pattern as well as in Atomic Magnetic Force Scanning Electron microscopy (AMF). These 2D patterns can be used to build up 3D colloidal crystals. Other forms or approaches e.g. for Lipid A or the autovaccines exploit the complex phase diagram of colloidal mixtures (*Faunce* et al., 2001). When colloids of different sizes are allowed to crystallise together, they can form a variety of different crystal structures depending on the sphere size and its

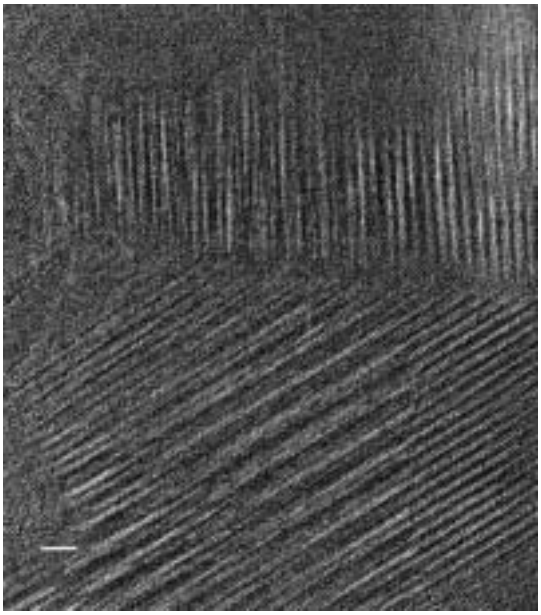

Figure 11: TEM image of unprocessed resolution of autovaccine showing three sets of lattice rows. The image shows also at least two domains of different orientations (likely twinned), and superimposed lattices of two domains. The bar scale is 25 nm (*Faunce* and *Paradies*, unpublished results).

polydispersity index, and particle number density rather than mass. Figures 11 - 14 show molecular resolution images obtained from TEM diameters of 2.75 – 2.80 nm, separated by herringbone structures of 4.62 – 4.64 nm of considerably lower density, and of a rope of 2.75 nm again. It can clearly been seen that two crystalline sheets of crystals are mixed and meet at angle of ~60° by forming an arrow, where each electron dense rope is separated by a less dense region of constant distance of 4.6 nm. This can best be described as disordered areas of molecular order but without long-range correlation. Similar patterns are observed by AMF in the presence of magnetite. Figure 12 depicts the fine structure of an unprocessed 30 nm x 30 nm image of a crystalline array of autovaccines (Ø = 4.0 x 10^{-4}, whereas Figure 13 shows one crystalline layer of autovaccines molecules where a molecular lattice (presumably cubic, could also be rectangular) is clearly visible. The inserts correspond to the electron diffraction pattern obtained from the same specimen. Figures 12 and 13 show almost absence of lattice defects and the presence of regular crystalline order. The sharp and symmetric reflections obtained by electron diffraction within this two dimensional array confirm that both the positional and orientational order are long-range. The 12 strongest reflections have been tentatively indexed on the basis of a body centred cubic lattice (bcc) with lattice repeats of a = 0.573 ± 0.042 nm, or on a distorted hexagonal pattern with two distinct lattice repeats of 0.543 ± 0.051 nm by 0.59 ± 0.05 nm with an angle of tilt of ~57° (Figure 14). For both structures the basic structural motif seems to be a dimer of the autovaccines. From the lattice repeats and the bilayer thickness measured from Figure 10 of 4.6 nm, we propose that the molecular

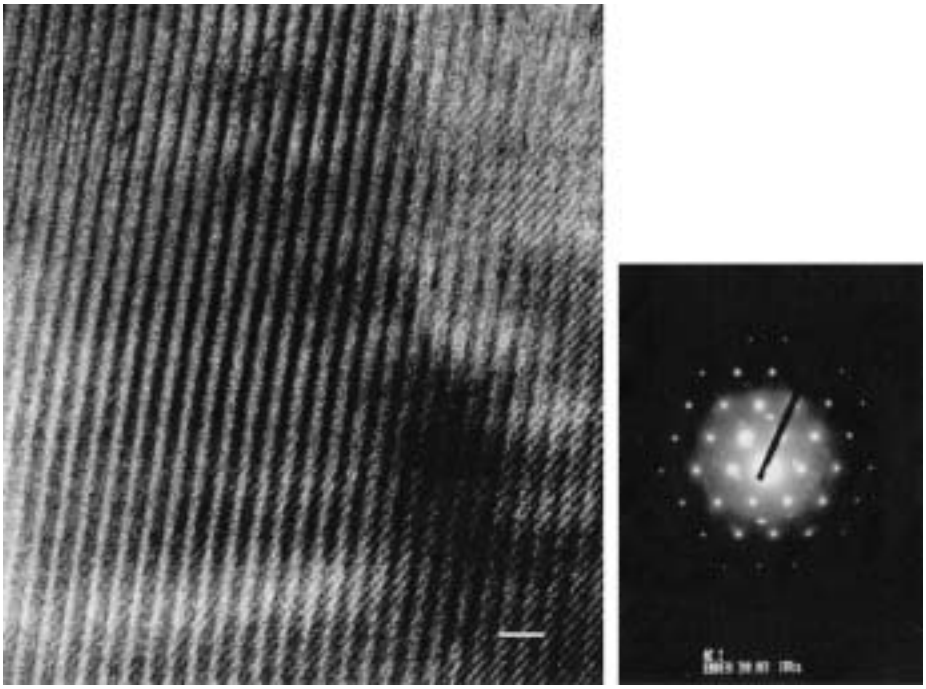

Figure 12: TEM image (left) and the corresponding electron diffraction pattern (right) of the image. Alternating rows of high and low zigzag molecules of autovaccines can be clearly seen. The bar size is 2.5 nm (*Faunce* and *Paradies*, unpublished results).

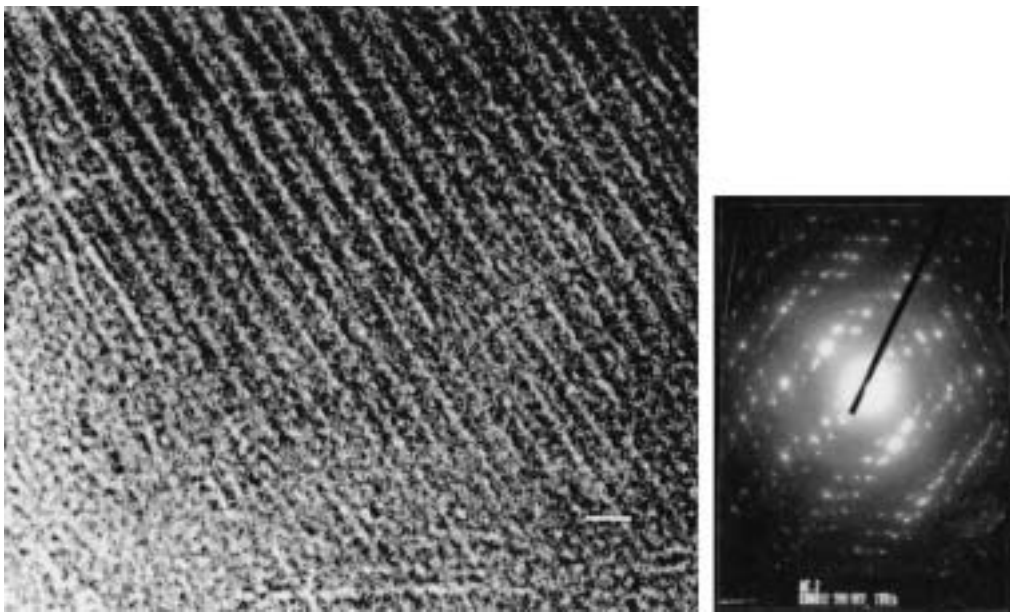

Figure 13: TEM image (left) and electron diffraction pattern (right) of superimposed crystalline lattices (unprocessed image of 33 nm x 33 nm. Three different lattices can be seen. The bar size is 2.5 nm (*Faunce* and *Paradies*, unpublished results).

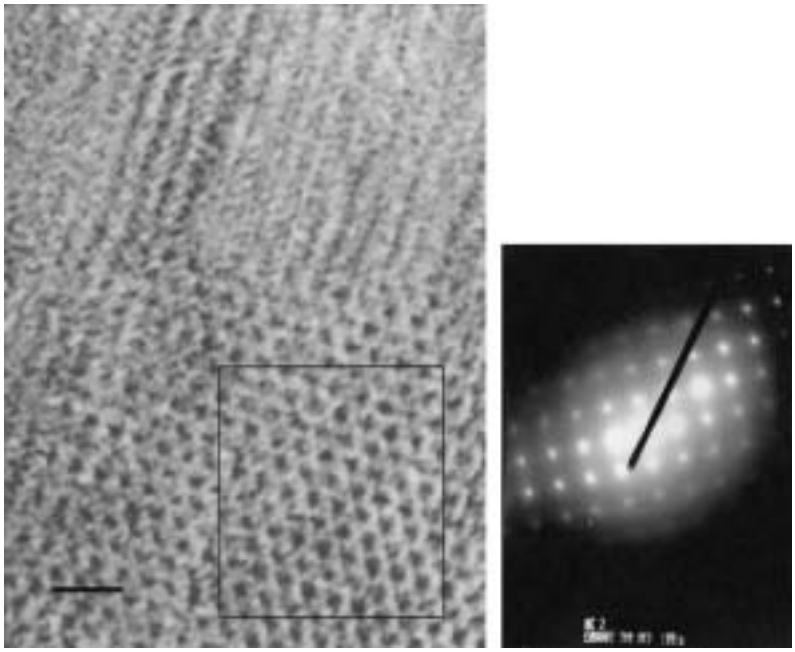

Figure 14: Highly ordered layer structure of autovaccines (left) in unprocessed molecular resolution and its electron diffraction pattern (right). A 30 nm x 30 nm image showing a defect molecular lattice (left). The sharp and symmetric reflections are indicative of long-range positional and orientational order (*Faunce* and *Paradies*, unpublished results)..

packing of the individual autovaccine molecule in the plane perpendicular to the long axis can be hexagonal (hcp), and that the autovaccine molecule are tilted toward nearest neighbours at angle of approximately 30°. From our data, it seems that the removal of the excess of counterions, e.g. NaCl, through tangential ultrafiltration (*Thies* et al., 2001; *Faunce* et al., 2001) plays a significant role in determining the multi-layered structure of the autovaccines and the formation of colloidal crystals. Particularly the influence of Ca^{2+}, Mg^{2+} and K^{+} ions play a dominant role in the formation and the substructure of the autovaccines as documented through the determination of the structure factors. In addition the particle number density determines whether a bcc or a fcc (hcp) lattice is formed as will be shown below. Apparently the acyl moiety of the autovaccines is, that determines the lattice parameters and symmetry that is compatible with close packing (*Kitaigorodskii*, 1961) gives the constraint imposed by specific counterions, and possibly the sugar backbone, too. Furthermore, the length of the fatty acid chains plays no severe role in the lattice parameters and symmetry, although it does have a limited effect on the details of the buckling superstructures for this class of autovaccines.

At high volume fraction ($4.15 \times 10^{-4} < Ø < 5.15 \times 10^{-4}$) and both ionic strengths (1.0 mM and 10.0 mM NaCl) and for $Ø = 4.75 \times 10^{-4}$ the x-ray spectra indicate the presence of long-range order (Figure 15). The assignment of the respective peaks to the crystal planes were made from comparison of the observed peak vectors according to

$$Q_{hkl} = \frac{2\pi}{\alpha}\left(h^2 + k^2 + l^2\right)^{1/2} \qquad (10)$$

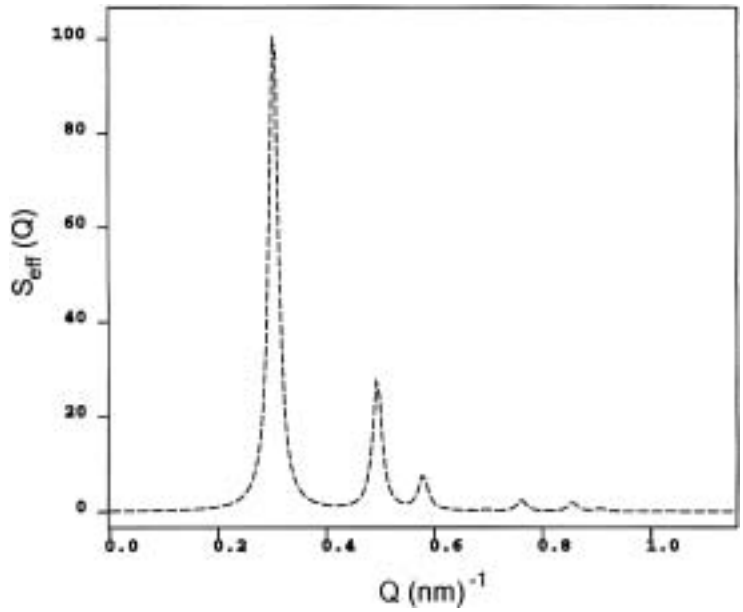

Figure 15: Small-angle-X-ray scattering, $S_{eff}(Q)$ vs. Q, obtained from autovaccines) dispersions at a volume fraction of $\varnothing = 4.75 \times 10^{-4}$ and 1.0 mM NaCl. The peak positions is assumed to be of a fcc type structure (*Faunce* et al., 2001).

where h, k and l are the Miller's indices and a is the unit cell size. The few peaks were assigned to be reflections originating from a face-centred cubic (fcc) lattice with a unit cell dimension of a = 57.25 ± 1.5 nm. The Bragg distances observed from the Q values using the Bragg equation $d = 2\pi/Q$ agreed with the corresponding values calculated from the sphere concentration. Due to the very few diffraction lines of the X-ray spectrum it is not possible to determine an appropriate space group unequivocally. Even with increasing volume fraction, no higher order peaks were found in the spectra and showed no definitive structure, e.g. at 1.0 mM NaCl and $4.15 \times 10^{-4} < Ø < 5.15 \times 10^{-4}$, strongly suggests that an ordered structure is present under this condition. However, no higher order peaks are found. The indication of long-range order behaviour of Lipid A (diphosphate) dispersions suggests that the degree of polydispersity associated with this charged system is tolerated. *Pusey* (1980) calculated the degree of polydispersity where colloidal crystals appear, and it did not exceed 0.07-0.11 (see above). Assuming that the time to form these structures of Lipid A (diphosphate) is related to the degree of polydispersity, it can be inferred that the increase in time of forming these colloidal crystals increases the degree of polydispersity.

The formation of colloidal crystals at volume fraction between $3.75 \times 10^{-4} < Ø < 4.15 \times 10^{-4}$ in the presence of 1.0 mM NaCl has also been observed and analysed by SAXS. Within this volume fraction regime the presence of long-range order can also be seen, but surprisingly the diffraction peaks are at different position (Figure 16). This X-ray spectrum was indexed on the basis of a bcc lattice with a = 36.14 ± 1.3 nm. From the X-ray spectra obtained at 1 mM NaCl and $Ø = 4.05 \times 10^{-4}$, it was assumed that the first peak corresponds

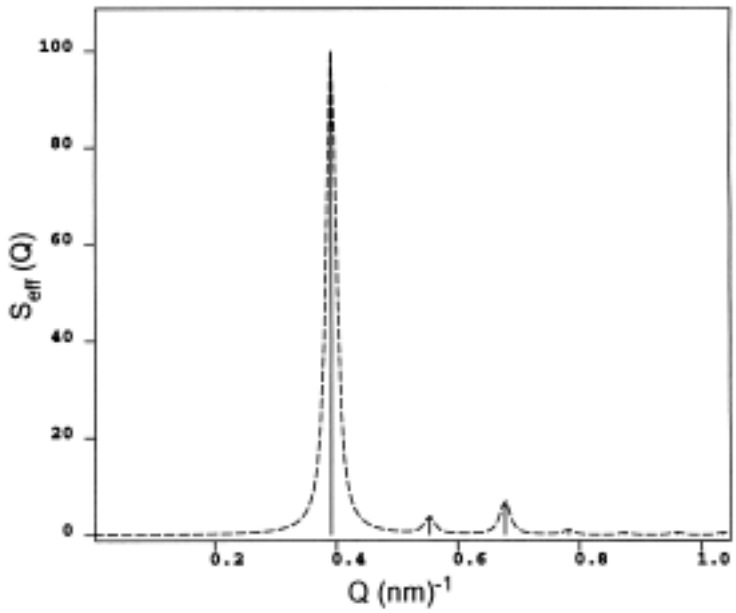

Figure 16: Small-angle X-ray scattering spectra, $S_{eff}(Q)$ vs. Q, obtained from autovaccines dispersions at a volume fraction of $\varnothing = 4.05 \times 10^{-4}$ and 1.0 mM NaCl. The peak positions is assumed to be of a bcc type structure (*Thies* et al., 2001, *Faunce* et al., 2001).

to that of the closest packed plane in the bcc structure, e.g. (110) having a spacing of 0.2458 nm^{-1}. Assuming that this first peak corresponds to the (110) planes, $d = d_{110}$, and for a bcc lattice, there should be two molecules per unit cell, and since the concentration is of Lipid A (diphosphate) is known, the molecular weight can be calculated according to

$$MW = (\sqrt{2} \cdot d_{110})^{1/3} \cdot c/2 \qquad (11)$$

From Equation (11) we calculated a molecular weight of 10.05×10^6 g/mol, which is consistent with the value found from light scattering and osmotic pressure experiments reported earlier. The molecular weight calculated from the fcc structure, which has four molecules in the unit cell, a value of 10.1×10^6 g/mol was found.

These two colloidal crystal forms apparently are in a sort of equilibrium when grown or appearing within a period of time of six to eight weeks. Increasing the subphase with Lipid A (diphosphate) at constant low ionic strength (1 mM or lower), we observe the formation of a fcc lattice in addition to the bcc lattice originally found. For both structures the diffraction lines are clearly distinguishable. Finally, after four weeks most of the colloidal crystals transformed to the fcc structure under this condition, if the concentration of the subphase of Lipid A (diphosphate) is kept constant ($\varnothing \cong 3.9 \times 10^{-4}$). Otherwise, a fraction of the fcc structure and the bcc structure will be present simultaneously.

It is noteworthy, that the quality of the diffraction data is strongly related to the sizes of the colloidal crystals of Lipid A (diphosphate) grown under deionised conditions. The relative intensity distribution (Figures 15 and 16) of the calculated and observed Bragg reflections, particularly those of the weak ones compared to the first order hkl

Bragg reflections, e.g. (110) or (111), are sensitive to variation in size of these colloidal crystals. The ultimate size and shape of the Lipid A (diphosphate) aggregate in form of a colloidal crystal, determines the geometrical arrangement, e.g. whether they have rounded, spherical or ellipsoidal or facetted shapes (hexagonal in shape), or as little cubic crystals, all observed in electron micrographs (TEM and SEM), and cannot not finely established presently but it is most likely.

Especially, for $0.1 \leq Q/Q_{110} \leq 0.75$ the Q dependence of the liquid phase and the crystalline bcc phase is very similar, and the peak in S(Q) for the liquid is close to $Q = Q_{110}$. This suggests a similarity in the structure of the liquid and the bcc crystal phase. But, taking the bcc structure, Q_{110}, to be at the first peak in S(Q), we would expect the second peak located at $Q = \sqrt{2} \times Q_{110}$, however, we notice a first minimum in S(Q) in the liquid state. Although we observe higher orders in the bcc structure, we only see a shallow peak in the liquid structure factor at $Q = 1.67 \times Q_{110}$, an indication that the wave vector dependence of the liquid dispersion of Lipid A (diphosphate) exhibits some common features with that for the longitudinal 110 lattice vibrations in the bcc crystal. It has been demonstrated by a number of authors (*Monovoukas* and *Gast*, 1989; *Okubo*, 1994; *Sirota* et al., 1982), that the bcc lattices are seen for systems which interact via long-ranged interactions, and this seems particularly true at low ionic strengths. A typically and sustained problem in colloidal systems is how to lower the ionic strengths. On the other hand, this system at 10 mM NaCl seems to be closer to the one for hard spheres than for one with the absence of ions, perhaps such a system is more sensitive towards the degree of polydispersity, and the observed degree of polydispersity can be one reason that the formation of long range order of a fcc lattice is inhibited, hence favours the formation of a bcc lattice (*Larsen* and *Grier*, 1997, *Cotter* and *Clark*, 1987). Furthermore, the very recent contribution by *van Roi* and *Hansen* (1997) offers an additional explanation, showing theoretically the coexistence of fluid-fcc and the fluid-bcc structures at very low ionic strength, is on energy terms very similar, since the free energy difference between fcc and bcc is much smaller than that between the fluid, and it is not possible to distinguish between fcc or bcc precisely. It is most likely, therefore, that the observations reported here are due to the existence of long-lived metastable colloidal crystallites at very low ionic strength.

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RESULTS OF AN OPEN, NON-PLACEBO CONTROLLED PILOT STUDY INVESTIGATING THE IMMUNO-MODULATORY POTENTIAL OF AUTOVACCINE

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SUMMARY

Autovaccines, a fundamental concept within Microbiological Therapy, are prepared from autologous, human, non-pathogenic, „rough" variants of *E. coli* derived from the stool flora of individuals according to a highly standardised procedure (SymbioVaccin, Germany). These autovaccines are mainly used to treat chronic inflammatory disorders associated with impaired immune reactions resistant to standard therapeutic treatments. Although immunomodulatory effects of outer membrane components or cell wall fragments of gram-negative bacteria on innate or adaptive immunity are widely accepted, mechanisms of actions of these autovaccines remained largely obscure, despite some recent publications about other autovaccine preparations of different origin. Therefore a pilot study was conducted with 78 outpatients from a physician in general practice. The patients suffered from variable disorders, ranging from recurrent respiratory infections to diffuse gastrointestinal complaints. To investigate whether the application of autovaccine affects non-specific and specific parameters of the immune system, the patients received their autologous bacteria parenterally in increasing doses. Before application and 4 to 6 weeks after application of autovaccine, blood samples of the patients were taken to investigate a range of immunological parameters such as acute phase proteins, serum antibodies and cytokines. The results revealed, that autovaccines were able to modulate significantly the release of three potent immunoregulatory cytokines but induces only slight changes in specific humoral immunity From these results it may be concluded that the autovaccine mainly acts antigen non-specifically on the cytokine level rather than inducing a high level of specific antibodies against autovaccine. Further studies with more detailed kinetic measurements of cytokines will have to verify these results.

INTRODUCTION

Autovaccines in the context of this paper are derived from human, non-pathogenic rough variants of *E. coli* isolated from the faecal flora of each patient. From the manufacturer of autovaccines (SymbioVaccin GmbH, Herborn, Germany), these bacteria were prepared according to a highly standardised procedure followed by heat-inactivation for 2h at 70°C (*Zielinski* et al., 1998). Autovaccines are used to treat chronic inflammatory disorders either associated with impaired resistance to infection or with hyperactivation of the immune system resistant to standard therapeutic strategies (*Rusch*, 1986). In fact, these heterogeneous indications underlying many sometimes unknown dysregulations of the immune system will require well-balanced immunoregulatory actions of autovaccines still unproved by relevant clinical observations up to now.

Although the application of autovaccines have a long traditional use going back to the beginning of the 20th century (*Wright* and *Douglas*, 1904), the underlying mechanisms of action are far from being clear. However, several recent reports described another form of autovaccine therapy inducing a specific immunisation, which has been shown for example by *Zaluga* (1998). This group examined immunological effects of an autovaccine preparation composed of *Propionibacterium acnes*, isolated from the skin of patients. These authors found a significant improvement in 47.6% of patients accompanied by functional changes in the immune system as for example a generation of specific antibodies against structural antigens of *P. acnes* (*Zaluga*, 1998). These observations were confirmed by others (*Rubisz-Brzezinska* et al., 1994) and agreed with reports demonstrating a therapeutic successful use of those autovaccines in children with severe nasal sinusitis (*Okrasinska-Cholewa*, 1994) or purulent otitis media (*Wilczynski* et al., 1995). Therefore the present study sought to investigate the immunomodulating potential of autovaccine preparations of autologous origin to clarify, whether there will be similarities or differences in immunological effects compared to those other autovaccines described recently. Thus, to elucidate the influence of the autovaccine on the immune system, an outpatient (n=78) collective recruited by a physician in general practice was used for the following study to examine changes in immunological parameters. The patients received the autologous bacterial preparation derived from their own stool flora and immunological parameters were determined before application and 4 to 6 weeks after application of the autovaccines.

As this was only a preliminary pilot study no placebo control was included. Because it was not known, which immunological effects, if any, would be elicited by the autovaccine, a broad panel of investigations was performed. Patients' peripheral blood leukocytes (PBLs) were used to determine *ex vivo* cytokine release by specific enzyme immunoassays (ELISAs). Among the cytokine family, granulocyte-macrophage-colony stimulating factor (GM-CSF), interferon-gamma (IFN-γ), interleukin-1beta (IL-1β), interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-10 (IL-10) and tumour-necrosis-factor alpha (TNF-α) were examined. In addition, non-specific serum parameters characterising inflammatory processes such as neopterin, C-reactive protein, soluble interleukin-2 receptor (sIL-2R), β2-microglobulin and interleukin-1 receptor antagonist (IL-1RA) were also deter-

Table 1: Demographic data of the participating patients

Number of patients	78
Patients available for two blood donations	60
Time span between sample donation	31 days (range 21-56) 25% percentile 28 days 75 % percentile 35 days
Female/male ratio	55/23 total 41/19 (second blood donation)
Age, median (range in parentheses)	36.5 (16-74) 25% percentile, 30 years 75% percentile, 44 years
Number of patients with previous history for autovaccine therapy	41/60 (68.9%)

mined. Finally, serum samples of patients tested before and after autovaccine therapy were used to detect a potential increase of specific antibodies of the IgG, IgM or IgA isotype against antigens of the autovaccines, using an ELISA technique.

MATERIALS AND METHODS

Recruitment of patients

The study population comprised 78 outpatients from a primary care physician (P.N.) at Berlin-Charlottenburg and were recruited for the present study during a time span between end of 1995 and beginning of 1997. Patients' informed consent was obtained to draw two blood samples. The demographic data of the patients are shown in Table 1. Further details were recently published (*Rusch* et al., 2001).

Test substances

The autovaccines used for application to the patients were prepared by the manufacturer from the stool flora of each patient. For the stimulation of PBLs from each individual donor, the corresponding autologous autovaccine preparation was used at 6 different concentrations ($3x10^2$ – $3x10^6$ bacteria/ml). A second control consisted of a polyclonal stimulus. This lectin mixture contained pokeweed mitogen (PWM), phytohaemagglutinin A (PHA) and concanavalin A each at a concentration of 1 ng/ml (all purchased from Seromed, Berlin, Germany). Lipopolysaccharide (1 ng/ml) derived from *E. coli* 055:B5 was obtained from Sigma/Aldrich Chemicals, Munich, Germany.

Preparation of peripheral blood leukocytes from the patients

For the investigation of the immunological effects of autovaccines, peripheral blood leukocytes (PBLs) of the patients were prepared as described recently (*Rusch* et al., 2001). Briefly, blood was taken immediately before application of autovaccine and 4 to 6 weeks later. For each patient, only a limited number of blood could be drawn, usually 30 ml, of which all cytokine determinations and preparation of serum or plasma had to be performed. After separation of cells by density centrifugation over Ficoll-Hypaque (Pharmacia fine chemicals, Freiburg, Germany) and several washing steps; the cell number was adjusted to $1x10^6$ cells/ml with cell culture medium.

Ex vivo treatment of patients PBLs with the test substances

To further investigate the role of the respective autologous bacteria used for the treatment of the patients, six different concentrations of each autovaccine were used for the additional *ex vivo* stimulation of the patients PBLs. The final concentration of autovaccine in the cell cultures were $3x10^2$, $3x10^3$, $3x10^4$, $3x10^5$, $1,5x10^6$ and $3x10^6$ bacteria/ml, together with $1x10^6$/ml PBLs. A polyclonal stimulus was also used, to determine the level of immunological reactivity of the patients' PBLs under these experimental conditions. Negative controls included medium treated cell cultures and all experiments were performed in duplicate wherever possible, using 24-well cell culture plates (Nunc, Wiesbaden, Germany). The incubation period of the cultures (37°C, 5% CO_2) started with the addition of the PBLs from the patients. Cells were incubated over a period of 6h for the determination of TNF-α. IL-1β, IL-2, IL-4 and GM-CSF were measured after 24 h; IFN-γ, IL-6 and IL-10 were measured after 48 h of incubation. To the indicated time points, cell culture supernatants were harvested by centrifugation (1200 rpm, 10 min., 4°C). After aliquotation, supernatants were stored at –80°C (4 weeks at least) before the determination of all cytokines with specific sandwich ELISA tests.

Preparation of serum for the determination of specific antibodies and markers of the acute phase response

Serum was prepared from whole blood after complete coagulation at room temperature for 3h according to *Rusch* et al. (2001). Aliquots were stored frozen at –25°C until determination of acute-phase markers and specific antibodies. β2-microglobulin and neopterine (IBL diagnostics, Hamburg, Germany), IL-1RA (R&D Systems, Wiesbaden, Germany) and sIL-2R (Biosource, Ratingen, Germany) were all determined by specific ELISA tests. Because the sensitivity of the commercially available ELISA test for CRP was too low, CRP was determined with an ELISA developed by Affina Immuntechnik GmbH (sensitivity of < 0.03 µg/ml CRP).

ELISA procedures: Cytokines

The details of the ELISA procedures were described recently by *Rusch* et al (2001). All cytokines were determined with specific Sandwich ELISA-tests, using commercially available antisera. For coating of the plates the following antisera were used: Murine anti-human IL-1β (2µg/ml, Genzyme, Munich, Germany), rat anti-human IL-2 (2µg/ml, PharMingen, Hamburg, Germany), murine anti-human IL-4 (1 µg/ml, PharMingen, Hamburg, Germany), murine anti-human IL-6 (1µg/ml, Boehringer Mannheim, Germany), rat anti-human IL-10 (1µg/ml, PharMingen, Hamburg, Germany), goat anti-human IFN-γ (kindly supplied by Prof. Dr. Noll / MDC, 5.2 µg/ml), murine anti-human-TNF-α (1 µg/ml, PharMingen, Hamburg, Germany) and rat anti-human GM-CSF (1 µg/ml, PharMingen Hamburg, Germany).

The appropriately diluted standards and samples, both diluted with complete cell culture medium as follows: the IL-2, IL-4, IL-10 and GM-CSF standards were purchased from PharMingen (Hamburg, Germany), whereas IL-6 and IL-1β were supplied by Boehringer Mannheim (Germany). The TNF-α standard was supplied by Calbiochem (Heidelberg, Germany) and the IFN-γ standard was kindly supplied by Prof. Cheperanov (Moscow, GUS). After incubation for 1 h at RT and three to four washes (each with 200 µl PBS/0.05% Tween 20) the second anti-

Table 2: Acute phase response markers in 39 serum/plasma samples available for testing before and after autovaccine application. The data are presented as median and range

Acute-phase protein	Before autovaccine application	4-6 weeks after autovaccine application	p-value
CRP (µg/ml)	0.55 (0.05-6.62)	0.41 (0.03-5.42)	0.042*
SIL-2R (pg/ml)	87.4 (28.5-1535.0)	79.2 (35.8-1737.2)	0.187*
ß$_2$-Microglobulin (mg/l)	0.84 (0.66-1.54)	0.85 (0.67-1.18)	0.807**
IL-1RA (pg/ml)	332.9 (177.7-726.6)	311.4 (127.1-1021.1)	0.572*
Neopterine (nM/L)	4.7 (2.6–10.4)	4.5 (2.8-9.2)	0.728*

*p-value calculated with the Wilcoxon-Test, Median
** p-value, calculated with the paired t-test, Mean

bodies were added as described recently, followed by the washing steps (*Rusch* et al., 2001). Thereafter, the substrate solution (Tetramethylbenzidine, TMB, Fluka via Sigma/Aldrich chemicals, Munich, Germany) was added (100 µl/well) and the plates were incubated at RT in the dark. The reaction was stopped with 50 µl 2N H_2SO_4 (Merck, Darmstadt, Germany). The optical density was measured at 450 nm in a microplate reader (Titertek Multiscan®, Flow Laboratories, Meckenheim, Germany).

Acute phase proteins and determination of specific antibodies and total immunoglobulin

The determination of CRP in patients serum/plasma samples and the determination of specific antibodies was performed as described recently (*Rusch* et al., 2001).

Statistics

The calculation of the significance of the data focused generally on an ANOVA principle including the comparison of the mean values ± standard deviation (S.D.) for cytokines for all 60 donors. Unpaired and paired t-test were used to compare cytokine values, acute phase markers and immunoglobulin differences between the different patients in the case of normal distribution of the data. Not normally distributed data were calculated with the Rank Sum test according to Wilcoxon and to Mann-Whitney. P-values between 0.001 and 0.05 were considered to be statistically significant.

RESULTS

Acute phase proteins

At the end of the study, plasma or serum samples from 39 patients were available for measurement of non-specific serum markers of inflammation (Table 2). No statistically significant differences between values obtained before autovaccine application and 4 to 6 weeks after therapy with autovaccine were seen. However, the median value for CRP was reduced about 23.5% compared to the concentration found before application of autovaccine. This reduction in CRP reached borderline significance (p= 0.042).

Specific antibodies and total immunoglobulin

The determination of total immunoglobulin demonstrated that an applica-

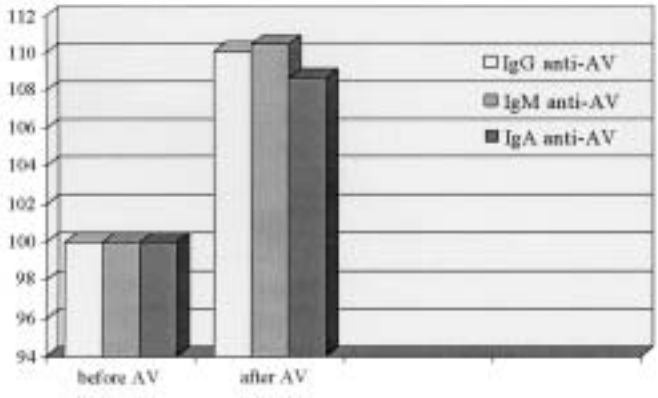

Figure 1: The percent values of autovaccine-specific immunoglobulins determined from 39 pairs of serum/plasma samples (taken the pretreatment values as 100%). Total antibody levels were measured by laser nephelometry. They did not change above pre-treatment values (data not shown). Autovaccine-specific immunoglobulins of the different isotypes were detected with an ELISA as described in materials and methods. Only at least 10% increase in specific immunoglobulins was seen.

tion of autovaccines during the observed time span of 4 to 6 weeks did not influence the total levels of the immunoglobulins IgG, IgM or IgA in plasma samples derived from the patients (the identical 39 pairs of samples as shown in Table 1, data not shown). There was no difference in antibody levels compared to pre-treatment. Only a very slight increase of specific antibodies against autovaccine of at least 10% was detected for the isotypes IgG, IgM and IgA. This is shown in Figure 1.

Influence of autovaccine on *ex vivo* cytokine release by patients PBLs

The comparative analysis of all cytokine mean values (±S.D.) for differences between the first examination (U1 = before autovaccine treatment) and the second time point (U2 = 4 to 6 weeks after application of autovaccine) revealed the following results: Figure 2 summarises the cytokine values for 60 patients under therapy with autovaccine after additional *ex vivo* stimulation of their PBLs with the respective autologous bacteria. The *ex vivo* stimulation of patients' PBLs resulted in a significant decrease of the cytokines GM-CSF ($p = 0.004$) and IFN-γ ($p= 0.007$). In contrast, the cytokine IL-1β was significantly increased at U2 ($p = 0.04$). Although IL-6 showed a tendency to increase after application of autovaccine, this enhancement reached no statistical significance ($p = 0.058$). All other cytokines measured remained unchanged under application of autovaccine, when the mean values of U1 and U2 were compared, whereby IL-4 remained below the detection level (see Figure 2).

DISCUSSION

This open pilot study with 60 patients demonstrated for the first time, that autovaccines prepared from autologous bacteria according to a standardised procedure are able to modulate the human immune system preferentially in

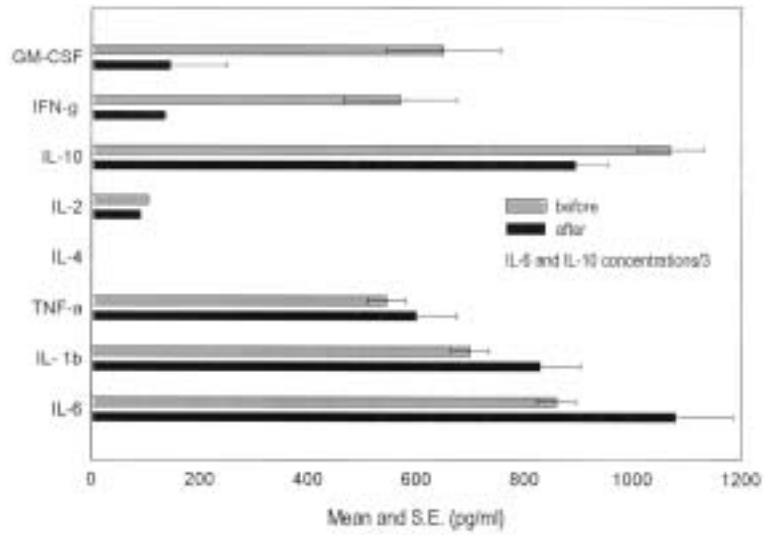

Figure 2: The cytokine mean values ± S.E. in pg/ml measured from cell culture supernatants from peripheral leukocyte cultures of 60 patients, before autovaccine application and 4 to 6 weeks after autovaccine application. The PBLs from each patient were treated *ex vivo* with the respective autovaccine and cytokines in the culture supernatants were determined as described in materials and methods. Statistically significant differences in cytokine production by patients' PBLs were observed with IFN-γ ($p<0.007$) and GM-CSF ($p<0.004$) as both mediators were significantly diminished after application of autovaccines. In contrast, the cytokine IL-1β was found to be enhanced ($p=0.04$). Please note that the concentrations of IL-6 and IL-10 had to be divided by the factor 3 for reasons of better graphical illustration.

an antigen-non-specific way, affecting the key „communication" molecules within in the immune response, the cytokines. Based on the literature concerning the immunological effects of other autovaccine preparations (*Zaluga*, 1998; *Rubisz-Brzezinska* et al., 1994; *Okrasinska-Cholewa*, 1994; *Wilczynski* et al., 1995), differences as well as similarities emerge between those autovaccines and the bacterial preparation tested in this study. Some of the bacterial preparations described elsewhere were for example obviously capable of inducing high levels of specific antibodies whereas the autovaccines examined herein failed to do so. On the other hand, thy cytokine profiles of patients PBLs were profoundly affected. The question is what causes these immunological differences between different preparations of autovaccines?

One explanation might come from the different origin of the bacterial strains used for the preparation of both types of autovaccines. Whereas for example *Zaluga* et al. (1998) used skin-derived autologous strains of *Propionibacteriaceae*, the autovaccines used in our trial were derived from the autologous faecal flora of the individual patients. The gastrointestinal flora nowadays is accepted as playing a major role not only in the pathogenesis of inflammatory gut diseases but also in the modulation of physiological reactions of the immune system (*Sartor*, 1997). Present knowledge on functions of the indigenous microflora of the gut includes profound effects on anatomical, physiological and immunological development of the host (*Berg*, 1996). Thus, it may not be a surprise that the individual faecal autologous bacteria of each

patient failed to induce a dramatic increase in specific antibodies above normal levels of pre-existing „natural“ antibodies.

However, even the small rise of Autovaccine-specific immunoglobulin isotypes opens the possibility for the interaction of different types of Fc-receptors with complexed gut-derived autologous *E. coli* antigens, which could initiate potent inflammatory pathways when not handled appropriately by the immune system. Generally receptors for the Fc domain of IgG (FcγR) represent a crucial link between the humoral and cell mediated immune responses. The ligation of these receptors can trigger a variety of immune effector functions (*Van der Winkel* and *Caperl*, 1993; *Daeron*, 1997; *Ravetch* and *Bolland*, 2001). So far the structural diversity of the different classes of FcR and their variable capabilities to deliver activating (FcγRI, FcγRIIa, FcγRIIIa) as well as inhibitory signals (FcγRIIb), as described by *Dijstelboem* et al. (2001), depends on the presence of an immunoreceptor-Tyrosine-based Activation Motif (ITAM) or an immunoreceptor Tyrosine-based Inhibitory Motif respectively. Therefore it is reasonable to assume that the control of cellular activities of both the innate and the adaptive immune system provides an efficient means by which FcR mediates immunoregulatory activities in controlling non-specific and even specific inflammation. This might have consequences for ongoing or memory T-cell and B-cell responses in terms of a down regulation of excessive activation. For example the inhibitory FcRγII was described to set thresholds for B-cell activation upon cross-linking with surface Ig, a mechanism whereby immune complexes can suppress the production of antibodies. In view of the inflammatory potential of non-cleared immune complexes this observation suggest for a profound role of FcR mediated immunoregulatory processes and constitutes an ideal link for the autovaccine to control innate and adaptive immunity.

In view of the outstanding role of the gastrointestinal immune system and the autologous flora in maintaining a certain state of tolerance against harmless luminal and food antigens but inducing an active immune response against infectious agents the tight control of peripheral immune responses should not be underestimated. With regard to cellular activation this recently demonstrated clearly by the experimental work of *Duchman* et al. (1995), showing tolerance to the gastrointestinal microbial flora but an enhanced peripheral immune response once tolerance was broken and conditions of impaired mucosal barrier. The report of *Kimura* et al. (1997) agreed very well with these observations. A large body of evidence derived from a vast amount of experimental animal data lead to the suggestion, that the gastrointestinal flora may also participate in the generation of mucosal inflammation (*Elson* et al., 1995; *Strober* and *Kelsall*, 1998), so that a tight control of immunoregulatory circuits (*Strober* et al., 1997) controlling the immune responses in the gut towards potential harmful infectious or harmless dietary antigens are of utmost importance.

In view of the cytokine profiles measured with patients PBLs in this trial, IL-1β, IL-6 and IFN-γ were reported to exert profound pro- as well as anti-inflammatory and immunoregulatory activities within the immune response (*Billau*, 1996; *Borish* and *Rosenwasser*, 1996; *Barton*, 1997; *Murphy* et al., 2000). Notably, in view of the pattern of cytokines influenced by the autovaccine treatment of patients it seems noteworthy to mention that in our study classical „pro-inflammatory“ cytokines, normally readily released in a

co-ordinated manner upon contact with bacteria and immune cells, such as TNF-α, IL-1β and IL-6, seem not to be upregulated simultaneously. It is generally accepted that in gram negative bacteria the Lipid A portion of LPS is the main component in stimulating these cytokines (*Brandenburg* et al., 1996). Our observation of immunological differences between the autovaccines described elsewhere in the literature and the autovaccine tested in this study, the latter stimulating rather the release of cytokines than inducing a specific antibody production, might be a hint for structural differences between those bacterial preparations and our autovaccine. Due to the amphiphilic nature of the LPS complex important structure-function relationships of endotoxines or the free Lipid A molecule exist (*Schromm* et al., 2000). Thus it may be conceivable that the *E. coli* strains used for the autovaccine preparation, representing particularly „rough" variants, may possess structural modifications of the Lipid A-core-polysaccharide complex leading to distinct biological responses of human leukocytes. Two recent studies with the application of purified endotoxines of normal *E. coli* to human volunteers showed only partial agreement with the cytokine profile measured with the autovaccines in our study (*Zimmer* et al., 1996; *Lauw* et al., 2000). Both reports described a marked downregulation of TNF-α, IL-2 and Interferon-γ shortly after low-dose intravenous endotoxin administration to healthy human volunteers. On the other hand, a concomitant short rise in IL-4 and IL-10 was observed. These observations led the authors to suppose a down regulation of cell mediated immunity. These reports may support the idea of structural differences between highly purified *E. coli* LPS preparations and the autovaccine used herein. However, taking into account the broad time span for measurement of *ex vivo* cytokine release of patients' PBLs, which was due to the preliminary character of the present study and the limited amount of blood available, differences in cytokine profiles after more detailed kinetic studies cannot be excluded. Further clinical examinations are needed to confirm these preliminary results and most importantly to obtain more information on the immunomodulating role of this highly interesting bacterial preparation.

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THE AUTOVACCINE: LINK BETWEEN INNATE AND ADAPTIVE IMMUNITY

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SUMMARY

Autovaccines are prepared in a special well-standardised manufacturing procedure by the SymbioVaccin GmbH at Herborn from rough non-pathogenic autologous *E. coli*, which were derived from the patients own faecal flora. Autovaccines, being an integral component of the Microbiological Therapy are being used to treat chronic non-specific inflammatory disorders and are applied to patients mainly parenterally (i.c. or s.c.). Common molecular and cellular pathways by which bacterial cell wall components of different Gram-positive and Gram-negative pathogens interact with immunocompetent cell types are summarised, although defined „receptor" recognising structural elements of the autovaccine are unknown at present. However, the recently discovered family of the so called Toll-like receptor proteins and the elucidation of common signalling pathways shared by Toll-like receptors and important cytokine proteins makes it likely that the autovaccines use some of them to exert their immunoregulatory functions. Based on recent experimental work with a whole blood culture system, demonstrating precise immunomodulatory effects of the autovaccine, some cardinal functions of anti-inflammatory and pro-inflammatory cytokines like IL-4, IL-10 or interferon-γ were briefly outlined. These examples clearly underline the close connection of the cytokines affected by the autovaccine with the innate as well as the adaptive immune system, for the cells producing them constituted lymphoid cell types (T-cells, macrophages, B-lymphocytes) as well as non-lymphoid cells (mast cells, basophils, fibroblasts, Langerhans cells, dendritic cells, endothelial cells) orchestrating the immune response to viral and bacterial invaders.

The intriguing reconstitution production by auotovaccines of the interferon-γ in lymphocytes taken from an individual unable to respond to classical T-cell receptor mediated stimulation, is commented. Finally, an immunoregulatory model is proposed, by which the autovaccine, as a Lipid A analogue but avoid of any toxic properties associated with the Lipid A, may influence the innate and adaptive immune response in a remarkable way. This raises justified hope for the future to use autovaccines as a general anti-inflammatory and immunomodulating drug for the treatment of non-specific inflammation often accompanying dysregulated immune responses in humans with diseases like autoimmunity, cancer or IgE-mediated allergies.

THE TOLL LIKE RECEPTOR FAMILY: AN IMPORTANT LINK BETWEEN INNATE AND ADAPTIVE IMMUNITY

Toll-like receptors recently described in mammals are related to the *Drosophila* toll proteins and function to help the innate immune system recognising pathogen-associated molecular patterns (PAMS), that are expressed on infectious agents, but not on host cells. The search for homology of *Drosophila* Toll proteins with those in the mammalian system led to the discovery of the human Toll proteins by *Medhitov* (1997) and *Janeway* and *Medzhitov* (1999). Subsequently, different subtypes of TLRs were characterised in more detail, illustrated by Figure 1**.**

Based on the similarity in the cytoplasmic portions (designated the Toll-IL-1R or TIR domain), TLRs are related to the IL-1 receptor. However, the extracellular portions of the different TLR subtypes differ in length of their extracellular domains, typically composed of so-called leucine rich repeats (LRRs). In contrast, the IL-1R contains three immunoglobulin-like domains. The genes of these proteins are dispersed throughout the genome and despite their sometimes overlapping recognition of both gram-positive and gram-negative bacterial cell walls, the sequence homologies between different TLRs are not extensive: for example the extracellular domains of human TLR4 and human TLR2 are only 24% identical (*Akira* et al., 2001; *Beutler*, 2000; *Anderson*, 2000). Searching human and mouse databases revealed at present about 10 members of the TLR family, expressed in a cell type specific manner and presumably differentially regulated under conditions of inflammation by the environmental cytokine milieu (*Cario* and *Podolsky*, 2000; *Cario* et al., 2000).

As shown in Figure 1, TLRs are triggered by different agonistic stimuli which may induce after binding different signal transduction pathways (*Rabehi* et al., 2001; *Akira* et al., 2001).

For example agonists for TLR-4 include Gram-negative bacterial cell wall components, heat shock proteins, LPS, the plant product Taxol, viral proteins and flagella-derived proteins. In contrast, the subtype TLR2 was described to react predominantly with bacterial cell wall compounds specific for Gram-positive bacteria such as muramylpeptides or lipoteichic acids. Additionally, bacterial ligands present in both Gram-negative and Gram-positive bacterial cell walls such as lipoproteins and glycolipids bind to TLR2 as well as mannosylated phosphatidyl-Inositol and different lipoproteins from *Prophyromonas*, *Leptospira* and *Borrelia* species (*Akira* et al., 2001). Furthermore TLR2 recognises whole bacteria, yeast cell walls and peptidoglycans. The findings that TLR-2 could recognise some special LPS-subtypes expressed by *Leptospira* spp. and *Prophyromonas gingivalis* underline that subtle differences in the lipid-A structure might use different TLRs. Concerning the function of TLR6 (not shown in Figure 1) this subtype was reported to synergise with TLR2 in the recognition of Gram-positive bacteria, because cytoplasmic portions of the TLR2 functionally pairs with that of TLR1 or TLR6 stimulating cytokine production (*Akira* et al., 2001).

TLR5 recognises flagellin, a 55 kDa monomer protein derived from bacterial flagella. Thus, flagellin constitutes another important virulence factor of both Gram-positive and Gram-negative cell walls. Experimental evidence that TLR5 is involved in the recognition of flagellin came from expression of the flagellin gene into non-flagellated *E. coli* demon

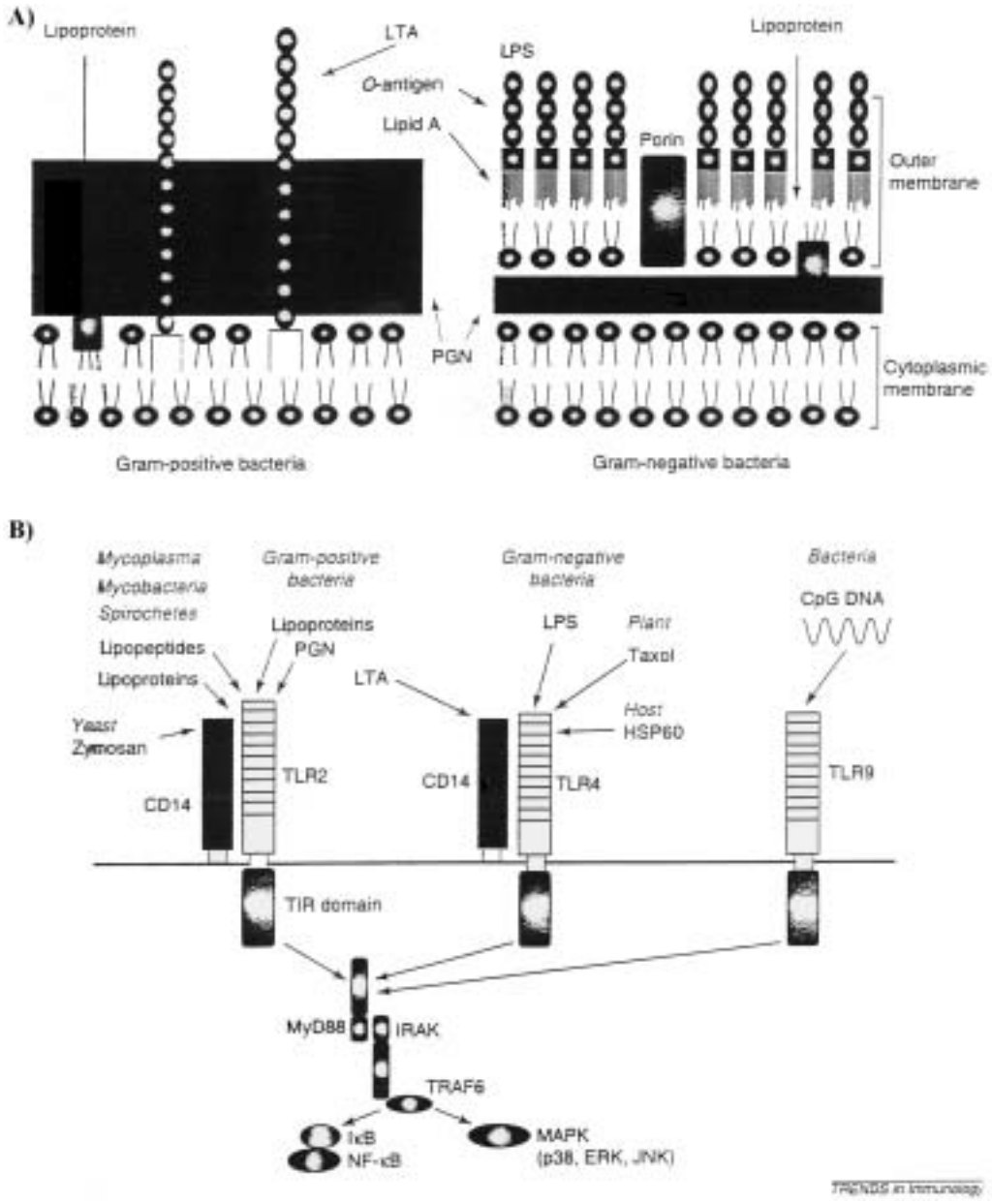

Figure 1: Subtypes of Toll like receptors.

strating that these bacteria were able to activate the TLR5, whereas deletion of the flagellin genes *from S. typhimurium* abrogated TLR- stimulating activity. At least TLR9 bind to bacterial-derived CpG oligonucleotides (*Wagner*, 2001). Again, this has been demonstrated by genetic engineered mice lacking the TLR9 gene, being completely unresponsive to stimulation by bacterial derived CpG oligonucleotides. In contrast, both null mutations for either TLR2 or TLR4 did not affect the ability of CpG oligonucleotides to enhance

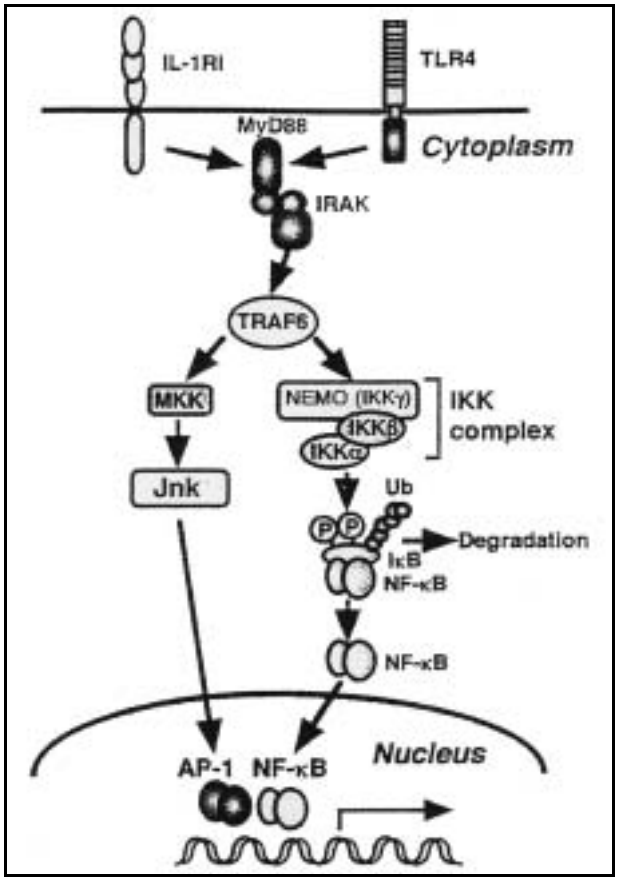

Figure 2: Signalling pathways mediated by Toll-like Receptor 4 in comparison to the IL-1R.

cytokine production by several cell types (B-cells, dendritic cells, monocytes) making it likely that this TLR subtype functions mainly as a sensor for bacterial DNA or DNA fragments (*Akira* et al., 2001).

INTRACELLULAR PATHWAYS INVOLVED IN SIGNALLING BY TLRs MAY BE DIVERGENT AS WELL AS CONVERGENT

Toll like receptors as type 1 transmembrane proteins are evolutionarily conserved between insects and humans, representing therefore the long searched „missing link" in the generation from an initially antigen non-specific immune response to the development of highly specific T-cell and B-cell mediated effector memory responses. The endogenous signalling pathways used by agonist-stimulated TLRs are partially unravelled (Figure 2). TLR4 signalling needs a small protein physically associated with the extracellular domain of TLR4 on the cell surface, that is the MD-2 protein. It was identified when TLR4 overexpression in a human fibroblast cell line called HEK293 did not automatically led to LPS responsiveness assuming that other molecules beside

TLR4 and CD14 may be essential for TLR4-mediated signalling. This was identified as the secreted protein MD-2 (*Viriyakosol* et al., 2001).

Considering the physical interactions of TLR4 with LPS it is not clear, whether TLR4 interact directly with the different bacterial cell wall components or whether CD14 transported LPS generates a signal able to initiate binding to TLR4, which resembles the pathways of the Toll protein activation cascade described for *Drosophila*. However, cross-linking experiments suggests that LPS is recognised in close proximity to CD14, MD-2 and TLR4. So far, the CD14 protein acts as a principal LPS binding protein so that it is reasonable to assume that these complexes interact with TLR4 in close association with the secreted MD-2, the latter increasing the affinity of LPS binding by stabilisation of the LPS/CD14/TLR4 complex. Indeed, this has recently been experimentally verified by the experimental work of Jiang et al. (2000) demonstrating, that LPS induced physical proximity between CD14 and the TLR4 prior to nuclear translocation of the NF-*k*B.

What may be of utmost importance concerning the partially overlapping signalling pathways of agonist stimulated TLRs and cytokine receptors is the observation, that the structural similarities between the IL-1R and TLRs in their cytoplasmic regions results in the use of common components for downstream signalling events, shown in Figure 2.

Binding of the respective ligands e.g. Interleukin-1β to IL-1R or LPS to the TLR4 was followed by recruitment of a cytoplasmic protein called myeloid differentiation antigen 88 (MyD88) through the TIR domain common for both receptors (*Akira* et al., 2001). The adaptor protein MyD88 links the TLR and the IL-R to the IL-1R associated kinase (IRAK) being a serine-threonine kinase linked to the pelle kinase of *Drosophila*. Upon phosphorylation of IRAK this kinase dissociated from the receptor complex and interacted with another adaptor protein also common for both pathways, the so-called TNF receptor-associated factor 6 (TRAF-6). Upon this step both pathways split into divergent signalling events distally: One activated the c-Jun NH_2 -terminal kinase (JNKs) or p38-mediated pathways whereas the other involved the degradation of the inhibitor of the NF-*k*B (=I*k*B) which binds normally to the cytoplasmic NF-kB family of transcription factors to hinder their import to the nucleus. Phosphorylation steps of the inhibitor of NF-*k*B is mediated by the I*k*B Kinase complex (=IKK) complex, containing several isoforms known as IKKα, β, and γ-isoforms which were shown to exert quite different functions (*Hatada* et al., 2000) in view of their extent of phosphorylative capacity. Meaning that the IKK-β is more potent that the IKK-α. Moreover IKK-β displayed a higher kinase activity towards the I*k*B-alpha subunit. The biological importance of this signaling pathway is illustrated by the fact that IKK-β homozygous deficient mice die as embryos and show massive liver degeneration due to hepatocyte apoptosis. Upon activation of the IKK-complex the inhibitor of NF-*k*B is sequestered to a ubiquitin-proteasome pathway to release the NF-kB p50-Rel dimers followed by their translocation to the nucleus and binding to their corresponding promotor regions of NF-*k*B inducible genes. So far the most intensively studied pathways of the IKK activation include those originating from the stimulation of the TNF-α IL-1β receptors (*Hatatda* et al., 2000).

Of note, MyD88 independent pathways has been reported recently with MyD88 homozygous deficient mice (*Akira* et al., 2001). These animals were completely unable to respond to

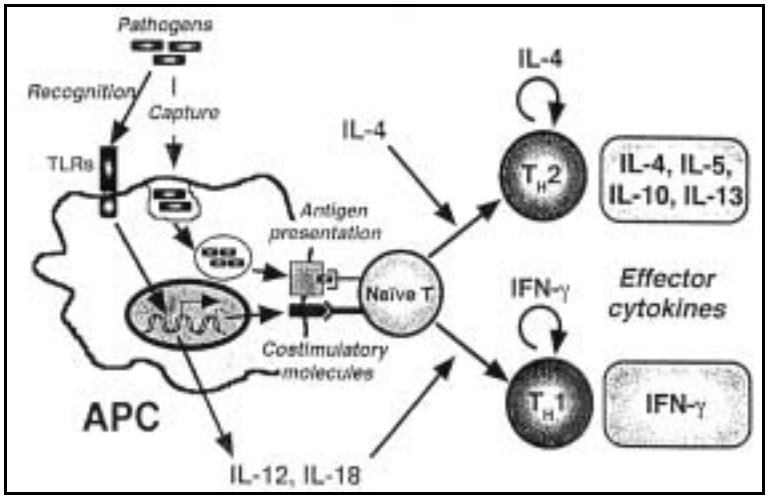

Figure 3: Impact of bacterial cell wall components on the polarisation of T-Helper cell sub-populations.

IL-1, IL-18, LPS, lipopeptides and peptidogylcans indicating that MyD88 being indispensable for signalling by these bacterial cell wall compounds. However subtractive hybridisation cloning identified genes in mouse macrophages that were induced by LPS independently of MyD88. These genes include the so-called IFN-γ inducible genes among them the IP-10 (IFN-inducible protein 10), the glucocorticoid attenuated response gene 16 (GARG 16) and the IFN regulated gene 1 (IRG-1). The expression of these genes need TLR4 but downstream activation pathways may involve IFN regulatory factor 3 (IRF-3) and NK-*k*B according to *Akira* et al. (2001).

IMPACT OF BACTERIAL CELL WALL COMPOUNDS ON THE GENERATION OF A POLARISED T-HELPER CELL RESPONSE

Additional studies on TLR signalling revealed the participation of phosphatidyl-inositol 3 Kinase (PiP3 kinase) in TLR2 signalling which may also involved in T-cell receptor triggered activation of T-lymphocytes, generally being accompanied downstream by the activation of the calcineurin pathway (*Robey* and *Allison*, 1995; *Putney* et al., 1986; 1993; *Whitney* and *Sutherland*, 1972; *Pai* et al., 1994). TLR-2 signalling events were also reported to be mediated by G-protein sensitive pathways for example the small GTPase „ras“ (*Heldwein* et al., 2001), linking T-cell activation through the mitogen activated family of protein Kinases (MAP-Kinases). This latter very important family of signalling molecules found in cells of the innate and adaptive immune system include the extracellular related kinases (ERKs), the p38 MAP Kinases and the JNKs (*Rincon*, 2001). Thus it may be conceivable that intracellular signalling events mediated by TLRs upon binding of their respective bacterial ligands and for example the autovaccine may result in signalling cascades important for both the innate immune response (classical pro-in-

flammatory bacterial-induced cytokines like TNFα, IL-1β, or IL-8) and the adaptive immune response (Il-4, IL-5, INF-γ) (*Genot* and *Cantrell*, 2000).

Getting the puzzle together a very simplified scheme results shown in Figure 3: Here the ligation of TLRs and/or the presentation of bacterial antigens by professional phagocytes profoundly promote through monocyte activation the polarisation of the well known T-helper cell subpopulations into the Th1 or Th2 differentiation pathway, reflected by the long held paradigm that bacterial infections initiate a strongly polarised Th1 response according to *Romagnani* et al. (1994).

Hence, Figure 3 illustrate the impact of bacterial pathogens for the regulation of the T-helper lymphocyte development either by agonistic stimulation of the Toll-like receptors on antigen presenting cells (APCs) or their capture by phagocytosis resulting in the generation of cell mediated specific immunity. Accordingly, Figure 3 mainly focused on the instructive role some cytokines will have on the commitment and differentiation of the different T-helper cell subpopulations (*Romagnani*, 1996; *Dinarello*, 1999). By interaction of bacterial cell wall compounds or whole bacteria with TLRs, cytokines such as interleukin-12 or interleukin-18 are induced, which may skew the further development of naive CD4 T-cells into a predominant Th1 like differentiation pathway. Over the past several years the processes by which naive resting CD4 T-helper cells expand and differentiate into much larger populations of highly active effector T-cells and smaller populations of memory T-cells have been the subject of very intense research (*Jancovic* et al., 2000; *Swain*, 1999).

Triggering of the T-cell receptor by antigens presented by different APC subtypes profoundly affects the spatial and time-dependent interactions of the T-cell receptor/MHC-antigen complex, mediated by the stepwise upregulation of various co-stimulatory molecules subsequently to TCR ligation, leading to the generation of the „immunological synapse“ (*Lanzavecchia* and *Sallusto*, 2000). The model of the immunological synapse may be a useful one illustrating some structural similarities between neuronal and immunological signalling transduction events. It postulates that the sequential upregulation of co-stimulatory molecules and their binding to their respective ligands on APC subtype not only profoundly dictates T-cell proliferation, maturation and differentiation but also increased avidity of the TCR (*Margulies*, 2001; *Viola*, 1996).

In addition, the surrounding cytokine milieu at the time of antigenic contact like the presence of IL-4, synthesised by activated mast cells or basophils or the presence of IFN-γ, secreted by natural killer cells (NK-cells) through macrophage derived IL-12 respectively, may further direct the polarisation into either the Th2- or Th1-pathway respectively, whereupon activated effector cell populations continue to secrete their predominant cytokines as described by Romagnani et al. (1996). However, care should be taken to oversimplify this scheme for the following reasons: The polarisation of the immune response culminating in cell mediated, antiviral or humoral immune responses against diverse infectious agents should not be regarded as a stable phenomenon. This implies that the commitment of a polarised T-helper cell population may be also reversed dependent on the microenvironment in the tissue (*Aebischer* and *Stadler*, 1996).

Accordingly, it has been suggested that CD4 polarisation may represent distinct developmental stages in T-cell differentiation and maturation pathways rather than a lineage-dependent maturation pathway, which can further be

dramatically influenced by a variety of other parameters tissue (*Aebischer* and *Stadler*, 1996): Common hypotheses on the factors inducing and regulating T-helper cell commitment and activation include among several still unknown factors the duration and the strength of T-cell receptor activation (*Iezzi* et al., 1999), the class and number of co-stimulatory molecules upregulated (*Sperling* and *Bluestone*, 2001), the nature of the antigen-presenting cell (*O'Garra* and *Murphy*, 1996; *Liu* et al., 2001; *Banchereau* and *Steinman*, 1998). The characteristics of the antigen or pathogen itself (physical structure, high vs. low antigen doses, parenteral vs. mucosal penetration) do play a prominent role in the decision process of the immune system to ensure the most effective way for elimination of the pathogen. All these variables might have a profound impact on the outcome of an immune response in view of tolerance induction or specific immunity.

From a biological point of view it is more reasonable to propose that in most instances the generation of an appropriate immune response against pathogens often depends on a mixed T-helper cell response (*Aebischer* and *Stadler*, 1996), so that only an over-activation of either T-cell population may be considered to have severe consequences for the host, particularly in view of the development of chronic inflammatory processes. Hence, a tight control of the immune response to infectious antigens by means of the regulation of cellular activity seems of utmost importance for the organism to avoid chronic inflammatory disorders or autoimmune diseases. Out of the numerous mechanisms underlying this control, the soluble mediators known as cytokines act as important immunoregulatory molecules. Their overproduction as well as their failure has been considered in the past to be associated with several important disease entities in humans, such as asthma or atopic diseases (*Brod*, 2000; *Barnes*, 2000; *Biedermann* et al., 2001; *Djukanovic*, 2000).

CYTOKINES INFLUENCED BY THE AUTOVACCINE IN WHOLE BLOOD CULTURES AND THEIR RELATION TO CHRONIC INFLAMMATORY DISEASE PROCESSES

The most popular functions of those cytokines whose synthesis were remarkably influenced by the autovaccine in the whole blood culture model is summarised in Table 1: It could be demonstrated that the autovaccine profoundly modulated the release of monokines as well as T-cell derived cytokines. Among them were IL- 4, IL-5, TNF-α, IL-10, IL-12 and IFN-γ. All cytokines, being profoundly modulated in their release by the autovaccine, are produced by lymphoid and non-lymphoid cell types (*Hunter* and *Reiner*, 2000). Thus their modulation by a bacterial immunomodulator reflects in an ideal way the important role of the autovaccine as a link between innate and adaptive immunity. IL-4 is considered to be the hallmark of those cytokines selectively produced in a Th2 type dominated immune response. It drives for example the differentiation of naive B-cells into IgE producing plasma cells. Therefore Th2 like T-lymphocytes are generally accepted to participate in type 1 allergic responses of the skin and the airways. However, CD8 T-cells were also reported to produce IL-4 (*Brown* and *Hural*, 1997). Originally, IL-4 has been described as a B-cell differentiation factor after antigenic stimulation.

Table 1: Short characteristic of immunoregulatory actions of cytokines whose synthesis was found to be modulated by the autovaccine in the whole blood culture system

Cytokine	Producers	Key functions in the immune response	References
IL-4	CD4-T-cells, mast-cells, basophils	Facilitates Th2 cell polarisation Activates Mast cells IgE-isotype switch in naive B-cells	Borish et al., 1996 Brown et al., 1997
IL-5	CD4-T-cells, eosinophils, Mast-cells	Eosinophil activator mast-cell activator	Borish et al., 1996 Teran et al., 1999
IL-10	Th1-cells, Th2-cells, T-reg-cells, Monocytes, macrophages, B-cells	Downregulates Th-1-cells and inhibits macrophage functions Growth factor for cytotoxic T-cells and mast cells	Borish et al., 1996
IFN-γ	NK-cells, Alveolar macrophages CD4-T-cells CD8-T-cells	Pro-inflammatory, anti-inflammatory and immunosuppressive actions dependent by the cellular activation state	Billau et al., 1990 Billau, 1996

IL-4 is mainly secreted by antigen-activated CD4 T-lymphocytes but mast cells, a subset of T-cells (the so called NKT-1 cells) and the basophils could release IL-4 (*Brown* and *Hural*, 1997). IL-4 stimulates the expression of co-stimulatory molecules like the B7-antigens, induced CD40 expression on B-cells and synergises with other B-cell activating cytokines like IL-2, IL-5 and IL-6 to increase antibody production. However, IL-4 possesses also anti-inflammatory properties as it inhibits monocyte production of IL-1, TNF-α and PGE_2.

Beside its action on B-cells and its pro-inflammatory effects during the initiation and maintenance of an allergic response, IL-4 controls the activation and differentiation of cytotoxic T-lymphocytes, which are essential for anti-tumour and antiviral immunity, so that IL-4 may be regarded as an important anti-tumour cytokine. Moreover, antibody-dependent cellular cytotoxicity and a downregulation of monocyte activation were general features attributes to IL-4 (*Borish*, 1996). During an allergic response, IL-4 enhances the recruitment of activated CD4 T lymphocytes into the airway epithelium by upregulating adhesion molecules on vascular endothelial cells.

In view of its potent pro-inflammatory role in allergy, IL-4 shares similarity with another mainly Th2-derived cytokine namely IL-5. IL-5 synthesis was found to be also markedly downregulated by the autovaccine in human leukocyte whole blood cultures. Beside its originally described growth promoting and differentiating effects on B-lymphocytes, IL-5 is a well known mediator of eosinophil activation (Borish, 1996; Teran, 1999). *In vitro* IL-5 was reported to induce chemo-attraction, trans-endothelial migration and superoxide production by human eosinophils and initiates their degranulation and prolonged their survival. The overexpression of the IL-5 gene in mice was accompanied by a fulminant eosinophilia, which can be blocked by a monoclonal antibody against IL-5.

In humans IL-5 mRNA was found to be upregulated in bronchial biopsies of asthmatic individuals and IL-5 protein was detected in the broncho-alveolar fluid of asthmatics (*Borish*, 1996; *Hamid* and *Minshall*, 2000). IL-5 is supposed to interfere with eosinophil survival by upregulation of the expression of anti-apoptotic molecules and the overexpression of the NF-*k*B transcription factors. IL-5 profoundly synergises with the cytokines IL-3 and GM-CSF in the recruitment of other non-lymphoid cell types profoundly involved in the generation and maintenance of an allergic response in the case of type 1 allergies. These are the mast cells and the basophils.

The overlapping and partially synergistic functions of IL-4 and IL-5 are good examples for the close interplay between different cytokines in disorders with an underlying dysregulation of the immune system and clearly underlines the clinical relevance and clinical importance of immunotherapeutic interventions. Targeting synthesis of different cytokines may result in a real immuno-modulating effect instead of a mere inhibition achieved by the suppression of only one cytokine (*Djukanovic* et al., 2000; *Biedermann* et al., 2001; *Creticos*, 2000). For the same reason, the modulation of the synthesis of interleukin-10 by the autovaccine constitutes an important means to mediate immunoregulating effects.

Interleukin-10 was originally described in the mouse system as a „cytokine synthesis inhibitor" predominantly acting on Th1 type T-lymphocytes and macrophages. However, there are important differences regarding the cellular source of IL-10 between different species: In the mouse system IL-10 is mainly produced by CD4 Th2 lymphocytes, whereas in the human system IL-10 can be synthesised by LPS activated monocytes/macrophages, by antigen activated Th1 and Th2 T-helper cells, by so called T-regulatory cells 1 (Tr1) and in an antigen non-specific way by mast cells and by B-lymphocytes.

IL-10 inhibits as an example interleukin-5 release by activated Th2 like cells thereby interfering with their growth and differentiation. Of note, IL-5 release was obviously suppressed by IL-10 in a different manner in response to different stimuli as demonstrated experimentally by Akdis et al. (2000), whereas in PMA/anti CD28 activated CD4 T-cells IL-5 release was abrogated, T-cells stimulated by the combination of ionomycin/PMA remained resistant to the inhibition by IL-5. This suggests that IL-10 suppression may target the activation signals generated by the costimulatory molecules B7/CD28**.**

In addition IL-10 downregulates the activation of macrophages, being one of the most important cell types linking innate and adaptive immunity. This inhibition was shown to be mediated either at the level of antigen-presentation by downregulation of co-stimulatory molecules or by the inhibition of the release of macrophage derived pro-inflammatory cytokines. Among them TNF-α, IL-1β, IL-6 or growth factors like GM-CSF or IL-3 were inhibited by IL-10. Likewise the activation of mast cells with their release of IL-3 and GM-CSF is negatively influenced by IL-10. Alle these actions are useful events to suppress the eosinophilic response generally observed in allergic asthma, leading in the past to the concept of IL-10 as an anti-allergic cytokine (*Pretolani* and *Goldman*, 1997). Most importantly IL-10 was found to interfere with the IFN-γ synthesis by activated CD4 Th1 helper lymphocytes participating in the activation of macrophages.

Particularly IFN-γ is considered to be one of most pleiotropic cytokines within the immune system, whose synthesis and expression in different

organs should be controlled very carefully, due to its highly pro-inflammatory effects on macrophages at one hand and its antiproliferative role on activated B-lymphocytes at the other hand.

By this two-sided effects of IFN-γ the complexity of activities of this cytokine become apparent. For example it can induce a striking induction of specific antibody production in activated B-cells but can also suppress antibody production. During infectious diseases, the proper activation of macrophages by IFN-γ helps the body to eliminate the infectious agent, but excess macrophage activation may cause severe acute or even chronic inflammation in target organs being sometimes fatal to the host (*Billau* and *Dijkmans*, 1990; *Billau*, 1996).

Accordingly, the cell type in the immune system being largely affected in its activation profile is obviously the monocyte/macrophage although other cell types like endothelial cells, dendritic cells or Langerhans cells may response to IFN-γ in some way. Thus important functions generally associated with immunoregulatory actions of IFN-γ are the dramatically enhancement of macrophage functions, for example intracellular killing of bacteria during cell mediated immune responses and lysis of tumour cells. Therefore a modulation of the IFN-γ response is considered to be ideally suited to affect innate as well as adaptive immunity. Considering the actions of IFN-γ on monocytes and macrophages, the activation state of the responding cell type dictates markedly the outcome of the response. In so far IFN-γ is often considered to function as a „priming signal". In view of the activation of Th2 T-lymphocytes during a type 1 allergic response, IFN-γ counteracts IgE response due to its antiproliferative effect on activated B-cells. Thus the balance between IL-4 and IFN-γ production may considered to be an important immunoregulatory pathway valuable for the downregulation of an overshooting Th2 dominated response (*Creticos*, 2000). Even in disease free intervals of allergic diseases such as allergic rhinitis, persistence of low level inflammation may cause over time a dangerous change in functions of the immune system so that a timely intervention in this process might be regarded as clinically very relevant to improve disease symptoms (*Ricca* et al., 2000).

SOME SPECIAL EFFCTS OF THE AUTOVACCINE ON LYMPHOCYTES ARE NOT SHARED BY OTHER CLASSICAL BACTERIAL DERIVED IMMUNOMODULATORS SUCH AS LPS

Clearly, some but not all cytokines modulated by the autovaccine, such as TNF-α, IL-10 or IL-6, can also be induced by the LPS molecule itself. So what are the real difference in the activation of the leukocytes by autovaccine or LPS? The complexity in the cytokine network increases as nearly every cell type in the body can respond to LPS with an upregulation of genes encoding a vast amount of growth factors, hormones or lipid mediators. Cellular responses upon LPS are dependent on the activation state and the surrounding cellular milieu, the type of the cell, number and affinity of receptors on the cell membrane and much more. So it is not surprising that Lipid A as the biological and toxic moiety of the LPS complex can beyond critical concentrations lying in the nanogram range induce fatal overactivation of the immune system resulting in the known sepsis syndrome and multi-organ failure. Not

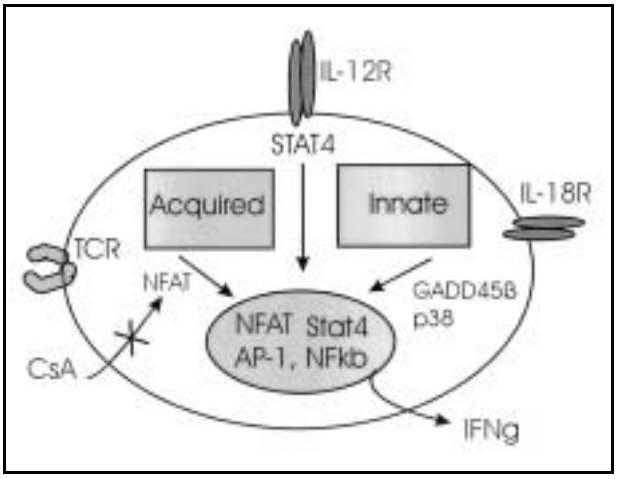

Figure 4: Innate and acquired pathways of Interferon-γ production.

so with the autovaccine: this molecule derived from rough *E. coli* has virtually any pyrogenic activity in rabbits and no apparent toxicity despite similarities in its structure with the Lipid A molecule.

So it may reasonably be assumed, that the responses of the immune system to autovaccine vs. Lipid A were only partially be comparable but even more complex with regard to the regulatory functions of autovaccine. Structurally, both autovaccine and Lipid A are partially related, but in view of the activation of the immune system, important differences emerged, demonstrated by the different dose-response curves in whole blood cultures of individual donors stimulated with autovaccine and the highly different inter-individual responses on the autologous bacteria compared to the homologous bacteria.

Generally it has been thought that IFN-γ may exclusively be produced by NK cells upon IL-12 released by macrophages or by TCR triggered T-lymphocytes in an antigen-dependent manner (*Billau* and *Dijkmans*, 1990). Considering the observed effects of the autovaccine being able to restore the Interferon-γ production by T-lymphocytes unable to respond to TCR ligation, some recent data may help to explain these findings.

For example this reconstitution of the IFN-γ production may agree with a recent report in the literature, that IFN-γ could also be induced in an antigen-independent manner by the combined actions of both IL-12 and IL-18 on T-lymphocytes, independent on the triggering of the T-cell receptor (*Yang* et al., 2001). Interleukin-18 being a member of the IL-1 family (*Akira*, 2000) signalling presumably through the same pathway like IL-1, shares intracellular similarities with the TLR-4 pathway, so that bacterial stimuli like autovaccine may also induce IL-18. However, an activation by autovaccine of IL-18 has not been tested yet.

The scheme illustrated in Figure 4 proposes therefore only a hypothetical pathway by which autovaccine could have restored the IFN-γ synthesis in T-cells from an individual, which could not activated by conventional T-cell agonists like anti-CD3 antibodies together with anti-CD28 antibodies. According to *Yang* et al. (2001) and *Nakanishi* et al. (2001), the combined action of IL-12 with IL-18 through their

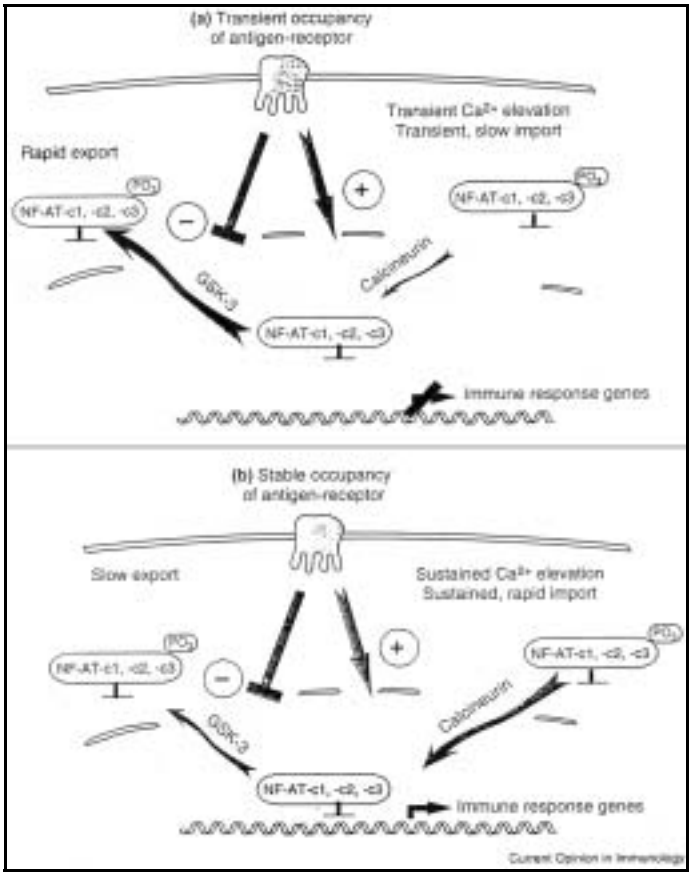

Figure 5: Hypothetical model how autovaccine Herborn might influence the calcium influx of T-lymphocytes.

corresponding receptors expressed on T-cells triggered the subsequent expression of a special cytoplasmic molecule of the MAP/ERK-kinase family e.g. GADD 45ß, which obviously bypasses the need for the classical pathway of a STAT-4 mediated IFN-γ production by macrophage derived IL-12.

The cytoplasmic expression of this molecule should enable CD4 positive T-helper cells to directly activate the NF-*k*B / AP transcriptional pathway for the activation of the IFN-γ gene in the case the cytokines IL-12 and IL-18 are *both* present.

As it has been demonstrated in the whole blood culture model that IL-12 was very efficiently modulated by the autovaccine and due to the fact that NK cells constitutively express both functionally active IL-12R and the IL-18R when freshly isolated, NK-cell derived IFN-γ could not be ruled out to explain the effects of autovaccine on IFN-γ production. However, the low number of NK-cells in whole blood argues against this possibility. In addition, the role of the macrophage as an IFN-γ-producing cell type is far from being clear: So far only one report by *Fenton* et al. (1997) described, that human alveolar macrophages were able to release IFN-γ after infection with *Mycobacterium tuberculosis* (*Fenton* et al., 1997).

Figure 5 should illustrate the role of autovaccine as a calcium-modulating

agent in the process of T-cell activation as a function of the duration of the subsequent activation of the calcium dependent phosphatase calcineurin. Assumed that autovaccine could restore defective TCR triggering by it s influence on the calcium influx into the cell, the figure based on the recent observations of *Neilson* et al. (2001).

In T-lymphocytes the transcription factors of the NF-ATc family are normally present in the cytoplasm in different phosphorylation states which hinders their nuclear import and binding to the corresponding promotor regions of respective cytokine genes on DNA, for example IL-2. The nuclear export of the transcription factors is mediated in this model by a member of the glycogen synthase Kinase 3 (GSK-3) which is constitutively active in T-cells. Under physiological conditions T-cells are often occasionally and transiently activated through their traffic from blood to lymph nodes and back during their search for potential harmful antigens. Occasionally the NF-ATc members may then enter the nucleus but are rapidly exported to hinder unfavourable activation of clonotypic T-cells in case of only low affinity binding of antigens.

Due to the observation that autovaccine binds strongly to calcium ions, the observed failure of the IFN-γ production upon ligation of the TCR by anti-CD3/anti-CD28 may presumably be substituted by the calcium-targeting effects of autovaccine, whereby too low intracellular calcium concentrations may be enhanced. Under low intracellular calcium concentrations the cytoplasmic phosphatase calcineurin is only marginally activated to dephosphorylate the NF-ATc transcription factors resulting in their enhanced export to the nucleus by the GSK-3. Assumed that the autovaccine could substitute for a prolonged calcium signal in an at present unknown way, the calcium concentration intracellularily may reach sufficient levels to allow an enhanced import of NF-ATc transcrption factors into the nucleus, simultaneously accompanied by a lower GSK-3 activity. This model was proposed by *Neilson* et al. (2001), according to observations by the authors that a mutation in human T-cells induced a failure to respond to specific TCR triggering or to ionomycin with a subsequent mobilisation of intracellular calcium stores. These special T-lymphocytes were supposed to be defective in the mobilisation of calcium ions. Interestingly, T-cell from these patients express all molecules required for T-cell stimulation at a normal level on the cell membrane but there seem to be a substantial abnormality in lymphocyte activation in the duration of the calcium signal. Thus defective calcium influx and/or inability of the T-cell to maintain sufficient prolonged intracellular calcium levels may have contributed to the observed defect in this special lymphocytes (*Pai* et al., 1994; *Ricca* et al., 2000; *Whitney* and *Sutherland*, 1972).

FUTURE WORK

How may these important features of the autovaccine be integrated into a hypothetical regulatory model of action?

It may be suggested (see Figure 6) that the autovaccine Herborn might interact similar to the classical LPS molecule with Toll-like receptor subtypes on a responsive cell type. However this interaction may be influenced by the aggregation state of the autovaccine (number and structure of „monomers“) and neighbouring receptors on immune cells potentially occupied by the autovaccine in a somewhat different manner such as

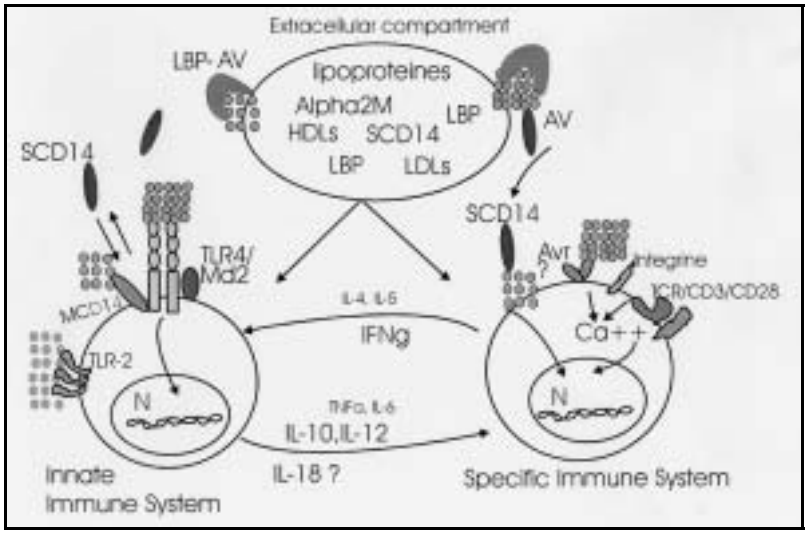

Figure 6: Model for the proposed immunoregulatory actions of autovaccine summarised in the context of the particular molecular structure of the autovaccine and its cytokine modulating capacities.

mCD14, integrins or lectin receptors like mannose binding protein. Additionally, lipoproteins in plasma, soluble CD14 and the Lipid A binding protein (LBP), generally known to regulate and control the traffic and biological activity of LPS in the blood stream (*Kitchens* and *Munford*, 1998; *Kitchens* et al., 1999; 2001; *Kitchens*, 2000) may also interfere with functions of the autovaccine on immunocompetent cells, resulting in the highly interindividual immunomodulating actions measured in the whole blood culture system. The downstream signalling events used by the autovaccine are largely unknown at present, so that in the context with this paper the proposed regulatory models of the actions of autovaccine illustrated in Figures 4, 5, and 6 are a mere speculatory one at present. Furthermore, surface-active properties of the autovaccine or electrostatic interactions of the autovaccine may also be taken into account and should and not be underestimated. Assuming different behaviour of aggregated and monomeric molecules, „monomeric“ molecules might profoundly interact for example with the so-called „lipid rafts“ in the eukaryotic cell membrane. This could have an impact on membrane fluidity thereby changing the conformation of raft-associated signalling molecules. To this end the autovaccine manufactured by the SymbioVaccin GmbH represents a remarkable class of bacterial-derived immunomodulators which to the present knowledge has not been described earlier in the literature with that individual specificity on lymphoid cells. From an immunological point of view it is highly interesting that a lipo-oligosaccharide preparation such as the autovaccine could initiate such a remarkable interindividual specific response on T-cells and on macrophages, without being strictly antigen-specific in the classical immunological sense. It will be of utmost importance for future work that this bacterial preparation, inducing probably a classical Th1 response in the whole blood culture system will be clinically evaluated for its effects in well designed, randomised clinical trials in very carefully selected indications.

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BACTERIAL PEPTIDES AS IMMUNOMODULATORS

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SUMMARY

Mucosal surfaces are habitats for the physiological microflora and are closely related to the mucosal immune compartment (mucosa-associated lymphoid tissue, MALT). Recently, considerable evidence has been accumulated showing that defined members of the physiological microflora express/liberate low molecular weight substances (peptides) which apparently are essential for the adequate immune response of the host. Biochemical analysis of microbial substances (originating from *Propionibacterium avidum* and other microorganisms) revealed reproducible chromatographic fractions with immunopotentiating and antitumour activities.

INTRODUCTION

The importance of the physiological microflora has recently been shown since, apparently, it guarantees the adequate function of organs such as gastrointestinal (GI) tract, skin and immune system (*van der Waaij*, 1985; *Roszkowski* et al., 1988; *Pulverer* et al., 1990a). The attention of many physicians had been focused primarily on the therapy of infections without sufficient notice being given to side effects, e.g. microbial dysbiosis and immunocompromisation, respectively. Data derived from experiments involving the GI tract of humans and animals provide some outline of the immune responses associated with the intestinal mucosal compartment. The mucosa-associated lymphoid tissue (MALT), the primary source of immunological function, extends beyond the intestine and consists of the gut-associated (GALT), bronchial-associated (BALT) and duct-associated lymphoid tissues (DALT). Thus, virtually every mucosal surface of the body has the ability to respond to and to induce effector cells capable of protecting the host from potentially harmful organisms or antigens (*Kagnoff*, 1987; *Kagnoff* et al., 1987; *Sim*, 1995). Our understanding of these defence mechanisms and how they equip the host for its continuing conflict against pathogenic organisms and potentially harmful substances deposited on mucosal surfaces has a wide range of biological and medical applications. For example, studies on mucosal immunity might lead to more effective methods of immunoprophylaxis against infections, neoplastic, and auto-immune diseases (*Araneo* et al., 1996).

PHYSIOLOGICAL MICROFLORA PROVIDES IMMUNOMODULATING SUBSTANCES

Previous studies suggested that the physiological microflora exert a stimulus on certain immune functions, since antibiotic decontamination of experimental animals resulted in immunosuppression and modification of anti-tumour immunity (*van der Waaij*, 1982, 1988; *Gorbach* et al., 1988; *Roszkowski* et al., 1984, 1985, 1993). In the course of investigations certain members of the BALB/c-mouse GI-tract microflora (e.g. *Bacteroides* sp., *Clostridium* sp., *Lactobacillus* sp., and *Propionibacterium* sp.) were found to liberate low molecular weight substances (MW < 6.500 D). To substantiate the assumption that microbial substances might prime basic immune responses, cultivation procedures were established to provide optimal conditions for their generation and release. In BALB/c mice, antibiotic decontamination of the GI-tract reproducibly resulted in considerable immunosuppression, apparently due to the lack of a specific stimulus. The substitution of low molecular weight substances from microorganisms of the GI-tract, such as *Bacteroides* sp. and *Propionobacterium* sp., to digestive-tract-decontaminated animals (route and interval of administration analogue to the antibiotic) reconstituted the cellular function (peritoneal macrophage phagocytic activity) and lymphatic tissue weight (thymus and spleen). To confirm the hypothesis that the human physiological microflora interacts with the immune system, certain bacteria of human origin were tested for their ability to liberate immunomodulating substances. Two species (*P. acnes* and *S. saprophyticus*) could be shown to release considerable amounts. Substitution of those substances (liberated from bacteria of human origin) to antibiotic-decontaminated (and immuno-compromised) BALB/c mice reconstituted the function of their immune system.

Sephadex chromatography revealed a uniform arrangement of peaks for microbial substances of different origin including those liberated from strains of BALB/c mouse GI-tract microflora (*Bacteroides* sp., *Clostridium* sp., *Lactobacillus* sp., *Propionibacterium* sp.) and those from *P. acnes* and *S. saprophyticus* of human sources (*Pulverer* et al., 1990b). Apparently, the generation and release of microbial substances seems to be a unique property of various members of the physiological microflora resulting in a moderate but constant priming of the immune system (mucosa-associated lymphoid tissue, MALT).

To investigate the immunomodulating potency with another well-established experimental model (Scollay et al., 1984a; Reichert et al., 1986a) substances from *P. acnes* and *S. saprophyticus* were administered to hydrocortisone-treated BALB/c mice. Hydrocortisone-resistant thymocytes have generally been used to investigate the functional maturity since the vast majority of thymocytes surviving the administration of hydrocortisone are of a mature phenotype (*Reichert* et al., 1986a). Intrathymic T-cell differentiation is a process in which immature thymocytes expand and develop by undergoing complicated maturational events leading to the acquisition of immunocompetence and subsequent emigration to the periphery (*Scollay*, 1984; *Scollay* et al., 1984b; *Lefrancois* and *Puddington*, 1995). This thymic microenvironment is thought to exert local influences, which may contribute to the T-cell maturation process (*Reichert* et al., 1986). Quantitative analysis re-

vealed a significantly decreased number of thymocytes after hydrocortisone treatment in BALB/c mice. However, administration of microbial substances apparently stimulated the cell proliferation and maturation, since the number of thymocytes increased significantly compared to non-treated animals.

Administration of microbial substance (released from *S. saprophyticus* or *P. acnes* of human sources) to non-treated BALB/c mice also manifested some immunopotentiation which positively correlated with a remarkable increase of thymus weight. However, weight gain of spleen was less pronounced (*Pulverer* et al., 1990b). It has been shown that T-lymphocyte antigens undergo characteristic changes in their surface density expression as T-cells mature in thymus and lymphoid tissues (*Ledbetter* et al., 1980; *Micklem* et al., 1980; *Reichert* et al., 1986a,b). Quantitative investigations on CD-3 (pan T-cells), CD-8+ (T-cytotoxic/suppressor cells), CD-4+ (T-helper/inducer cells) expression has been facilitated by the use of monoclonal antibodies. Directly fluorescence-conjugated anti CD-3+, anti CD-4+, and anti CD-8+ monoclonal antibodies were each used alone and in combination in FACS (fluorescence-activated cell sorter) staining experiments. The T-cell receptor first appears during thymic ontogeny (Ceredig et al., 1983; Fitch , 1986). Roughly 80% of thymocytes are CD-8+/CD-4+ and a small proportion are CD-8-/CD-4- cells belonging of these thymocyte subsets are thought to be immature (*Micklem* et al., 1980; *Scollay* et al., 1984a,b; *Scollay*, 1984; *Reichert* et al., 1986b; *Lefrancois* and *Puddington*, 1995). In contrast, approximately 15% of thymocytes and nearly all peripheral T-cells express the mature CD-8-/CD-4+ (T-helper/inducer) or CD-8+/CD-4- (T-suppressor/cytotoxic) phenotype (*Ceredig* et al., 1983; *Scollay* et al., 1984a). Administration of microbial substance (liberated from *P. acnes* or *S. saprophyticus*) to BALB/c mice apparently provides a stimulus for the development of lymphoid cells. Accordingly, the numbers of T-helper/inducer cells evidently increased in thymus after injections of microbial substance, whereas T-cytotoxic/suppressor cells did not undergo considerable changes. A calculation of the helper/inducer-suppressor/cytotoxic cell ratio suggested that the administration of microbial substance preferably stimulated the proliferation of T-cells with helper-inducer phenotype (*Pulverer* et al., 1990b). The exact mechanisms for this selection process have not yet been clarified; however, a variety of growth factors and interleukins similarly affect effector tissues (*O'Garra*, 1989; *Heumann* et al., 1994; *Takada* et al., 1995). A further characterisation of the involvement of antigen receptors and/or other cell surface molecules during T-cell development and their activity will provide additional insight into events that determine the T-cell repertoire.

PROPIONIBACTERIA AND ITS COMPONENTS: POTENT IMMUNOMODULATORS

Bacteria (especially *Propionibacterium* species) and their products are known to be highly effective in stimulating the immune system (*Pulverer* et al., 1985). Three species (*Propionibacterium acnes, P. granulosum, P. avidum*) appeared to be of special medical interest, and after evaluating the immuno-active potential of a great number of strains (*Lefrancois* and *Puddington*,

1995). *Propionibacterium avidum* KP-40 and *P. granulosum* KP-45 were selected for further experimental and clinical studies. For practical reasons (e.g. cultivation procedure, biological and immunological standardisation) *P. avidum* KP-40 was preferably introduced for clinical evaluation, although its immuno-active capacity is absolutely identical to *P. granulosum* KP-45.

Recently it was shown that the efficiency of propionibacterial immunomodulation is related not only to the type of tumour involved but also to the bacterial strain used and the route and timing of administration (*Szmigielski* et al., 1982). After optimising these preliminaries, treatment with propionibacteria proved to be of considerable clinical benefit, inducing potent immunostimulation. These data on the obvious efficacy of propionibacterial treatment encouraged the initiation of a prospectively randomised clinical multicentre trial in colorectal carcinoma patients where overall survival, relapse rate, relapse-free interval, metastasis, quality of life, immune response were beneficially modified a single preoperative administration of *Propionibacterium avidum* KP-40 (*Isenberg* et al., 1995).

The obvious therapeutical benefit of *Propionibacterium avidum* KP-40 treatment in neoplastic disease induced further experimental studies. Recently, we investigated and confirmed its stimulating effects on the non-specific immune system (*Pulverer* et al., 1985). During these investigations we were able to determine the effect of *Propionibacterium avidum* KP-40 on thymocyte proliferation, maturation and emigration into peripheral blood using a murine model. Single intraperitoneal administration of the optimal immunomodulating dose of *Propionibacterium avidum* KP-40 (1 mg per mouse, as determined in preceding studies) to BALB/c mice resulted in enhanced thymus weight and accelerated thymocyte maturation (generally leading to emigration of these cells into peripheral blood), followed by enhanced proliferation of immature cells. Furthermore, we found that absolute counts of peripheral blood lymphocytes (PBL) and monocytes (PBM) were significantly enhanced as well as the expression of activation markers (e.g. interleukin (IL)-2 receptors on PBL; MAC-3 antigens on PBM) with peak values 6 days after *Propionibacterium avidum* KP-40 injection.

To evaluate the anti-tumour/anti-metastatic effect of *P. avidum* KP-40 induced immunomodulation, BALB/c-mice were intravenously challenged with RAW 117-H 10 lymphosarcoma cells and checked for liver tumour colonisation as described elsewhere (*Scollay*, 1984). Compared to control group the number of liver colonies was significantly lower in *Propionibacterium avidum* KP-40 treated mice. The above mentioned experiments were analogously performed with chromatographic purified components (peptide fractions 1-3 and 10) of *P. avidum* KP-40 and yielded comparable results.

The ability of bacteria to modulate the immune response to non-related antigens is well documented. Propionibacteria are amongst the most potent immunomodulators stimulating cell populations involved in (non-) specific resistance. Generally, the activated immune system provides protection from infectious pathogens and spread/growth of malignant cells through mechanisms of recognition and elimination. Accordingly, propionibacteria and its defined low molecular weight substances could be shown to be effective in the treatment of infections and neoplastic diseases in experimental and clinical settings. Further studies are currently being performed to confirm these promising data

CONCLUSION AND FUTURE ASPECTS

Mucosal surfaces are habitats of the physiological microflora and are closely related to the mucosal immune compartment (mucosa-associated lymphoid tissue, MALT) which interacts with the systemic immune compartment on separate levels of host defences (*Tomasi* et al., 1980; *Bienenstock* and *Befus*, 1984). It is the first line of defence and has the ability to block antigen-access to the systemic compartment of the host by producing local responses (*Walker* and *Isselbacher*, 1977; *Challacombe* and *Tomasi*, 1980). However, antigens (e.g. microbial substances) can gain access to the MALT and trigger (local) immune responses. In addition, some antigens are able to produce systemic tolerance (*Challacombe* and *Tomasi*, 1980). If these particular antigens gain access to the local immune system, a suppression of the systemic immune response may be induced by suppressor cells which were activated in the MALT and then translocated to the systemic immune compartment (*Walker* and *Isselbacher*, 1977; *Richman* et al., 1981).

Recently, considerable evidence has been accumulated showing that the physiological microflora liberates/expresses low molecular weight substances which, apparently, prime certain immune responses. Investigations on suppression and reconstitution of immune functions depending on the presence of the physiological microflora (respectively on microbial substances liberated from members of the physiological microflora) favoured the hypothesis that symbiotic microorganisms (respectively defined microbial substances) are essential for adequate immune functions. Since those events are generally stimulated and regulated by T-helper/inducer cells, this activity may be explained by the production of growth and differentiation factors. These properties of interleukins and related molecules (e.g. microbial substances) indicate a key role in the positive and negative regulation of antigen-specific cellular and humoral immune responses and in the ontogeny of the immune system. Preliminary investigations suggested that microbial substances may be considered to be potential growth factors (e.g. for fibroblasts, epithelial cells, bone marrow cells, tumour cells) as well as differentiation factors (e.g. for lymphoid cells, bone marrow cells). Accordingly, a wide range of biological and medical applications of these substances should be considered, e.g. (1) specific immunomodulation (with special emphasis on anti-infectious and anti-neoplastic immunity, (2) therapeutical administration of growth and differentiation factors (interleukin-like molecules) (3) specific adjuvant in patients treated with decontaminating antimicrobial drugs (omnispectrum therapy) and, last but not least, (4) a contribution to current knowledge on interactions of the physiological microflora and immune responses.

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AUTOSPECIFICITY IN *ESCHERICHIA COLI* AUTOVACCINES: EFFECTS ON THE HUMAN IMMUNE SYSTEM *IN VITRO*

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SUMMARY

The Autovaccine Herborn (AV) consists of a lipid fraction (Lipid A like structures) of the outer membranes of *E. coli* rough strains isolated from the stools of those patients that are to be treated with this individualised preparation. According to previous results the major pharmacological effects of this particular type of autovaccine seem to be mediated through the activation of cells of the immune system (leukocytes).

To evaluate the degree of individuality in the immuno-modulatory effects of the AV, 5 different autovaccines were prepared from the stools of 5 healthy subjects by a particular manufacturing process developed by the Institute for Microecology, Herborn. Each of these autovaccines was tested in whole-blood cultures together with the blood of each of the five stool-donors. For this purpose the cells were co-activated in separated cultures with stimuli specific for a) phagocytes (Zymosan), b) T-lymphocytes (antibodies to CD3 and CD28) or c) B-lymphocytes (Pokeweed mitogen). As readouts for AV activities the production of a series of cytokines was chosen.

A rather homogeneous type of response was observed when the AVs and the blood for the cultures came from different donors. Roughly, this response resembles a Th1 type reaction (i.e., inducing TNF-α, IL-6 and IFN-γ but suppressing IL-5 as a Th2 cytokine). In addition a pronounced increase in the secretion of IL-10 was observed, regardless of whether Zymosan or PWM was used as stimulant.

In contrast, when the five autovaccines were tested together with the blood of the same donor the stool sample was obtained from ("autologous" situation), much more heterogeneity in the production of the different cytokines was to be seen. The basic pattern of a Th1 response still was present, yet the ratios sometimes changed dramatically. For example, in some instances even the synthesis of TNF-α, a mediator usually produced in high amounts in response to Lipid A, did not show any influence by the autologous AV.

Our results provide first clear evidence for the postulated individuality in the reactions of the human immune system to the special, lipid-type of autovaccine (Autovaccine Herborn) tested in the study presented below.

INTRODUCTION

During the fifties of the past century the name "autovaccine" has been introduced to characterise bacterial preparations, for example derived from dermal flora, that were meant to specifically immunise the host against facultative pathogenic bacteria that can cause recurrent local inflammations (*Müller*, 1950). A few years later the Autovaccine Herborn (AV) was created and introduced to human medicine (*Kolb*, 1959). This was long before some decades later the concept of antigen-specific cellular reactions of the immune system was developed and such reactions could clearly be differentiated from those of non-specifically acting cells (the so-called natural immune system).

Today it is quite clear that the antigen-specific cells predominantly respond to polymers of amino-acids and/or sugars. Only rarely they recognise lipid structures specifically enough to elicit a selective antibody or T-cell response (*Porcelli*, 1998). The AV on the other hand consists of a highly purified fraction of lipids isolated from intestinal bacteria. This was shown by a thorough chemical characterisation during the past years which demonstrated the biologically active molecules of AV to be lipid structures that resemble very closely the Lipid A of the outer membrane of Gram-negative bacteria (*Thies*, 2001). Therefore, the postulated concept of the AV inducing an individual-specific immunomodulation is questionable.

The present study was conducted to address this issue. Five different autovaccines, prepared from the faeces of healthy volunteers were tested for immunomodulatory activities in cell cultures prepared from the whole blood of each of the stool donors.

MATERIALS AND METHODS

Preparation of the autovaccines

Stool samples were collected from 5 healthy volunteers who at the time of stool collection had to be clinically free of any symptoms of acute or chronic inflammatory diseases, gastro-intestinal complaints or any other type of immune activating disorders. No drug intake or vaccination was allowed within the last four weeks before stool. All samples were sent to the Institute of Microecology at the day of collection. In this laboratory the AVs were prepared from rough strains of *E. coli* cultivated on selective agars. The stock solutions of the AVs from all 5 donors were then prepared by a standardised procedure from a suspension of 10^9 germs/ml and shipped back to EDI (Experimental & Diagnostic Immunology) GmbH.

Whole blood cultures

Cultures of whole-blood samples of each of the donors from which the AVs were performed as described elsewhere (*Schmolz* et al., 2001). Briefly, whole-blood (anti-coagulated with heparin) was suspended in RPMI 1640 together with the stimulants, which were either Pokeweed Mitogen (PWM, Sigma, Munich), an antibody mixture to CD3 (R&D Systems, Wiesbaden) and CD28 (DKFZ, Heidelberg) or Zymosan (Sigma, Munich) that was opsonised with human AB plasma. RPMI 1640 medium served as control in non-stimulated cultures.

The cultures were incubated (37°C, humidified atmosphere with 5% CO_2) for 24 or 48 hrs, depending upon the type of stimulation and the mediator to

be tested (Zymosan-activated cultures: 24 h; PWM and antibody stimulations: 48 h). Thereafter, all plates were centrifuged and the supernatants were harvested and stored at -20°C until testing for mediator synthesis in standard ELISA assays.

Test for immunomodulatory activities of AVs

Serial dilutions (1:3) were prepared from these lipid suspensions by adding Hanks' Balanced Salt Solution (HBSS). These dilutions were tested in cultures of freshly isolated whole blood (see above). The immunomodulatory effects of the different autovaccines were determined by measuring cytokine synthesis (standard enzyme immunoassays): Activities on monocytes were detected in Zymosan stimulated cultures by measuring the mediators TNF-α, IL-6, IL-10 ("IL-10 Zy") and IL-12; the effects on T lymphocytes were tested in anti-CD3/antiCD28 stimulated cultures by quantifying IFN-γ (Th1 cells) and IL-5 (Th2 cells). Finally, influences of AVs on B cell activation were derived from PWM supernatants in which also IL-10 ("IL-10 PWM") was determined.

Calculations

When comparing the cytokine syntheses of individual blood donors, a frequently occurring problem is the huge heterogeneity in the capacity of the leukocytes of the single donors in the production of the cytokines. Thus, it was decided to normalise the data by setting the stimulated controls to the level of 1.0 and calculate stimulation indices using the following formula:

$$\text{Stimulation Index (SI)} = \frac{\text{cytokine concentration of sample culture (pg/ml)}}{\text{cytokine concentration of control culture (pg/ml)}}$$

In some cultures a discontinuous dose-response curve was observed. This prevented a determination of half-maximal effective concentrations and thus a direct comparison of the overall activity of the individual AVs. To enable this, the area under the curve (AUC) of each activity curve was determined. For calculating "relative activities" of the homologous AVs compared to the autologous ones, the AUC of the autologous AV was adjusted to 1.0 and the values of all relative activities of the different cytokines were plotted into net diagrams:

$$\text{Relative Activity (RA)} = \frac{\text{cytokine conc. of homologous AV}}{\text{cytokine conc. of autologous AV}}$$

RESULTS

The first series of experiments was performed to give a measure of the variation in activities AVs generate when they are tested on leukocytes of different donors (Figures 1-7). As could be shown, the degree of similarity between the dose-response curves among the AVs when tested on the whole-blood cultures of a single donor was striking (here exemplified by plotting the graphs of blood donor CR). All parameters tested showed clear and characteristic AV-dependent changes.

Instead, when examining the effects of AVs in cultures of blood donors that also gave the stools to prepare the respective AV ("autologous" situation), the picture changed dramatically. In

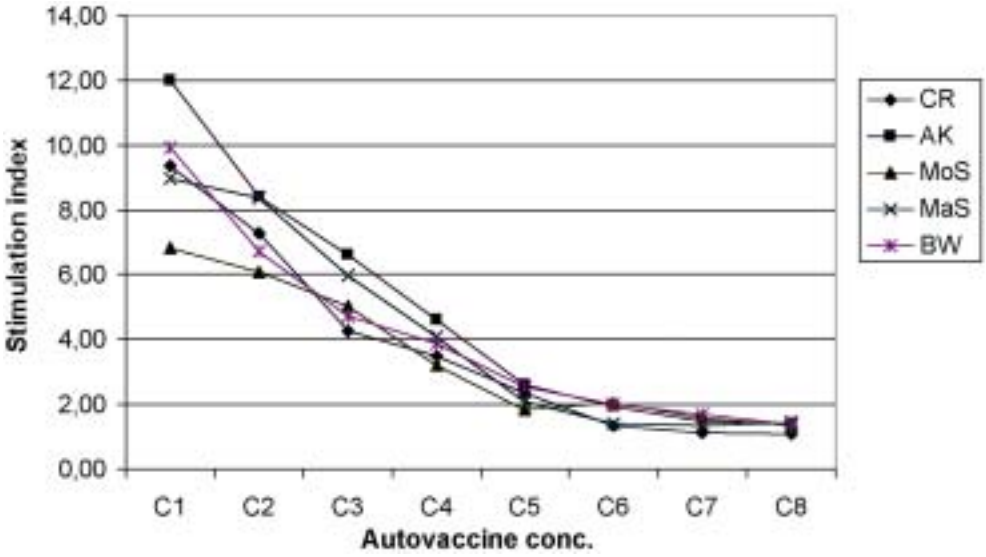

Figure 1: Test of AVs from 5 different donors in whole-blood cultures of one of the 5 donors (CR). The cultures were activated by a combined stimulus using antibodies to the surface antigens CD3 and CD28 (endpoint: release of IFNγ). AVs were tested in 8 different concentrations (C1-C8), starting with 10^9 cells per ml followed by serial dilution (1:3). CR, AK etc. = initials of the donors.

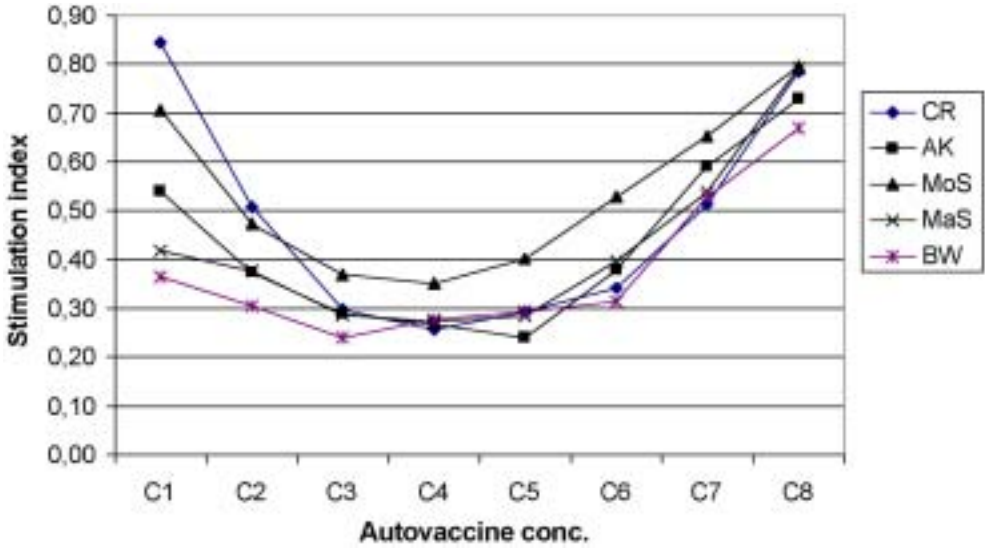

Figure 2: Test of AVs from 5 different donors in whole-blood cultures of one of the 5 donors (CR). The cultures were activated by a combined stimulus using antibodies to the surface antigens CD3 and CD28 (endpoint: release of IL-5). AVs were tested in 8 different concentrations (C1-C8), starting with 10^9 cells per ml followed by serial dilution (1:3). CR, AK etc. = initials of the donors.

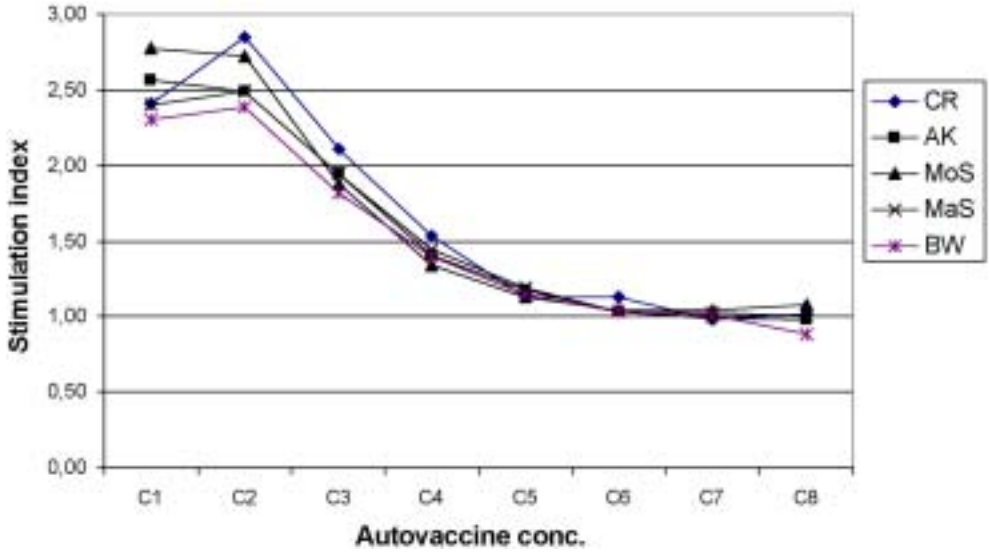

Figure 3: Test of AVs from 5 different donors in whole-blood cultures of one of the 5 donors (CR). The cultures were activated by a mitogen predominantly stimulating B-lymphocytes, PWM (endpoint: release of IL-10 (PWM)). AVs were tested in 8 different concentrations (C1-C8), starting with 10^9 cells per ml followed by serial dilution (1:3). CR, AK etc. = initials of the donors.

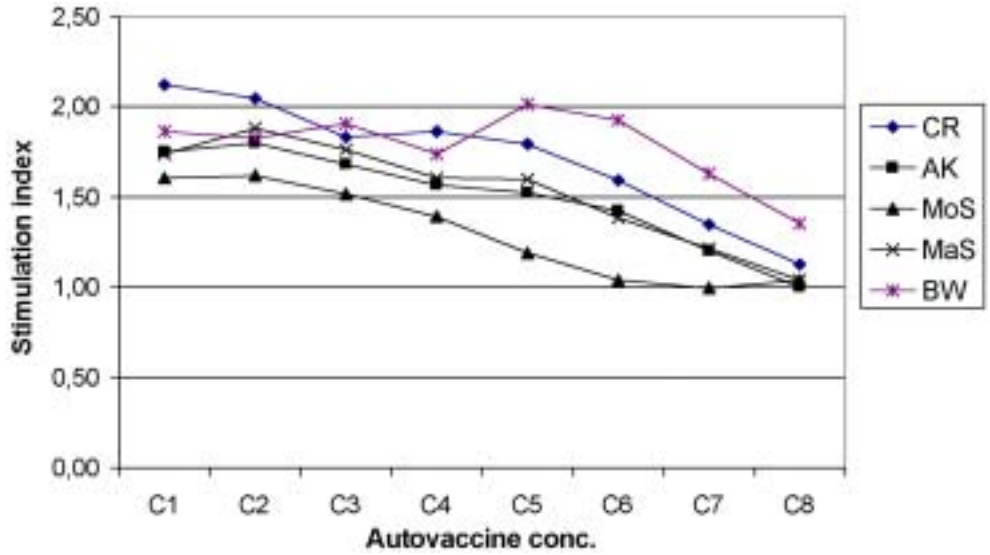

Figure 4: Test of AVs from 5 different donors in whole-blood cultures of one of the 5 donors (CR). The cultures were activated by a particulate phagocytosis stimulus, Zymosan (endpoint: release of TNFα). AVs were tested in 8 different concentrations (C1-C8), starting with 10^9 cells per ml followed by serial dilution (1:3). CR, AK etc. = initials of the donors.

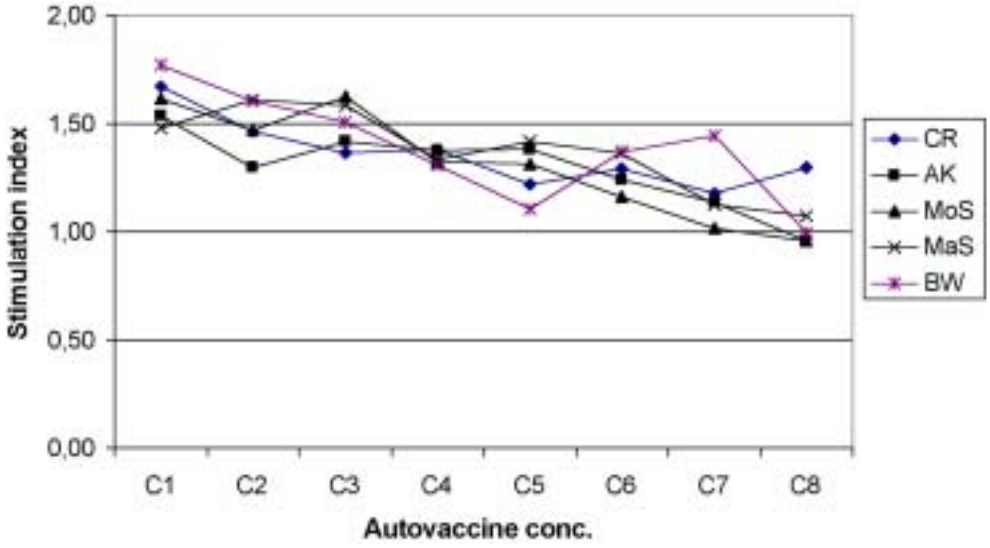

Figure 5: Test of AVs from 5 different donors in whole-blood cultures from one of the 5 donors (CR). The cultures were activated by a particulate phagocytosis stimulus, Zymosan (endpoint: release of IL-6). AVs were tested in 8 different concentrations (C1-C8), starting with 10^9 cells per ml followed by serial dilution (1:3). CR, AK etc. = initials of the donors.

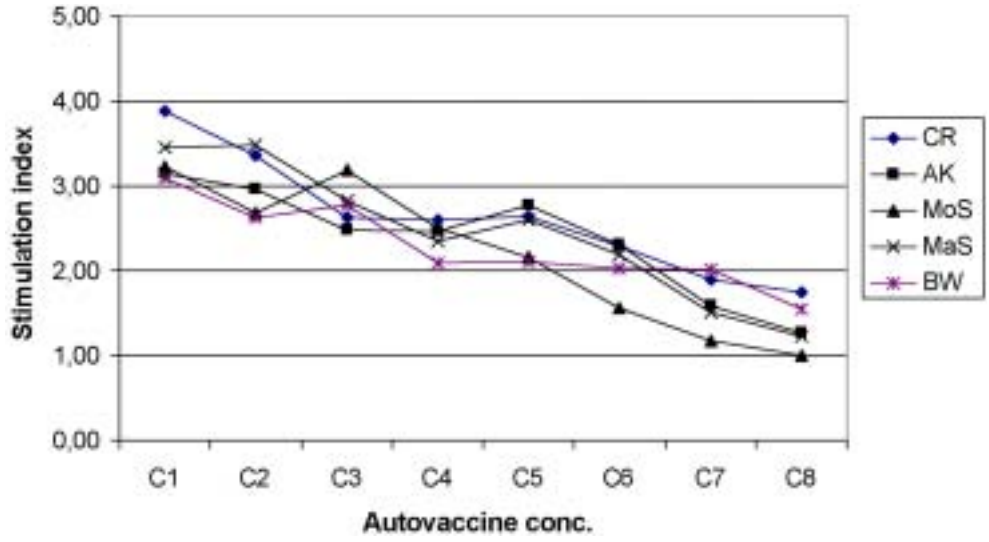

Figure 6: Test of AVs from 5 different donors in whole-blood cultures of one of the 5 donors (CR). The cultures were activated by a particulate phagocytosis stimulus, Zymosan (endpoint: release of IL-10 (Zy)). AVs were tested in 8 different concentrations (C1-C8), starting with 10^9 cells per ml followed by serial dilution (1:3). CR, AK etc. = initials of the donors.

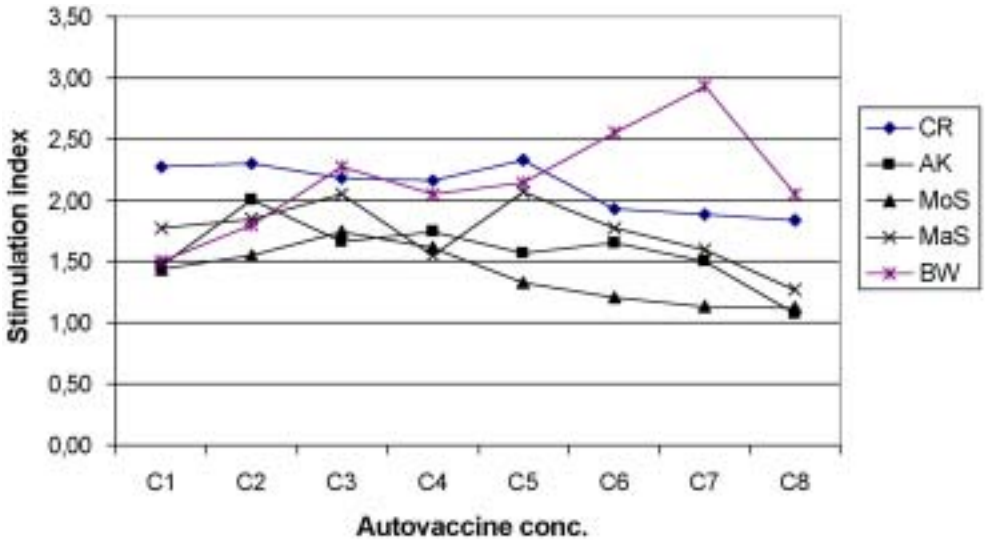

Figure 7: Test of AVs from 5 different donors in whole-blood cultures of one of the 5 donors (CR). The cultures were activated by a particulate phagocytosis stimulus, Zymosan (endpoint: release of IL-12). AVs were tested in 8 different concentrations (C1-C8), starting with 10^9 cells per ml followed by serial dilution (1:3). CR, AK etc. = initials of the donors.

these experiments the effects of the autologous AVs varied over a wide range in the donor group. For instance, the TNF-α synthesis of donor AK obviously did not change at all in the presence of his own AV whereas cultures of CR being incubated with the AV of CR showed a substantial increase in TNF-α production of more than 100% compared to the Zymosan stimulated control (Figure 8). On the other hand, the AV of BW seemed to inhibit the Zymosan triggered release of IL-12 at higher concentrations slightly without exerting any stimulatory effect at any of the concentrations tested. Yet, all other autovaccines at autologous culture conditions induced stimulations to a variable degree (up to 2.5-fold the concentration of the stimulated control, see Figure 11). Similar results could be found with the other monokines (IL-6 and IL-10Zy, Figures 9 and 10).

The same was true for the lymphocytic mediators which reacted with only very little variation when AVs from other donors were applied to the cultures. In contrast, autologous AVs gave rise to a rather broad spectrum of activities, especially regarding the release of IFN-γ and IL-10 (see Figures 12 and 14). IL-5 seemed to react at a much lower degree of variation (Figure 13).

As a last approach to identify individual-specific actions of autologous AVs, we compared the dose-response curves of each of the cytokines and each of the donors with those of the others. Transforming these data into web-like diagrams (see Figures 15-19) it was possible to compare the overall effects of each of the autologous AVs on the complete cytokine pattern to that of the homologous AVs. Compelling activity patterns could be revealed demonstrating a broad heterogeneity generated by the autologous AVs in the immune systems of individual donors, especially when compared to the homologous AVs.

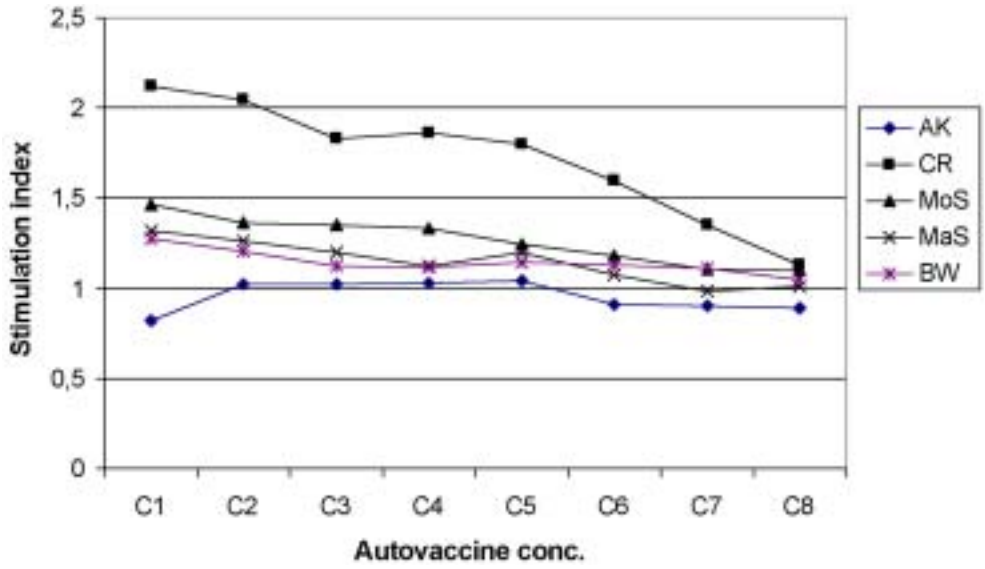

Figure 8: Test of AVs from 5 different donors in whole-blood cultures of those donors the AVs were obtained from (autologous test). The cultures were activated by a particulate phagocytosis stimulus, Zymosan (endpoint: release of TNFα). AVs were tested in 8 different concentrations (C1-C8), starting with 10^9 cells per ml followed by serial dilution (1:3). CR, AK etc. = initials of the donors.

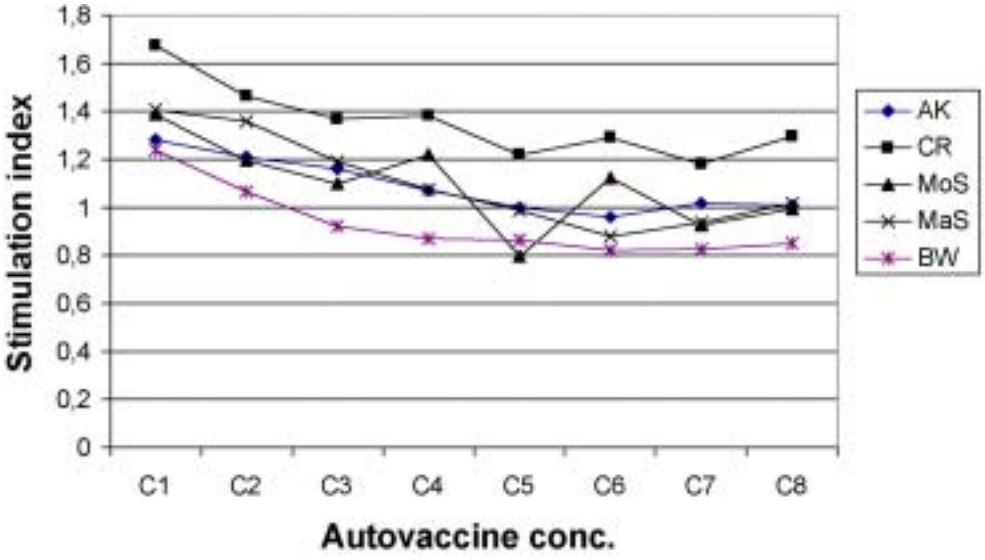

Figure 9: Test of AVs from 5 different donors in whole-blood cultures of those donors the AVs were obtained from (autologous test). The cultures were activated by a particulate phagocytosis stimulus, Zymosan (endpoint: release of IL-6). AVs were tested in 8 different concentrations (C1-C8), starting with 10^9 cells per ml followed by serial dilution (1:3). CR, AK etc. = initials of the donors.

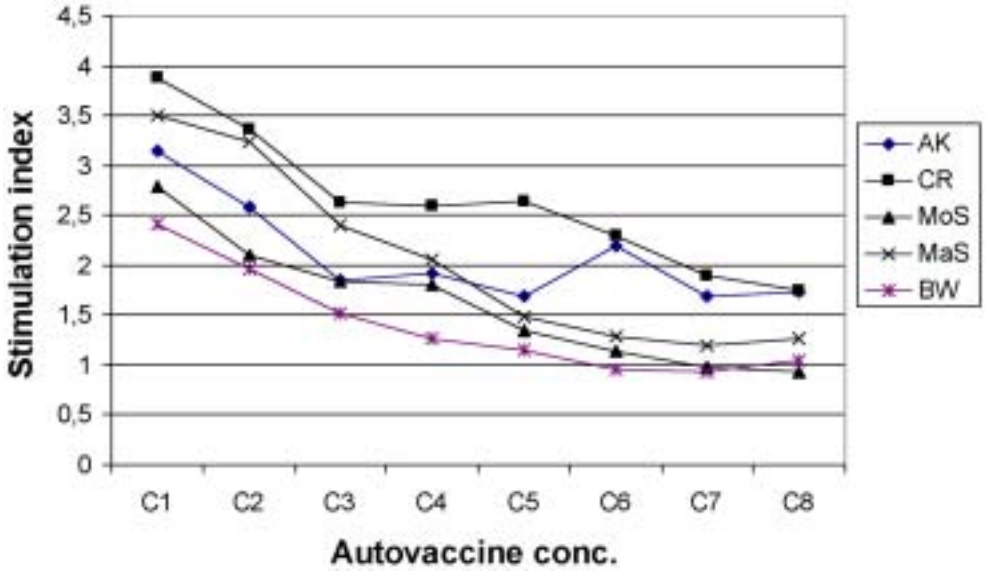

Figure 10: Test of AVs from 5 different donors in whole-blood cultures of those donors the AVs were obtained from (autologous test). The cultures were activated by a particulate phagocytosis stimulus, Zymosan (endpoint: release of IL-10). AVs were tested in 8 different concentrations (C1-C8), starting with 10^9 cells per ml followed by serial dilution (1:3). CR, AK etc. = initials of the donors.

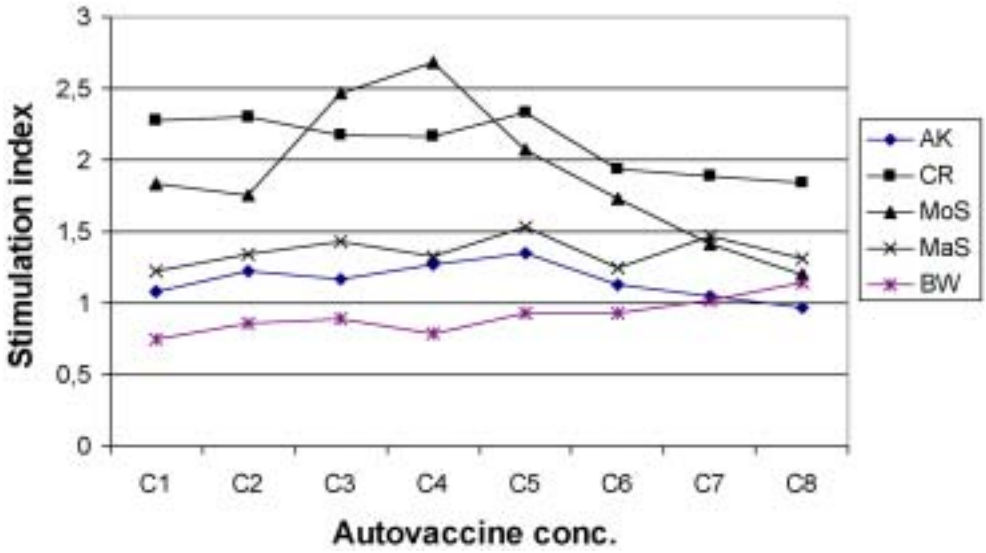

Figure 11: Test of AVs from 5 different donors in whole-blood cultures of those donors the AVs were obtained from (autologous test). The cultures were activated by a particulate phagocytosis stimulus, Zymosan (endpoint: release of IL-12). AVs were tested in 8 different concentrations (C1-C8), starting with 10^9 cells per ml followed by serial dilution (1:3). CR, AK etc. = initials of the donors.

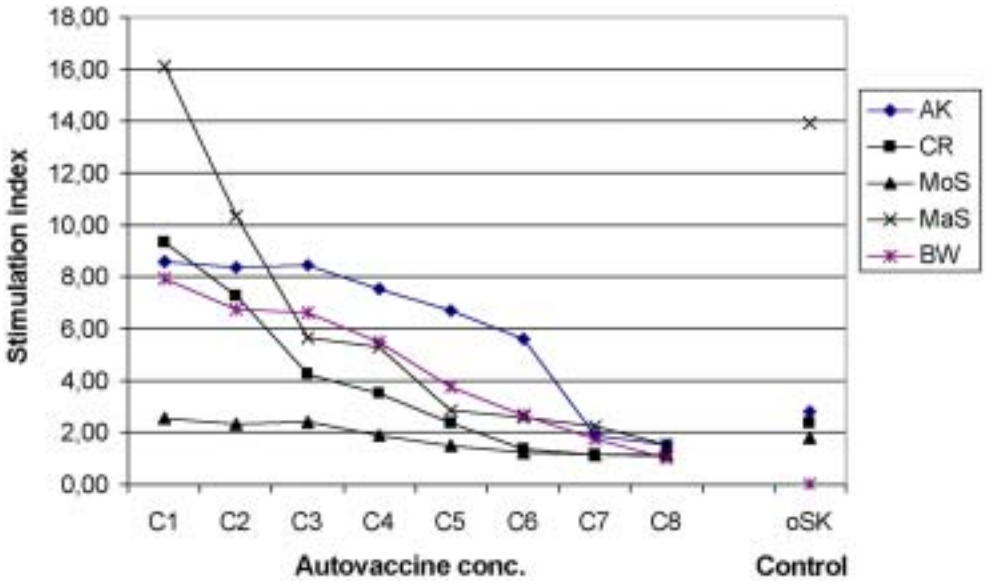

Figure 12: Test of AVs from 5 different donors in whole-blood cultures of those donors the AVs were obtained from (autologous test). The cultures were activated by a combined stimulus using antibodies to the surface antigens CD3 and CD28 (endpoint: release of IFNγ). AVs were tested in 8 different concentrations (C1-C8), starting with 10^9 cells per ml followed by serial dilution (1:3). CR, AK etc. = initials of the donors.

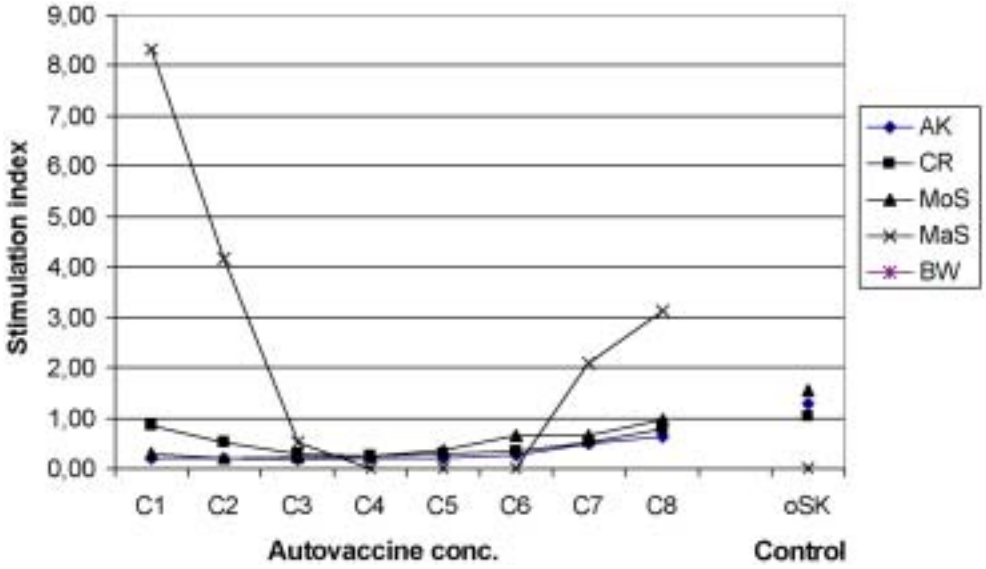

Figure 13: Test of AVs from 5 different donors in whole-blood cultures of those donors the AVs were obtained from (autologous test). The cultures were activated by a combined stimulus using antibodies to the surface antigens CD3 and CD28 (endpoint: release of IL-5). AVs were tested in 8 different concentrations (C1-C8), starting with 10^9 cells per ml followed by serial dilution (1:3). CR, AK etc. = initials of the donors.

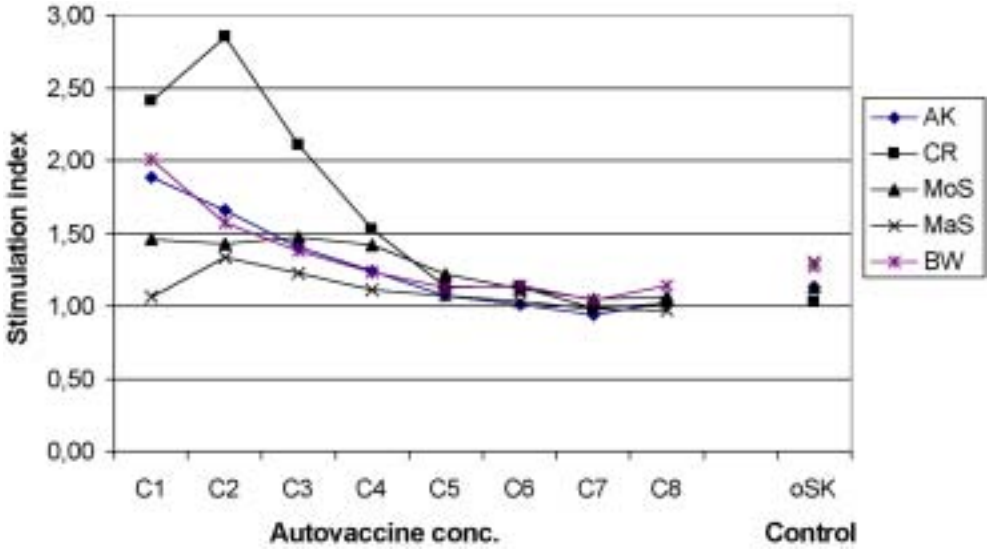

Figure 14: Test of AVs from 5 different donors in whole-blood cultures of those donors the AVs were obtained from (autologous test). The cultures were activated by a mitogen predominantly stimulating B lymphocytes (endpoint: release of IL-10). AVs were tested in 8 different concentrations (C1-C8), starting with 10^9 cells per ml followed by serial dilution (1:3). CR, AK etc. = initials of the donors.

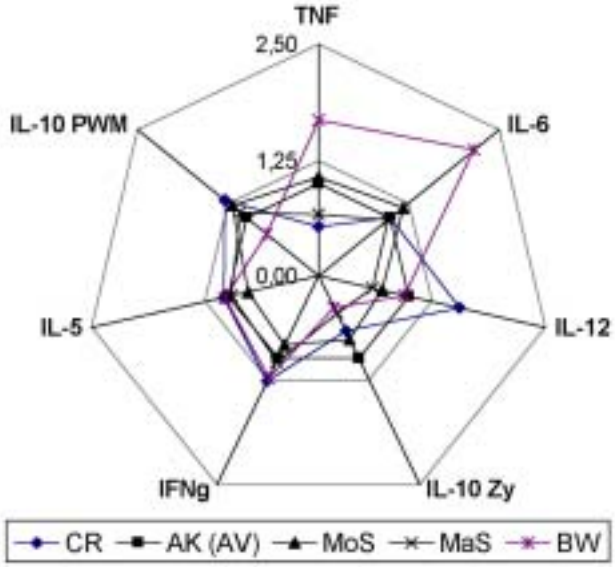

Figure 15: Cytokine response of the blood cells of donor AK to his autologous AV compared to the AVs of four other donors. For each of the cytokines the area under the curve (AUC) was calculated from the dose-response curve. Then the autologous AV was set to 1.0 and a ratio was formed between the homologous and the autologous AVs. CR, AK etc. = initials of AV donors; AK (AV) = donor of blood and AV (autologous situation).

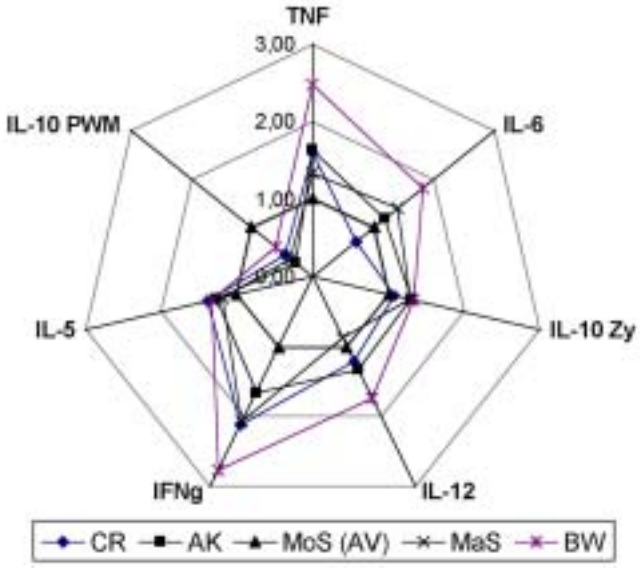

Figure 16: Cytokine response of the blood cells of donor MoS to his autologous AV compared to the AVs of four other donors. For each of the cytokines the area under the curve (AUC) was calculated from the dose-response curve. Then the autologous AV was set to 1.0 and a ratio was formed between the homologous and the autologous AVs. CR, AK etc. = initials of AV donors; MoS (AV) = donor of blood and AV (autologous situation).

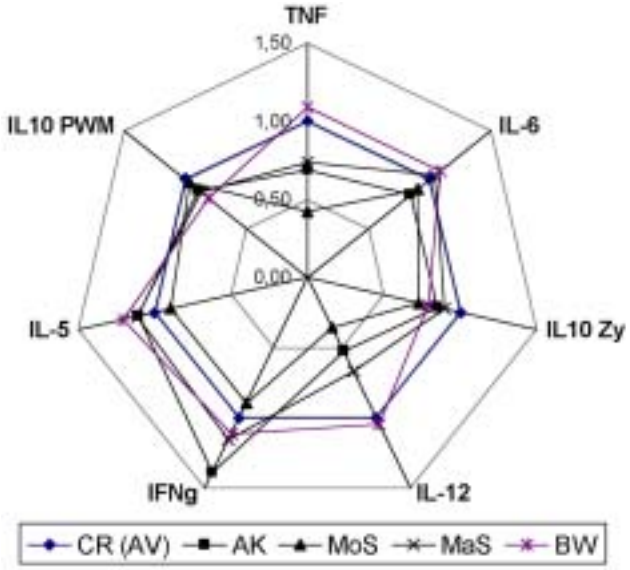

Figure 17: Cytokine response of the blood cells of donor CR to his autologous AV compared to the AVs of four other donors. For each of the cytokines the area under the curve (AUC) was calculated from the dose-response curve. Then the autologous AV was set to 1.0 and a ratio was formed between the homologous and the autologous AVs. CR, AK etc. = initials of AV donors; CR (AV) = donor of blood and AV (autologous situation).

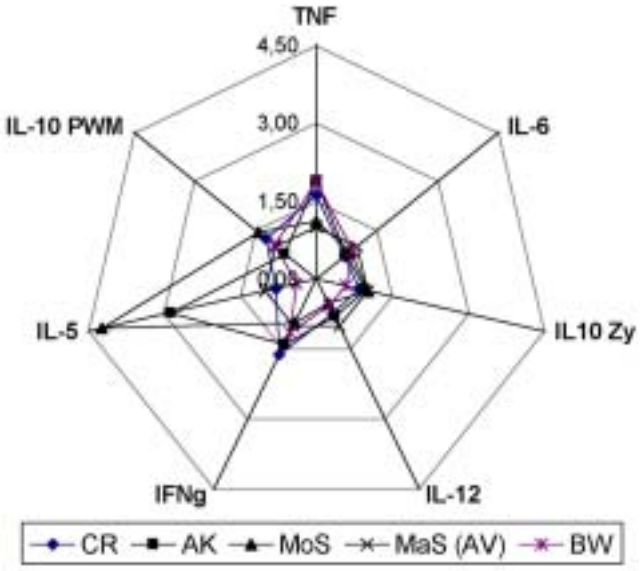

Figure 18: Cytokine response of the blood cells of donor MaS to his autologous AV compared to the AVs of four other donors. For each of the cytokines the area under the curve (AUC) was calculated from the dose-response curve. Then the autologous AV was set to 1.0 and a ratio was formed between the homologous and the autologous AVs. CR, AK etc. = initials of AV donors; MaS (AV) = donor of blood and AV (autologous situation).

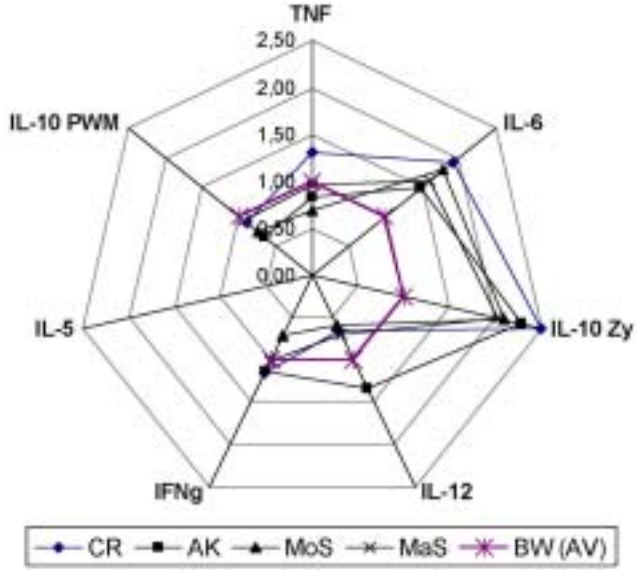

Figure 19: Cytokine response of the blood cells of donor BW to his autologous AV compared to the AVs of four other donors. For each of the cytokines the area under the curve (AUC) was calculated from the dose-response curve. Then the autologous AV was set to 1.0 and a ratio was formed between the homologous and the autologous AVs. CR, AK etc. = initials of AV donors; BW (AV) = donor of blood and AV (autologous situation).
The gap seen with IL-5 is the result of a cellular response that was too low to give reliable results, therefore, the data had to be omitted.

DISCUSSION

The Autovaccine Herborn (AV) is composed of bacterial lipids, the structure of which is very closely related to the well-known Lipid A of Gram-negative bacteria. Lipid A in turn is the central building block of the so-called lipopolysaccharide (LPS), also known as endotoxins. The immune system uses LPS as well Lipid A as common indicators of the presence of bacterial infection and, by means of specific cellular receptors, as trigger signals for antigen non-specific leukocytes, such as granulocytes and macrophages (*Jackson*, 1997; *Ozinsky* et al., 2000). Those receptors, besides displaying more or less selectivity for several such types of stimulating molecules, exhibit only a low degree of fine specificity. Thus, although different types of LPS molecules can bind to TLR4 or the LPS-binding protein, they nevertheless induce the same type of cellular reaction. In contrast to Lipid A, LPS often displays considerable structural variability, although not in its lipid moiety but rather because of differences in the polysaccharide "side-chain". In Lipid A the side-chain usually is truncated down to 1 or 2 sugar rests, leaving only little space for this type of variation (*Rietschel* et al., 1993; *Mueller-Loennies*, 1998). Taken together, these facts reduce the degrees of freedom to produce structural heterogeneity in Lipid A molecules dramatically. The fact that AV consists of a highly purified fraction of Lipid A raises the question if individuality can actually be found in the immunomodulatory activities of AVs, even if one takes into account that each of the patients usually harbours an individual set of *E. coli* rough strains in the gut.

To address this issue, a series of five AVs was prepared from the stools of different healthy volunteers, which later were also employed as blood donors to prepare whole-blood cultures to test their AVs for immuno-pharmacological effects. An extensive cross testing of each AV with the blood of each of the donors ensured a complete overview on the influences the AVs showed on the pattern of immunoregulatory mediators (7 different cytokines). The sets of results were obtained: a) data derived from cultures where AVs were tested in cultures from the same donor who gave the stool sample to prepare this very AV ("autologous" situation) and b) data from tests where AVs were added to cultures of blood of the other donors ("homologous" experiments). This allowed to detect the overall differences in the effects the AVs caused under both test conditions and by using these data characterise the degree of autospecificity AVs are able to produce.

Interestingly, when the different homologous AVs were tested on the blood of a single donor there was considerable homogeneity in the reactions they generated, indicating that generally there is not much individuality in the response of the antigen non-specific leukocytes towards AVs of different origin. The opposite seems to be true when testing AVs in an autologous system: Each test donor reacted to his/her own AV with a cytokine pattern that revealed distinct differences to what was seen in response to the AVs of other donors. In addition, every volunteer's leukocytes demonstrated a particular (set of) cytokine(s) that seemed to be influenced much stronger (or much weaker) by most if not all other AVs compared to the effects of the autologous AV.

Our *in vitro* experiments clearly demonstrated the autologous AVs to exert immuno-pharmacological activities showing pronounced individual-specific effects. Further experiments using either

healthy volunteers or patients who receive autologous or homologous AVs respectively will now have to prove in *ex vivo* experiments if this holds true also for the situation as it is *in vivo*.

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OLD HERBORN UNIVERSITY SEMINAR ON PROBIOTICS: BACTERIA AND BACTERIAL FRAGMENTS AS IMMUNOMODULATORY AGENTS

MINUTES AND OVERVIEW OF THE DISCUSSIONS

DIRK VAN DER WAAIJ

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DISCUSSION PARTICIPANTS (in alphabetical order):
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Charles L. Bevins:

Four potential roles for human defensins can be recognised:

1. Protection of the stem cells located just distal of the Paneth cells in the crypts of Lieberkühn in the small intestines
2. Microbes ingested with food and water may be confronted with a hostile environment created by defensins
3. Defensins may shape the intestinal microflora as they have a broad spectrum antimicrobial activity while some bacteria are more susceptible than others
4. The colon is more heavily colonised than the small intestines, which may be partly due to activity of defensins.

In addition to antimicrobial activity, defensins may have a signal function to the adrenals where they act as 'corticostatins' as they down regulate corticosteroid production. Furthermore, defensins are chemo-attractants to neutrophils and finally inside the crypts defensins would stimulate Paneth cells.

- Paneth cells are already during pregnancy present in the gut of the human foetus and thus at birth. Activity, however, is lower than at birth. In mice Paneth cell activity is turned on much later after birth namely at the time of weaning to become of adult type at six weeks of age.
- It is not yet known which bacteria in particular stimulate Paneth cells in particular; perhaps *Bacteroides* species are good candidates.
- Breast milk may have a stimulatory effect on Paneth cell function; however, this is still uncertain.
- Many human cancer cell types are sensitive to defensins, which may physiologically originate from neutrophils.
- Paneth cells develop/differentiate from crypt stem cells which to this end migrate downward and have a lifespan of about four weeks.
- Defensins are produced as double molecules which are later clipped by a trypsin which is simultaneously produced by Paneth cells.

Note: *Shigella* species seem to be able to shut down Paneth cell activity (by blocking or enzymatic breakdown).

Necrotising enterocolitis has been described in patients with a Paneth cell deficiency.

In human neutrophils, quite a number of defensins have been found but not in the mouse. Deficiencies but also

the opposite excess of defensins have been described in plants. Deficiencies exist also in man but the genome is not yet determined; in knock out mice, perhaps more than one genome is involved in Paneth cell activity.

Germain Trugnan:

The glycosylation is being studied *in vitro* and *in vivo* as one of the targets of resident bacteria in human intestinal cells. Glycosylation may play several roles in the gut such as modulating interactions with pathogens, changing cell proliferation and differentiation. Glycosylation changes may represent the mechanism by which resident bacteria from the gut may influence gut functions through a remote control process. The works presented demonstrated that *Bacteroides* species release soluble factors that specifically modulate galactosylation, *in vitro*. *In vivo* studies confirmed that a soluble factor of *Bacteroides* modulates galactosylation but also shown that sialylation and fucosylation of proteins and lipids were modified at the surface of the host cell. Small molecules of *Bacteroides* have been isolated and shown to carry the glycosylation modifying activity. In vitro data shown that increasing the surface galactosylation by the soluble factor of *Bacteroides* cause the cells to resist rotavirus infection.

Note: The signalling molecules can pass the mucus layer.

Lars Å. Hanson:

The human new-born has a small immune system. Therefore, it is amazing that it can manage the influx of bacteria from the outside world upon birth and keep it under control. In newborns, Th1 precursor cells function normally, there is a deficiency in neutrophil and monocyte function. This implies that although the new-born has to a certain extend a deficient innate immune system and a marginally functioning adaptive immune system, yet the new-born can control the microbial attack from the outside world. However the help from the mother via transplacental IgG antibodies and milk secretory IgA antibodies provides important support.

In the mother, microbes that get in contact with Peyer's patches in the gut, (may reach mesenteric lymph nodes and spleen and) stimulate specific secretory IgA production. Lymphocytes producing such secretory IgA also migrate to (reaches) the mammary glands via (with) the blood stream. Once in the mammary glands they produce the milk antibodies against the mother's intestinal flora. However, quite a number of neonates are raised successfully without breastfeeding, although their risk of repeated even lethal infections is considerably higher. How babies (manage) expand their immune system to take over the role of breastfeeding is still not fully known.

Important monitoring factors during pregnancy are the cytokines stemming from the mother's immune response to the paternal structures of the foetus. The mother's milk is also full of full of signals. This includes among else cytokines which affect (stimulate) insulin production, fatty acid metabolism, the central nerves system and of course lymphocytes in the neonate.

Mycobacteria (and endotoxines) are microbial stimulants, which play a crucial role as stimuli of the development of the immune system as has been found in

experimental animals.

In mothers suffering from *Myasthenia gravis* and in *Lupus erythematosis*, evidence has been found that their babies produce the same auto-antibodies. This is possible due to transplacental passage of anti-idiotypic antibodies which relate to the disease and which turn on a secondary response in the neonates.

Presumably, defensins are not playing an essential role in the shaping of the microflora since baby mice do not have defensins in the gut and yet develop their microflora much more gradually than occurs in man.

Tolerance in human babies develops almost directly after birth. This could be an important factor in the acceptance of the developing intestinal microflora. This capacity to tolerance is to avoid immunological reactivity to food proteins (either the cow's milk proteins in the milk-drinking mother's own milk or in case the neonate is given formula). It is safer to provide host defence against the microbes.

Note: Microbial signals coming from the skin and the gut, may reach the nervus vagus along peripheral receptors and thus reach the brain area to influence various brain functions.

Bengt Björkstén:

In several studies, differences in the composition of the intestinal microflora have been described between allergic and non-allergic individuals. Consistent in these studies was differences between and numbers of *Clostridium* and *Bifidobacteria* species. It is unknown whether this is the cause or an epi-phenomenon.

In infants with skin allergies, improvement has been claimed upon treatment with "Lactobacillus GG". However, the study was small and the infants were only studied for one month. The immunological implications of this kind of treatment are at present being studied.

Administration of antibiotics early in life and even during pregnancy, may play a role in development of allergy. Furthermore, the composition of the maternal vaginal microflora seems to be related to bronchial obstruction in four-year old children.

Prematurity as well as low birth weight is inversely related to development of atopy. High birth weight on the other hand may enhance the risk for atopy.

Breastfeeding appears to beneficially limit "infant wheezing" but not respiratory tract allergies like 'asthma'. At this point, there may be confusion about the term asthma. For example until some ten years ago, in Germany asthma was considered non-existing because a different definition of asthma was used.

Fatty acids in the diet would enhance cytokine production in the gut and therewith influence the risk for development of an allergy. However, this is till uncertain and certainly not a major factor. The intestinal microflora may modify long chain fatty acids, which may play a role in allergy.

In a recent study published in the Lancet, the possibility to prevent allergy was assessed in infants who were followed over the first two years of life. In the breast-fed group, the mother was treated with lactobacillus GG during and after birth; in the bottle-fed group lactobacillus GG was administered to the child (it was then no longer given to the mother). The authors claimed that this treatment in both groups would be comparable. In the groups with 'Lactobacillus GG' treatment, a 50% reduction

in atopy was found. It is unlikely that, if the conclusion is correct and both treatment groups were comparable, that the outcome would be confined to "Lactobacillus GG". It is considered likely that *L. reuteri* and *L. plantarum* would have a similar effect as "Lactobacillus GG". *L. plantarum* would adhere better to mucosal cells than *L. reuteri*.

For proper study designs, all children with an atopic disease should be sampled for a year and be followed up for two years. This is however very expensive in a double blind placebo controlled study design. In Cologne, Joseph Beuth is working on a computer model and is of the opinion that such an "expert model" would make an expensive study, as described above, unnecessary.

Rudolf Kunze:

In a pilot study the effect of auto-vaccines, (made from killed *E. coli* isolates from the patients included in the study) was studied. Blood samples were taken at 1 and 3 weeks after subcutaneous auto-vaccine administration in inflammatory bowel disease inflammatory bowel disease (IBD) patients. In the blood samples the concentration of IFN-α and GM-CSF (granulocyte/monocyte colony stimulating factor) were determined. It was found that both were down-regulated by auto-vaccine treatment. The question arose whether auto-*E. coli* would work better than a vaccine prepared from any *E. coli* strain and it was suggested that a non-vaccinated control group would be of help in subsequent studies.

Graham A.W. Rook:

Advocates of the "hygiene hypothesis" have suggested in the past that the striking increase in the incidence of allergies in the clean rich countries might be due to lifestyle changes leading to insufficient activation of Th1 (T helper 1) cells. In the absence of sufficient Th1 cell activity, there might be an increase in the activity of Th2 (T helper 2 cells) that mediate allergies. However this view of the hygiene hypothesis must now be changed. There is a simultaneous rise in the incidence of diseases mediated by Th1 cells, such as autoimmune type 1 diabetes and multiple sclerosis. Similarly there is also a rise in inflammatory bowel diseases (IBD: ulcerative colitis and Crohn's disease). This simultaneous rise in allergies (Th2), autoimmunity (Th1) and IBD (mixed Th pattern) implies that the problem lies *not* in Th1/Th2 balance, but in the balance of regulatory T cells (such as TR1 and Th3) to effector cells (Th1 and Th2). It is also possible that changes in the pattern of disorders of mood and behaviour (such as autism, depression, chronic fatigue syndrome) are also linked to the changes in cytokine balance that result from this disturbed ratio of regulatory to effector T cells. Recent epidemiological studies show links between some of these disorders and allergies or IBD, and cytokines such as Interferon-α and IL-1 and IL-2 have profound effects on mood.

It was suggested that a decreased exposure to mycobacteria in the modern lifestyle might be one factor because these organisms have been shown to evoke not only Th1 effector cells, but also, more importantly, IL-10-secreting regulatory T cells that can treat allergic manifestations. Thus mycobacteria from the environment, and probably other genera such as *Lactobacilli* found in the

bowel flora, may play a crucial role as "regulatory cell adjuvants", that are an evolutionarily determined necessity.

Kurt Zimmermann:

The central question discussed in view of the proposed health claims proposed for probiotics was whether live or dead bacteria were equally effective in modulating some important immune functions associated with improvement of diseases. Up to now, among functional foods, the community of probiotic bacteria has become increasingly important under scientific as well as under commercial aspects in the past decade.

It is generally accepted from the actual definition of probiotics, that these products should contain predominantly living microorganisms. Among the numerous strains marketed as probiotics, some „dominant" probiotic strains have emerged such as *Lactobacillus rhamnosus* GG, *Lactobacillus johnsonii* La1 and *Bifidobacterium lactis* Bb12, for which a substantial amount of literature claimed several important health effects. The majority of "health promoting effects" include, as yet mostly still unproven functions such as a "general improvement of well being". Also clear medically relevant effects have been reported in several recent publications like beneficial effects of probiotics in inflammatory bowel disease, allergy, cancer or prevention of intestinal tract diarrhoea as well as general GI infection. However, these probiotic-mediated effects in these serious medical indications remain to be proven with well-controlled clinical studies and the immunological mechanisms underlying the sometimes-observed clinical efficacy await further clarification

Assuming that a considerably important part in health promoting effects may result from interactions of the bacteria with the human immune system, it is still not yet clear which molecular mechanism(s) may underlie these interactions particular in view of the viability of the bacterial strains. It was discussed that interacting with different subtypes of TLRs, LPS or Lipoteichoic acids from Gram-negative and Gram-positive bacteria may induce different immunomodulatory functions. However, with regard to preventing infections in the GI tract, live bacteria may considered to be more effective than dead ones. But the different bacterial cell wall compounds, characteristic for Gram-negative and Gram-positive bacteria, may be in some kind as effective as live microorganisms in terms of cytokine induction.

This was reported during the meeting by experimental data to occur on the cytokine modulating capacity of a heat-inactivated bacterial preparation named Pro-Symbioflor® which contains a mixture of Gram-negative and Gram-positive human non-pathogenic strains, *E. coli* and *Enterococcus faecalis*, respectively. This preparation could induce a predominant TH_1-immune response in an *in vitro* model in blood. It was tentatively concluded that use of this probiotic preparation to counteract the overactivation of TH_2-lymphocytes seen in atopic allergy.

Note: The capacity of Pro-Symbioflor® to upregulate IL-12, IFN-γ and the immunoregulatory cytokine IL-10 *in vitro* contrasts some recent literature findings that Gram-negative and Gram-positive bacteria would induce preferentially cytokines like IL-12 and IL-10. Furthermore, reports dealing with the cytokine inducing capacity dependent on bacterial growth phase and their heat treatment obviously cannot be applied to Pro-Symbioflor®. This

makes likely, that important qualitative and quantitative differences exist among medical-probiotic strains of bacteria.

Henrich H. Paradies:

Autovaccines prepared from *E. coli* (named AutoColiVaccines in the contributions by *Schmolz* and *Ottendorfer*) can loose their kDO side chains (two or three units as determined by MALDI-TOF-MS and determination of the number of carboxyl groups), depending on initial concentration, decrease of pH (pH 5.6) and subsequent incubation at 70°C (5 minutes). The remaining material consists of free Lipid-A analogues of which 80% (w/w) is surprisingly unphosphorylated and 20% (w/w) is phosphorylated at C-1 of one of the glucosamine disaccharide. Pure free Lipid-A analogues aggregate into colloidal supermolecular structures depending entirely on particle number density rather than on absolute mass at extremely low ionic strength at ambient temperature as determined by light scattering methods and small-angle neutron scattering measurements (SANS). The formed colloidal crystals can grow to sizes as large as 1-5 μm, revealing highly ordered structures, showing either a bcc lattice with a = 41.5 nm at low number particle density, or a fcc lattice with a = 51.7 nm (SANS) at moderate number particle density, or e.g. inter-twinned ropes (TEM and HRTEM), which show elastic and extremely contractile properties as found by AFM and MFM (Magnetic Force Microscopy). These colloidal assemblies are extremely sensitive towards Ca-ions (nM, yielding compact structures) and Mg-ions (μM, giving rise to expanded and monolayer structures), which influence the elastic and contractile properties of LPS and free Lipid-A in Gram-negative bacteria like an ion-sensitive pump (osmometer) due to influencing the osmotic and thermodynamic properties (compressibility) of this dynamic system.

Note: Autovaccines obtained from patients suffering from various chronic diseases have been found to have different chemical structures for the free Lipid-A as well as for the covalent attachment of the number of the kDO units, and their sugar components within the kDO units, whereas the glucosamine disaccharide units are conserved and not altered at all. However, the number of acyl chains bound to the disaccharide moiety and partly the chain lengths can be significantly altered, e.g. shortened from C_{14} to C_{12}. The significant changes in the TNF-α, IL-10, IL-5, IL-12 and IFN-γ activities, which is upward or downward regulated in a concentration dependent manner in the presence of free Lipid-A including the influence of the kDO units.

Manfred W. Schmolz:

Five different AutoColiVaccines (ACV) were prepared from the stools of 5 individuals. When these ACV were tested in whole blood cultures of these subjects, the response of the leukocytes, measured by cytokine syntheses, depended strongly upon which ACV was applied to the cultures. The leukocytes responded to the ACV of other individuals with a much more homogenous cytokine pattern compared to the ACV of the donor from which they were taken. Generally the response followed a Th1-type mediator pattern, although when leukocytes saw the "autologous" ACV, there was large

variability. The leukocytes of some donors even refused to secrete TNF-α, which is one of the major cytokines released upon challenge with Lipid-A like molecules such as the ACV.

The question arose as to which mechanisms could modulate individual responses to a molecule that, when given to leukocytes from other donors elicited mediator productions exactly as could be expected when testing Lipid-A or LPS. Future experiments will have to elucidate if this is the result of an antigen-specific mechanism (such as antibodies neutralising Lipid-A in the cultures, Lipid-A specific regulatory T cells) or some up to now unknown signalling through Toll-like receptors and their adjunct co-regulatory proteins.

Doris Ottendorfer:

The discussion concerned immunoregulatory function of AutoColi-Vaccines (ACV) developed in Herborn. Potential receptors on T-lymphocytes and macrophages such as Toll-like receptors, scavenger receptors or glycolipid specific receptors (such as the CD1 antigens), that possibly were involved in responses to ACV, were discussed. All receptors were found able to influence the secretory and functional activities of different cell types of the innate and adaptive immune response. Several possible mechanisms were discussed by which the ACV could affect the cytokine response of monocytes, B-lymphocytes or T-cell subpopulations found *in vitro*. Assuming that different intracellular signalling pathways might be initiated upon the ligation of the different receptors, the recently shown immunoregulatory actions of ACV in the whole blood culture model appear quite extensive. It was speculated that the internalisation properties of the ACV might be altered by its physical state. The way by which different-sized "superstructures" of the ACV interact with leukocyte membranes could determine the observed cytokine response profile, particularly due to their dynamic and elastic mode of solution behaviour e.g. formation of ordered compact structures and specific monolayers in the presence of cytoskeletons, actin cells and adhesins by influencing the rheologic properties of the entire process. The author speculated that possibly these effects might be of value for treatment of chronic inflammatory diseases.

It was considered important in this respect that, if a rabbit is injected i.v. with an ACV preparation, which resembled in structure the core complex of the natural Lipid-A molecule of Gram-negative bacteria, no fever occurred in response. Presumably pure LPS would caused IL-1 release and therewith fever. It was proposed that upon i.v. injection of ACV an increase in IL-10 may contribute to this activity after administration of ACV This assumption is based on *in vitro* observations in the whole blood culture model which revealed a clear induction of IL-10 release by B-lymphocytes and monocytes.

Note: Intra-dermal injection of ACV does not stimulate general antibody production in human subjects, as antibodies to various antigens tested do not rise in titre in the blood. This is possibly due to the site of administration: the skin and its cellular composition i.e. dendritic cells, keratinocytes, 'skin-homing' T-cells and Langerhans cells. These cell types may play a role in the initiation and regulation of an immune response to intra-dermal applied bacterial components like those present in ACV.

Joseph Beuth:

Propionibacterium avidum and *P. agnes* as well as defined coagulase-negative staphylococci (CNS) were chosen from 200 bacterial strains because of their immunologic activity. The highly active strain (*P. acnes*) caused fibrosis, which was obviously an unwanted side effect so that *P. avidum* was selected for further studies. In mice the effect of different doses on the thymus was studied as well as its effect on tumours and infections (e.g. induced by *E. coli*). In colorectal cancer patients as well as in patients with malignant melanoma or breast cancer, *P. avidum* was i.v. injected with an apparent success. However, the study had to be discontinued because approval for the study was stopped.

In mice injection of whole LTA molecules caused side effects (e.g. wasting-like syndrome). An active peptide was extracted from the whole bacterium *P. avidum*. These peptide-induced effects on T-cells, cytotoxic T-cells, NK-cells and monocytes, however, were less pronounced than the whole cell vaccine.

From CNS, three peptides were isolated which were shown to stimulate B- respectively T-cells to proliferation and were therefore considered for further evaluation. These proliferation-inducing peptides may be used for induction of stem cell proliferation.

P. avidum peptide was further purified meanwhile. A heat-killed vaccine (whole bacterium, *P. avidum*) can be applied orally in capsules.
The cytokines induced are not toxic but stimulate cell proliferation (e.g. of T-cells) presumably due to the low size (molecular weight) of the peptide.