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18. FROM FRIENDS TO FOES

EDITORS:

PETER J. HEIDT
TORE MIDTVEDT
VOLKER RUSCH
DIRK VAN DER WAAIJ

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EDITORS:

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

Tore Midtvedt, M.D., Ph.D.
Department of Medical Microbial Ecology
Karolinska Insttute
von Eulers väg 5
S 171 77 Stockholm
Sweden

Volker Rusch, Dr. rer. nat.
Institute for Integrative Biology
Auf den Lüppen 8
D-35726 Herborn-Dill
Germany

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

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

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Authors

Josef Beuth, M.D., Ph.D., Institute of Naturopathy, Medical Faculty, University of Cologne, Robert-Koch-Straße 10, 50931 Cologne, Germany.

Charles O. Elson, M.D., Professor of Medicine and Microbiology, Division of Gastroenterology and Hepatology, The University of Alabama at Birmingham, 633 Zeigler Research Building, 703 S. 19th Street, Birmingham, AL 35294-0007, USA.

Paul W. Ewald, Ph.D., Professor of Biology, Department of Biology, University of Louisville, Louisville, KY 40208, USA.

J.S.H. Gaston, M.A., Ph.D., B.M., B.Ch., F.R.C.P., F.med.Sci., Professor of Rheumatology, University of Cambridge School of Clinical Medicine, Department of Medicine, Box 157, Level 5, Addenbrooke's Hospital, Hills Road,Cambridge, CB2 2QQ, UK.

Åsa Ljungh, M.D., Ph.D., Department of Medical Microbiology, Dermatology and Infection, Lund University, Sölvegatan 23, SE 223 62 Lund, Sweden.

Willem L. Manson, M.D., Ph.D., Department of Medical Microbiology, University Medical Centre Groningen, Hanzeplein 1, 9713-GZ Groningen, The Netherlands.

Glen D. Mithoe, M.D., Department of Medical Microbiology, University Medical Centre Groningen, Hanzeplein 1, 9713-GZ Groningen, The Netherlands.

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

HELICOBACTER: CHRONIC EFFECTS AND ROLE IN HOST MICROECOLOGY

TORKEL WADSTRÖM and ÅSA LJUNGH

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

SUMMARY

Helicobacter pylori is the first named species of the Helicobacter/Wolinella family, now including more than 20 species and about 10 candidate species. The organisms are all micro-aerophilic "mucinophiles" with a few exceptions. *H. pylori* is the prototype for a number of bile-sensitive species colonising the stomach of most mammals, including dolphins and whales. The low toxicity of the lipopolysaccharide (LPS) and a number of properties unique for these species determine how they may cause life-long infections. *H. pylori* carries the vacA toxin as well as a set of other virulence traits permitting optimal early colonisation of the host, e.g. in childhood. The cagA pathogenicity island (PAI) makes $cagA^+$ strains of *H. pylori* more virulent than cagA- strains to develop chronic active gastritis, gastric atrophy and pre-cancerous lesions in the host as well as in mouse and mongolian gerbil models. *H. pylori* as well as a number of entero-hepatic bile-tolerant species are camouflaged from the innate immune system of the GI epithelial cell surfaces, yet $cagA^+$ *H. pylori* transcribe NF-κB to the nucleus of these cells and of macrophages and other cells. At least *H. pylori* evades the host immune system by a number of responses such as molecular mimicry of the H–K adenosine triphosphatase and of gastric cell surface fucosylated antigens. The degree of inflammation is modulated by the IL-1β cytokine polymorphism and probably by a number of other host factors.

The co-evolution of *H. pylori* and man back to the origin of mankind is clearly defined with a sophisticated haemostasis between the *H. pylori* as a pathogen. Alternative scenarios in the 21st century in several parts of the world with a "clean" *Helicobacter*-free human stomach are addressed as well as recent reports of a newly discovered gastro-oesophageal microflora and the rapid increase in GERD, Barrett´s oesophagus, oesophageal cancer and obesity as well as changes in living conditions in Western societies.

INTRODUCTION

Helicobacter pylori lives in the mucus layer overlying the gastric epithelium and does not appear to invade tissues. However, the mucosa underneath the area of colonisation is invariably inflamed (chronic superficial gastritis; *Northfield* et al., 1994). Most infected persons do not show clinical manifesta-

Table 1: Entero-hepatic bile-tolerant *Helicobacter* species

Species	Comment
Helicobacter pylori	some strains are bile-tolerant
Helicobacter pullorum	common in chicken
Helicobacter bilis	common in rodents
Helicobacter hepaticus	common in rodents
Helicobacter cholecystus	common in hamster
Helicobacter canis	common in dogs
Helicobacter rappinii	certain subtype common in sheep
Helicobacter ganmani	anaerobic

tions of the inflammation. Studies that include human volunteers, experimental animal infections and treatment of patients with antimicrobial agents show that *H. pylori* plays a critical role in this inflammation and in these diseases. Much evidence suggest that *H. pylori* is an indigenous microbe of the human stomach and that most, if not all, mammalian species harbour related *Helicobacter* species with a long co-evolution of microbe and host (*Blaser,* 1998; *Richter,* 2001). *H. pylori* probably evolved from bile-tolerant enteric *Helicobacter* species colonising rodents and other mammals, including primates and man (*Fox* et al., 2001; Tables 1 and 2). The phylogenetic tree of proteobacteria includes *Sulphurospirillum*, *Arcobacter*, *Campylobacter, Helicobacter* and *Wolinellae* (*On*, 2001; Figure 1).

More than 20 species of *Helicobacter* are recognised today, with *H. heilmanii* as a second gastric species. This species and some others are highly fastidious and difficult or impossible to culture *in vitro* under micro-aerophilic or anaerobic conditions with *H. ganmani* as the prototype of the second group (*Robertson* et al., 2001). All species are highly motile and possess non-sheathed or sheathed flagellae enabling them to swim in the mucin layer (*Andersen* and *Wadström*, 2001). Suerbaum and colleagues (*Schreiber* et al., 2004) recently showed that *H. pylori* prefers a specific part of the gastric mucin layer, probably regulated by acid secretion, *H. pylori* urease and ammonia formation, a metabolite most toxic for the gastric mucosa. Urease-negative as well as catalase-negative mutants are unable to colonise and infect the mouse stomach, suggesting that ammonia production and the redox potential are crucial to initiate the infection. Several ge-

Table 2: Evidence that *Helicobacter pylori* infection of humans is of ancient origin.

- Extensive genetic heterogeneity
- Acid-secreting stomachs arose early (300 million years ago!) in vertebrates
- *Helicobacter* genus is highly prevalent in the stomach and gut of all vertebrates?
- *H. pylori*-like organisms are widely present in the stomach of primates
- High incidence among human populations in Asia and Africa of *H. pylori* (>80-90%)
- *H. pylori* is adapted to persist for lifetime in the human stomach

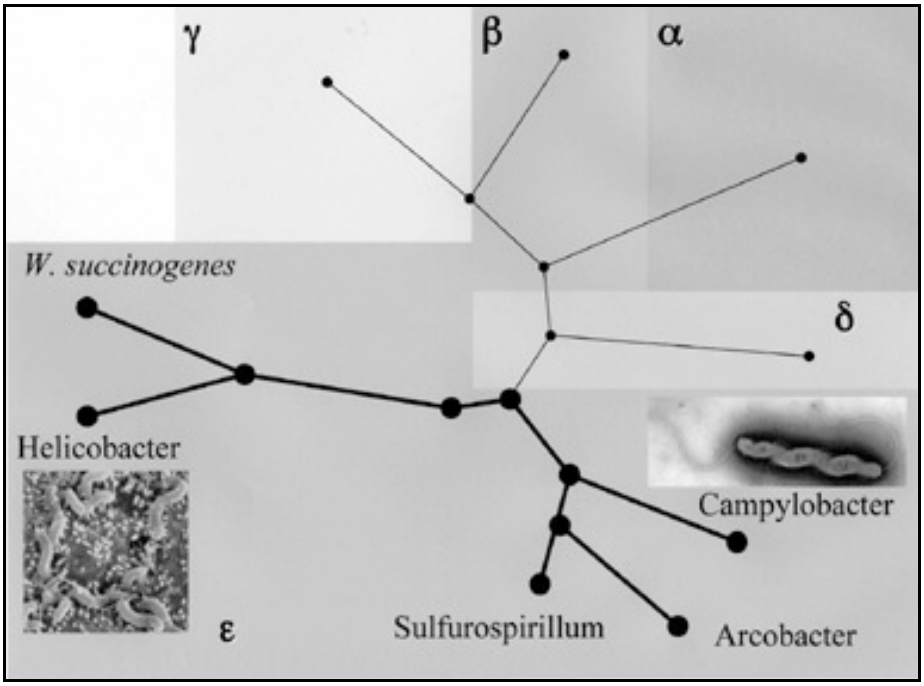

Figure 1: Representation of the phylogenetic tree of proteobacteria (modified from *On*, 2001).

netic studies of *H. pylori* isolates from a single human stomach show that these microbes are highly adaptive organisms, which partly explains that this pathogen can persist for decades in a single stomach inducing a low grade tissue inflammation (*Blaser* and *Atherton,* 2004). This adaptation involves mutations and recombination, and many strains may be classified as hypermutation phenotypes. *H. pylori* is able to maximise diversity of genetic sequences under strong selective pressure while maintaining alleles critical for its lifestyle (*Björkholm* et al., 2004).

Helicobacter-like organisms, resembling the syphilis spirochete, were reported by several pathologists in human and animal stomachs already in the period from1880 to 1890, including beautiful studies in dogs by Bizzozeroni in Italy, describing a species today named *H. bizzozeron* (*On,* 2001). However, its possible role as a gastric pathogen and not a post mortem "by-stander" was not addressed properly until *Marshall* and *Warren* (1984) in 1982 grew the first *Campylobacter pyloridis* (later *C. pylori* and renamed to *Helicobacter pylori* in 1989). By drinking viable *in vitro* cultured *H. pylori* cells, Marshall and colleagues showed that it induced acute achlorhydria and dyspepsia, which was suppressed or cured by a bismuth-antibiotic therapy.

Later, *in vitro* co-culture studies of *H. pylori* and gastric cancer epithelial (AGS) cells showed that strains containing the 35 to 40 kilobase cag pathogenicity island (PAI) flanked by specific 39 basepair direct DNA repeats induced a higher cytokine response (IL-8), and promoted an anti-apoptotic pathway aiding persistence of the organism in the gastric mucosa (*Crabtree,* 2001).

Another reason for its persistence is the molecular mimicry, in part due to the low biological activity of its lipopoly-

saccharide (LPS)(*Moran* et al. 2000*; Blaser* and *Atherton,* 2004). Molecular mimicry between *H. pylori* antigens and H^+,K^+-adenosine triphosphatase activates $CD4^+$ T cells in the stomach. This leads to gastric autoimmunity in genetically susceptible individuals via molecular mimicry (*Amedei* et al., 2003).

H. PYLORI PATHOGENESIS – A MULTIPLE STEP INFECTION TO CHRONIC GASTRITIS AND GASTRIC ATROPHY

Early development of mouse models has clearly given good opportunities to elucidate the *H. pylori* pathogenesis, and to develop alternative prophylactic and treatment schedules to standard proton pump inhibitor (PPI) and antibiotics (*Hamilton-Miller*, 2003). Mice given the vacuolating (vac) toxin orally developed ulcers. However, strains producing a vac toxin with an S1/m2 mid-region seem to bind poorly to specific cell lines and induce less tissue damage and cell membrane pores (*Blaser* and *Atherton*, 2004). Moreover, the S2 genotype is associated with a lack of the cag PAI and may induce a less severe gastric inflammation. Transient oral and gastric *H. pylori* colonisation occurs in children, as shown in a study from Dhaka, Bangladesh (*Casswall* et al., 1999). It is likely that *H. pylori* is a paediatric infection, "achieved" soon after weaning in all primitive societies (*Blaser,* 1988). Weaning habits such as maternal chewing of food and early rotavirus and other viral infections changing the gastric physiology influence the time of acquisition. Ongoing infection can be detected by faecal immunomagnetic bead based PCR or antigen detection methods (*Weingart* et al., 2004). A humoral as well as local immune response is rapidly induced. Antibody titres remain for several decades but interestingly, $cagA^+$ strains disappear more rapidly (*Perez-Perez* et al., 2002).

The *H. pylori* LPS is an anergic low toxicity endotoxin with a unique lipid A core structure (*Hynes* and *Wadström*, 2004). It stimulates only macrophage Toll-like receptor (TLR4) and not gastric TLR4 (*Bäckhed* et al, 2003). CagA-positive strains induce transcription of NF-κB in the epithelium through recognition of Nod1, an innate intracellular pathogen-recognition molecule, recognising soluble bacterial peptidoglycan fragments (*Kim* et al., 2004). How such molecules as well as other cell surface, extra-cellular and cell lysis molecules, including nucleic acids, are delivered to the gastric mucosa is poorly understood. Further studies are needed to define new possible interventions, such as probiotic-based strategies including anti-*Helicobacter* peptides and bacteriocins (*Hamilton-Miller*, 2003; *Lorca* et al., 2001).

The *H. pylori* infection down-regulates the immune response, suppresses T-cell proliferation and induces selective T-cell apoptosis (*Shirin* and *Moss,* 1998; *Lundgren* et al., 2003). The early gastric colonisation involves a Lewis B binding cell surface (HOP) protein as well as a number of other adhesins, such as sialic acid lectins (SAL´s) recognising cell surface mucin and glycolipid molecules (*Gerhard* et al., 2001; *Falk* et al., 2000). Inhibition studies with milk glycoconjugates (*Hirmo* et al., 1998; *Wang* et al., 2000a; *Wang* et al., 2001) and a probiotic strain of Lactic Acid Bacteria (LAB) could prevent, suppress or cure *H. pylori* infection in a mouse model (*Cruchet* et al., 2003).

H. pylori further induces a rapid, early neutrophilic activation by a specific molecule (HPNAP) (*Teneberg* et al., 1997). This induces a rapid cell uptake

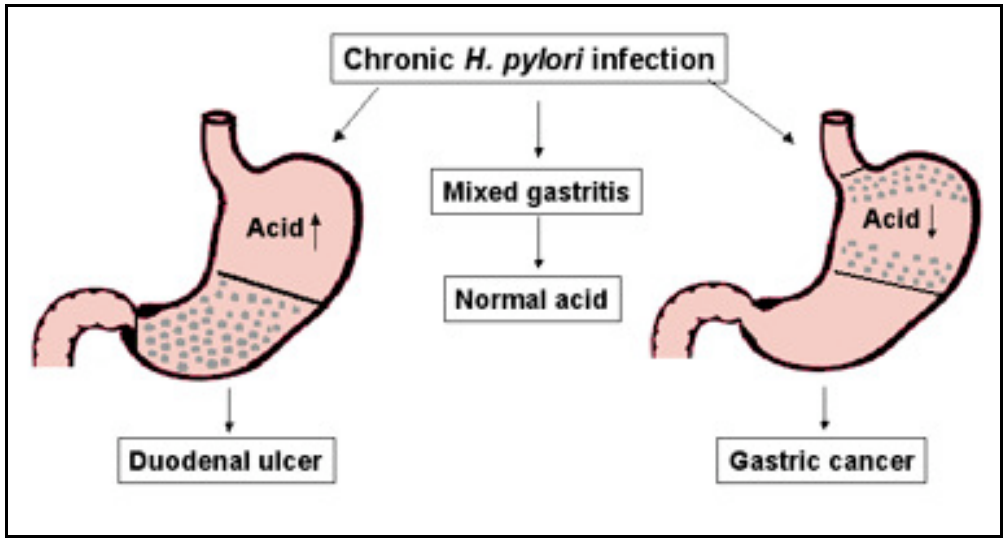

Figure 2: Divergent responses to *Helicobacter pylori* infection.

through lectino-phagocytosis by SAL´s and glycosaminoglycan (GAG) surface lectins. Other chronic infections, such as a specific helminth or parasite infection may modulate the Th1/Th2 immune response to a predominant Th2 response in black Africans, "the African enigma". This may reflect a genetic predisposition selected by malaria (*Fox* et al., 2000; *Bennedsen* et al., 1999).

A specific IL-1β polymorphism induced by *H. pylori* increases the risk of severe gastritis proceeding to gastric atrophy, hypochlorhydria and adenocarcinoma (*Blaser* and *Atherton,* 2004; Figure 2). Polymorphism of the TNF-α and IL-10 genes may have a similar modulating effect on the outcome of a chronic inflammation after one or two decades. A sophisticated somatostatin regulation of gastrin release, a growth factor for *H. pylori,* creates a feedback loop reversal after curing of an *H. pylori* infection (*Zhao* et al., 2003). A persistent increased tissue gastrin level increase the parietal cell mass and enhances the process of gastric metaplasia in the duodenum associated with *H. pylori* inflammation and duodenal ulcer disease (*Wang* et al., 2000b).

H. PYLORI IN THE 21ST CENTURY

H. pylori still infects the majority of children in the non-industrialised world, leading to pangastritis and stomach atrophy. Depending on food intake, i.e. a high or low level of fruit, antioxidants and possibly food carcinogens, the risk of gastric malignancies varies between 2.7 and more than 12-fold in various studies with high prevalences in Japan, Northern-China, the Baltic countries and other parts of Eastern Europe (*Forman* and *Graham,* 2004). However, in Western societies with a low incidence of *H. pylori* infection in children (< 2% today in Scania, Southern Sweden), the human stomach homeostasis and health should be studied since pangastritis leads to a reduction of gastric acid production (*Sande* et al., 2001; Figure 2). It is likely that an increased acid production is

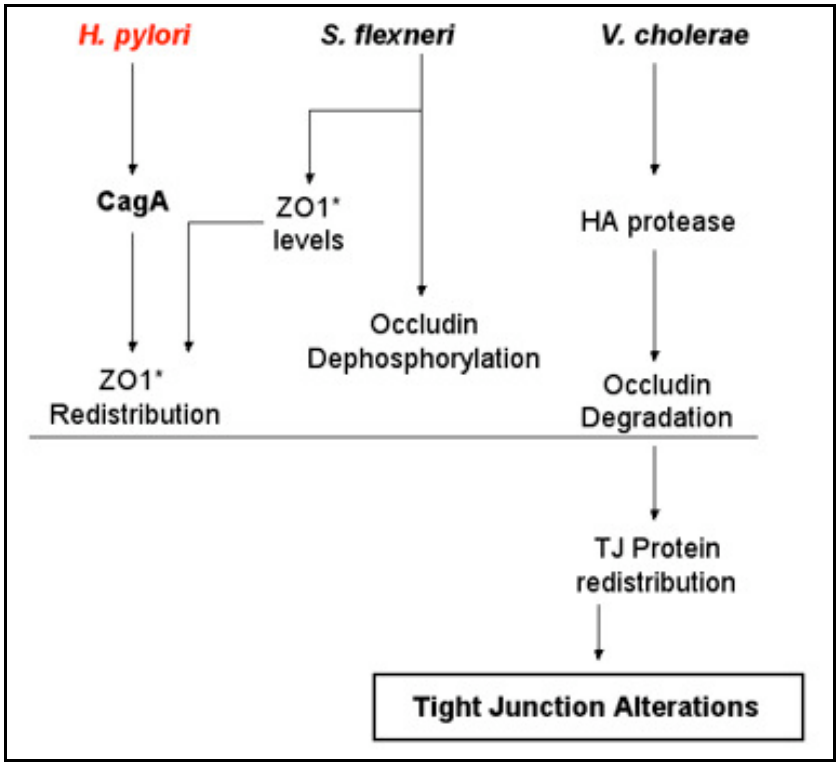

Figure 3: Disruption of tight junctions by microbes and microbial products.
*: ZO1= zona occludens 1.

associated with GERD-reflux oesophagitis and related conditions, such as Barrett´s oesophagus and pre-malignant epithelial changes (*Fitzgerald,* 2001).

Moreover, *H. pylori* infection is associated with elevated serum leptin levels (*Breidert* et al., 1999; *Matarese* and *Lechler,* 2004). A weight gain is common after *H. pylori* eradication (*Azuma* et al., 2001), possibly predisposing to adult as well as adolescent obesity. A high intake of antioxidant-rich food and food supplements can inhibit free reactive oxygen metabolites (ROM´s) and inhibit transcription of NF-κB and DNA mutations in the epithelium (*Wang* et al., 2000b). The relative role of food carcinogens, such as nitrosamines and water rich in nitrates, in gastric carcinoma development should be studied in various geographical regions of the world. Likewise, in patients on a long-standing PPI regime to suppress acid reflux (GERD) disease, gastric overgrowth by enteric microbes with potential carcinogen production (c.f. enterococci) should be investigated.

THE IL-10 -/- MOUSE AND *HELICOBACTER*-INDUCED GASTRITIS AND COLITIS

LAB of the upper mouse stomach form a barrier towards *Salmonella, Helicobacter* and other bacterial gastrointestinal pathogens. An early germ-

free (GF) mouse model to study anti-*Helicobacter* effects of *L. gasserii* was developed in Japan (*Kabir* et al., 1997). More recent studies indicate that GF mice are not readily colonised by *H. pylori* and enteric *Helicobacter* sp. (*E. Norin, H.-O. Nilsson,* and *T. Wadström,* unpublished observations). However, an IL-10 -/- mouse derived from C57-black mouse responded to *H. pylori* with a more severe gastric inflammation than Balb-c, and this mouse strain seems promising to optimise a mouse *H. pylori* gastric cancer model (*Kullberg* et al., 2003).

The IL-10 -/- mouse is susceptible to *H. pylori* as well as to natural and experimental *H. hepaticus* and other enteric *Helicobacter* species (*H. bilis, H. ganmani,* etc., see Table 1).

A first *H. hepaticus* colitis study in IL-10 -/- mice by Pena and co-workers (*Pena* et al., 2004) suggests that this model may become the model of choice to study effects of probiotic microbes as well as other therapies towards gastric and enteric *Helicobacter* infections. IL-10 is associated with several traits such as gut permeability regulation, which seems important for LAB as well as antioxidant anti-*Helicobacter* effects. Similar mechanisms were proposed for *Salmonella* and other enteropathogens, including *Vibrio cholerae* (Figure 3). The complete genome of *H. hepaticus* has been published (*Suerbaum* et al., 2003). This will provide a valuable tool to identify virulence genes.

However, in the near future well defined conditions to create and keep *Helicobacter*-free mouse colonies should be addressed, including a modified Schaedler flora to stimulate studies on chronic experimental models of inflammatory conditions and to avoid interference of murine *Helicobacter* induced inflammations in various experimental models. These include inflammatory bowel disease (IBD) in dextran sulphate and other chemically as well as microbe induced IBD-like syndromes.

CONCLUSIONS

The gastric as well as the intestinal epithelium is an interactive barrier that directs neutrophil movement. Specific peptides act via Toll-like receptors and induce NF-κB transcription with production of pro-inflammatory cytokines. *H. pylori* and several enteric *Helicobacter* species may disrupt tight junctions (TJ:s) (Figure 4) in a similar way as discussed for enteric pathogens such as *Shigella flexneri*. Ongoing studies in several laboratories aim at means to stabilise TJ:s, e.g. by probiotic and antioxidant treatment. This may also be an important step in inflammatory bowel disease (IBD) research in IL-10 knockout mice as well as in human patients. Further comparative studies on the pathogenesis of *H. pylori* and enteric IBD-inducing species (*Sturegård* et al., 2004) may reveal new preventive and curative methods for chronic gastric and enteric inflammations.

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PATHOGENESIS OF INFLAMMATORY BOWEL DISEASE: THE BACTERIAL CONNECTION

CHARLES O. ELSON[1], YINGZI CONG[1], ROBINNA G. LORENZ[2], and CASEY T. WEAVER[2]

[1]Division of Gastroenterology and Hepatology, Department of Medicine and [2]Department of Pathology, The University of Alabama at Birmingham, Birmingham, Alabama, USA

SUMMARY

The inflammatory bowel diseases are complex, idiopathic disorders whose pathogenesis is beginning to be understood largely due to the generation and investigation of many experimental models over the past decade. In most of these models, the enteric microbial bacteria are obligatory for disease expression, and in most, $CD4^+$ T-cells are the effector cells mediating chronic intestinal inflammation. Thus these disorders appear to represent disorders of host - microbial interactions in the intestine. One model, C3H/HeJBir mice that are a high susceptibility phenotype for spontaneous colitis, has been particularly informative regarding the 'bacterial connection' to IBD. C3H/HeJBir T-cells are highly reactive to enteric bacterial antigens, and such Th1 cells can mediate disease upon transfer to immunodeficient SCID recipients. Sera from C3H/HeJBir mice has been used to identify and clone 60 antigens that stimulate their pathogenic T-cells and B-cells. The adaptive response to the microbiota is thus highly selective, even in the setting of chronic intestinal inflammation. Interestingly enteric bacterial flagellins comprised the major group of these antigens, and about half of patients with Crohn's disease have IgG antibodies to them as well. There are emerging data from both experimental models and humans that the innate immune system plays a major role in the host interaction with the microbiota and that defects in innate immune cells, such as epithelial cells, dendritic cells, and macrophages, direct and shape the abnormal T-cell and B-cell immune responses to enteric microbial antigens that result in IBD.

INTRODUCTION

The term "inflammatory bowel diseases (IBD)" comprise two idiopathic chronic inflammatory diseases of the intestine, ulcerative colitis and Crohn's disease. Similar to other chronic inflammatory and autoimmune conditions, IBD appears to involve complex interactions among environmental, immune, and genetic factors. Over the past decade many new experimental models of chronic intestinal inflammation have been developed that are providing insights into the pathogenesis of these disorders (Table 1). Many of these models have resulted from either deletion of a gene or to insertion a gene into a mouse to generate an "induced mutant" strain. Hundreds of immu-

Table 1: Selected experimental models of inflammatory bowel disease.

Excess T-cell effector function	Deficient T-cell regulation	Defective innate immune response	Spontaneous
STAT4 Tg	CD45RB^{hi} transfer	mdr1a$^{-/-}$	C3H/HeJBir
IL-7 transgenic	IL-10$^{-/-}$	Keratin-8$^{-/-}$	SAMP1/Yit ileitis
TNF-α "knock-in"	IL-10Rß$^{-/-}$	Gai1$^{-/-}$	
CD40L transgenic	Macrophage-PMN	Conditional STAT3$^{-/-}$	
	STAT3$^{-/-}$	NF-κB p65$^{+/-}$ p50$^{-/-}$	
	IL-2$^{-/-}$	A20$^{-/-}$	
	IL-2Ra$^{-/-}$		
	BM-->Tge26		
	TGFß$^{-/-}$		
	TCRa$^{-/-}$		

nologic genes have been selectively deleted or transgenically over-expressed in mice. A small subset of these induced mutants have developed IBD in the absence of further manipulation. Acommon feature in these induced mutant mice that develop colitis has been that the normal intestinal microbiota is the stimulus driving the inflammatory disease and that CD4$^+$ T-cells are the effector cell mediating disease in most of them (for review see: *Elson* and *Weaver*, 2003). These experimental models have provided strong support for the immunologic hypothesis that IBD is due to a dysregulated mucosal immune (CD4$^+$) T-cell response to enteric bacterial antigens in a genetically susceptible host.

The intestine is the major interface between the host and the external environment. There have been major advances in revealing the mechanisms by which pathogens interact with the immune system, but little is known about how commensal bacteria in the intestine interact with the host and how the host response to them is regulated. It is clear that the microbiota has profound effects on the intestine and on the host. Such effects were highlighted by a recent study in which germfree mice were mono-colonised with *Bacteroides thetaiotaomicron*, a commensal that resides in the gut lumen and does not approach the epithelium itself. Such colonisation resulted in dramatic changes in gene expression in the epithelium and in the lamina propria. The concept is emerging that there is a circuit of interactions among the microbiota, the epithelium, and the immune system in the intestine, and that the communication continues throughout life. Moreover, abnormalities of this circuit can result in chronic intestinal inflammation.

One of the barriers to investigation of the interaction of the host with the microbiota is the latter's sheer complexity, comprising an estimated 500-1000 species. The aggregate number of genes associated with the microbiota, the "microbiome", has been estimated at 2-4 million genes (*Xu* et al., 2003). The microbiota has profound effects on the development of the intestine, including the epithelial layer (*Hooper* and *Gordon*, 2001; *Hooper* et al., 2001), the mucosal immune system (*Cebra*, 1999), and the enteric nervous system. In regard to the mucosal immune system, certain strains such as segmented filamentous bacteria are highly stimulatory and others are not (*Klaasen* et al., 1993; *Umesaki* et al., 1995). The reasons for these differences are presently unknown. One concept is that microbes that are resident in the

mucus layer are more stimulatory to the immune system, but data supporting this idea are lacking. Clearly bacteria that reside only in the lumen can have profound effects on the intestine, particularly on epithelial cells (*Hooper* et al., 2001). Information about the effects of the anaerobic microbiota is particularly lacking because of the difficulty in culturing them; yet these are the majority of the intestinal flora.

The molecular basis of the immune recognition of microbes is being defined (*Janeway* and *Medzhitov*, 2002). Innate immune and other cells have on their cell surface a set of receptors that recognise microbial products. These receptors are called pattern recognition receptors (PRR) in that they bind to and are triggered by selected microbial structures that form a molecular pattern (*Underhill* and *Ozinsky*, 2002). These microbial structures have been termed "pathogen-associated molecular patterns" (PAMPs) but the same molecular patterns are present on non-pathogenic commensal bacteria. The best studied of these PRRs is the Toll-like receptor (TLR) family which is comprised of at least 10 members (*Akira* and *Hemmi*, 2003). PRR are of ancient origin and appear to be conserved in insects, plants, and animals. The molecular patterns that they discern include lipopolysaccharide, bacterial DNA, bacterial peptidoglycan, flagellins, etc. Different TLRs can distinguish among different types of bacteria, for example TLR2 responds to Gram-positive bacterial ligands, whereas TLR4 responds mainly to endotoxin of Gram-negative bacteria (*Takeuchi* et al., 1999; *Yoshimura* et al., 1999; *Hirschfeld* et al., 2001; *Re* and *Strominger*, 2001). The TLRs are clearly important in host defence against pathogens but are also likely crucial in the host interaction with the intestinal microbiota (*Cario* and *Podolsky*, 2000; *Cario* et al., 2000; *Ortega-Cava* et al., 2003). NOD1/ CARD4 and NOD2/ CARD15 are intracytoplasmic PRRs that bind to the muramyl dipeptide component of bacterial peptidoglycan (*Girardin* et al., 2003; *Inohara* et al., 2003). When PRRs bind their ligand, they activate the NF-κB signalling pathway, which, in turn, activates many immune system genes. For most of the TLR family an important adapter protein, MyD88, is required for initiation of the signalling cascade through IRAK-1 (*Barton* and *Medzhitov*, 2003).

C3H/HeJBir AS MODEL SYSTEM TO STUDY INNATE AND ADAPTIVE IMMUNE RESPONSE TO THE MICROBIOTA

An experimental model that has provided insight into the host interaction with the microbiota is the C3H/ HeJBir mouse, a sub-strain of the common C3H/HeJ strain. The C3H/ HeJBir mouse was generated by a program of selective breeding for peri-anal ulceration and soft faeces (diarrhoea) that was observed sporadically in C3H/ HeJ mice due to focal chronic inflammation in the caecum and right colon. These lesions developed by 2-4 weeks of age, coinciding with bacterial colonisation, and largely resolved by 8-12 weeks of age (*Sundberg* et al., 1994).

Analysis of immune cells and function in colitic C3H/HeJBir mice vs. the parental C3H/HeJ strain has revealed few differences between the two strains. Both strains have mutations in TLR4 rendering them unresponsive to the effects of bacterial endotoxin. The major difference is that the C3H/ HeJBir substrain has increased levels of S-IgA in the intestine, high levels of serum IgG antibodies to commensal bacterial antigens, and increased T cell responses to intestinal microbial antigens.

B-cell response to microbiota

C3H/HeJBir mice do not respond to food or epithelial cell antigens, but have high titre IgG antibodies, mainly IgG2a, to antigens of the commensal bacterial flora (*Brandwein* et al., 1997). Serum from control C3H/HeJ mice has no reactivity to the enteric bacteria under the same conditions. A striking feature of the C3H/HeJBir serum antibody reactivity to the flora is that it is highly selective: Only a very small subset of antigens of the enteric bacterial flora is detected out of the thousands of bacterial proteins that are present. Trypsin treatment of the microbial preparation abolishes reactivity indicating that these are proteins or glycoproteins. The pattern of bands on Western blots was highly reproducible within different cohorts of C3H/HeJBir mice. When specific species of enteric bacteria were tested by Western analysis, there was no relationship between the relative abundance of a bacterial species and the number of bands detected. Quantitative analysis of antibody reactivity against *E. coli* outer membrane antigens demonstrated more than a 100,000-fold increase in reactivity in C3H/HeJBir serum IgG compared to C3H/HeJ. These data are consistent with an abnormal B-cell adaptive immune response to antigens of the enteric bacterial flora in the C3H/ HeJBir strain. The timing of the appearance of antibody relative to the development of caecal or colon lesions was not consistent with these antibodies playing a pathogenic role, however they did identify the antigens stimulating the pathogenic adaptive response (*Brandwein* et al., 1997).

T-cell response to microbiota

C3H/HeJBir $CD4^+$ T-cells failed also to respond to food or epithelial antigens but did respond vigorously to antigens of the commensal bacteria with both proliferation and cytokine production. The kinetics of the response was typical of an antigen-specific rather than a mitogenic or super-antigen response. The precursor frequency of bacterial-reactive, IL-2 producing $CD4^+$ T-cells was 1:2000 in C3H/HeJBir mice compared to 1:25,000 in normal C3H/ HeJ mice. Bacterial-reactive $CD4^+$ T-cells were detectable by 4 weeks of age in C3H/HeJBir mice, concomitant with the age of onset of disease. The cytokine pattern of these $CD4^+$ T-cells, mainly IL-2 and IFN-γ, was consistent with a Th1 subset response to the enteric bacterial antigens (*Cong* et al., 1998).

Transfer of disease by $CD4^+$ T-cells

To determine the pathogenic potential of these bacterial-reactive C3H/ HeJBir T-cells, adoptive transfer experiments were done. C3H/HeJBir $CD4^+$ T-cells were activated with commensal antigen-pulsed APCs for 4 days *in vitro*, then adoptively transferred into histocompatible C3H/HeSnJ $Prkdc^{scid}/Prkdc^{scid}$ (SCID) recipients. The SCID mice developed a focal colitis over the ensuing 2 months, lesions similar to that observed in the C3H/ HeJBir donor. Adoptive transfer of bacterial antigen-activated control C3H/HeJ $CD4^+$ T-cells, or of anti-CD3-activated C3H/HeJBir $CD4^+$ T-cells did not result in colitis in the SCID recipients, indicating that non-specific activation was insufficient. This was the first formal demonstration that $CD4^+$ T-cells reactive with conventional antigens of the commensal bacterial flora can mediate chronic inflammatory bowel disease.

A series of $CD4^+$ T-cell lines reactive with commensal bacterial antigens was derived subsequently from C3H/ HeJBir (Bir) mice by repeated cycles of stimulation with antigen-pulsed APCs followed by intervals of rest. All of these $CD4^+$ T-cell lines produce mainly Th1-type cytokines when re-stimulated *in vitro* and all are pauciclonal based on analysis of the TCRßV repertoire util-

ised. Most of these memory T-cell lines induced focal colitis uniformly after transfer to SCID recipients with increased levels of IL-12p40 and IFN-γ mRNA and protein detected in the lesions. Administration of anti-CD40L to SCID recipients of pathogenic Bir CD4 T-cell lines blocked the development of colitis. Thus interactions between CD40L on pathogenic $CD4^+$ T-cells with CD40 on mucosal APCs endogenously loaded with commensal bacterial antigens is critical for a sustained increase in IL-12 and thus progression to colitis (*Cong* et al., 2000).

Tr1 regulation of pathogenic bacterially-reactive T-cells *in vivo*

The above studies on T-cell reactivity to commensal antigens in C3H/ HeJBir mice, including the derivation of the long-term cell lines, utilised mice 10-12 weeks old that no longer had any evidence of gut inflammation. The presence of pathogenic $CD4^+$ T-cells in the absence of lesions implied that regulatory T-cells might be preventing the expression of pathogenic T-cell function *in vivo*. This would be compatible with results in other model systems such as the $CD4^+$ $CD45RB^{high}$ T-cells adoptive transfer system in which regulatory T-cells can prevent the effects of potentially pathogenic T-cells *in vivo*, at least in part by production of IL-10 (*Groux* et al., 1997; *Asseman* et al., 1999).

We asked whether T regulatory cells could be detected in adult C3H/HeJBir mice, and if so what were their properties. A C3H/HeJBir $CD4^+$ T-cell line, named Bir 8, was generated in the presence of IL-10 (*Cong* et al., 2002). The Bir 8 line produced high levels of IL-10, low levels of IL-4 and IFN-γ, and no IL-2, consistent with the phenotype of Tr1 cells (*Groux* et al., 1997). The Tr1 cells proliferated poorly to caecal bacterial antigen (CBA) stimulation compared to Th1 or Th2 lines with similar specificity, but proliferation of all three types was dependent on CD28-B7 interactions and was MHC class II restricted. CD40 blockade did not change IL-10 production significantly. Transfer of Bir 8/Tr1 cells into SCID mice did not result in colitis, although transfer of a pathogenic CBA-reactive $CD4^+$ Th1 cell line did induce colitis. Co-transfer of Bir 8/Tr1 cells with pathogenic $CD4^+$ Th1 cells prevented such colitis. Bir 8/Tr1 cells also inhibited the proliferation and IFN-γ production of a CBA-specific Th1 cell line *in vitro*. Addition of anti-IL-10 or anti-IL-10R mAb partially reversed this inhibition. Thus, CBA reactive Tr1 $CD4^+$ T-cells can be generated from C3H/HeJBir mice. These Tr1 cells inhibited pathogenic Th1 cells *in vivo* and *in vitro*. $CD4^+$ T-cells were isolated from the lamina propria of normal C3H/HeJ mice had properties of Tr1 cells, producing IL-10 only. These lamina propria $CD4^+$ T-cells inhibited a pathogenic Th1 cell line in the presence of CBA-pulsed APC, but not in the presence of anti-CD3 mAb, indicating the inhibition was CBA specific. Addition of anti-IL-10 or anti-IL-10R mAb partially reversed the inhibition. Thus, enteric bacterial antigen-specific T-cells with activity similar to Tr1 cells are present in the murine lamina propria, and can inhibit pathogenic $CD4^+$ T-cells, at least partially through production of high amounts of IL-10.

IDENTIFICATION OF THE ANTIGENS STIMULATING PATHOGENIC ADAPTIVE IMMUNE RESPONSES

A major limitation to progress has been the lack of information on the identity of the microbial products stimulating pathogenic T-cells and B-

cells. We have recently resolved this problem using serologic expression cloning. This strategy was based on our earlier observations that C3H/HeJBir mice develop IgG2a (Th1 dependent) antibodies to a limited, but reproducible set of bacterial protein antigens (*Brandwein* et al., 1997). Serum from C3H/HeJBir mice was used to probe a caecal bacterial DNA phage lambda library. A small number of immunodominant antigens was identified, cloned and sequenced. Unexpectedly, the major class of antigen was commensal bacterial flagellins, representing 25% of the 56 proteins cloned (*Lodes* et al., 2004). Two of these flagellins, CBir1 and FlaX, were studied in detail. Serum IgG anti-flagellin was identified in three different mouse models and in roughly half of patients with Crohn's disease, but not in patients with ulcerative colitis or in normal controls. CBir1 flagellin stimulated pathogenic Th1 cells in two mouse models and CBir1-reactive Th1 cells were able to induce colitis upon transfer to SCID recipients. Subsequently a similar serologic expression cloning has been done using serum antibodies from mdr1a$^{-/-}$ mice (unpublished data), and again, 25% of the antigens identified were bacterial flagellins. These data are consistent with the hypothesis that there is a limited set of immunodominant antigens in the microbial flora that activate pathogenic effector T cells and thus induce IBD. Some of these antigens may be peculiar to one strain or model, but others such as the flagellins appear to cross both strains and species.

Flagellins are bacterial proteins that assemble in long polymers to form the bacterial flagellum (*Samatey* et al., 2001). Flagellins have conserved amino and carboxy domains which are connected by a hypervariable region of variable length (*Eaves-Pyles* et al., 2001a). The conserved amino and carboxy domain form the polymerisation site that is necessary for bacterial motility (*Smith* et al., 2003). The amino and carboxy domains are sufficiently conserved as to allow phylogeny trees to be developed showing relatedness of different bacteria (*Winstanley* and *Morgan*, 1997). Flagellins are strong antigens and the immune responses to them are protective in certain intestinal infections such as with *Salmonella* (*McSorley* et al., 2000). Flagellins are the ligand for TLR5 (*Hayashi* et al., 2001) and have potent effects on the host, including a sepsis-like syndrome (*Eaves-Pyles* et al., 2001b). Because of their potent effects on innate immune cells they can serve as adjuvants for other antigens (*das Gracas Luna* et al., 2000; *McSorley* et al., 2002). Thus flagellins have both adjuvant activity and are strong immunogens, properties that might account for their strong representation in the expression cloning.

INNATE IMMUNE INTERACTIONS WITH THE MICROBIOTA SHAPE THE ADAPTIVE IMMUNE RESPONSE

The innate immune system appears to play a major, if not predominant, role in host interaction with the microbiota. For example, mice that have innate immunity but lack T-cells and B-cells such as SCID or RAG$^{-/-}$ mice are able to live in harmony with the commensal flora. MacPherson and colleagues have identified a T-cell-independent pathway stimulating S-IgA responses to commensal bacteria that involves direct interactions between bacteria bearing dendritic cells and mucosal B-cells (*Macpherson* et al., 2000; *Macpherson* and *Uhr*, 2004). The latter interaction does not stimulate serum IgG or spleen T cell responses. We have completed a study of the immune response to 20 re-

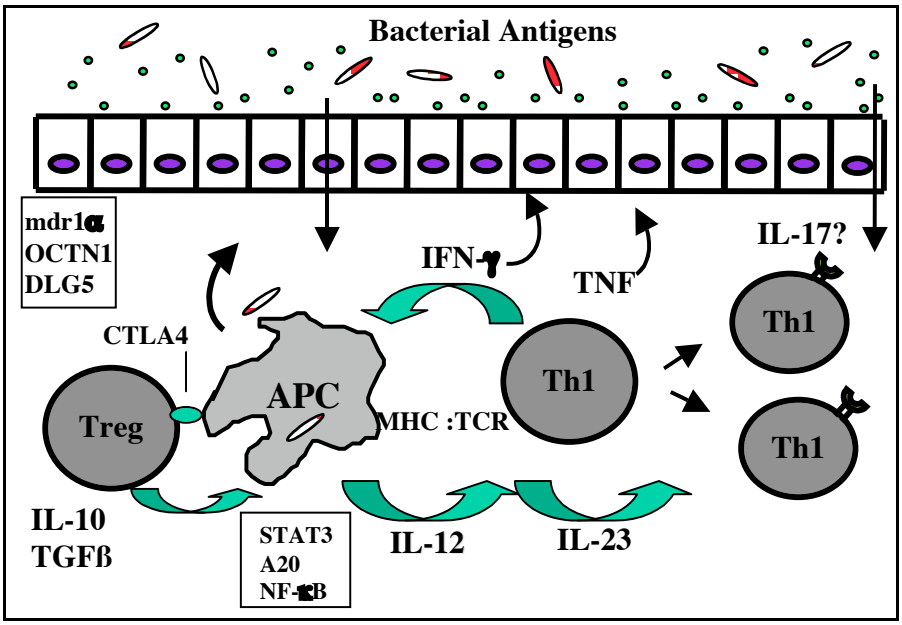

Figure 1: A schematic representation of the cells, cytokines and other factors that maintain normal intestinal homeostasis in the face of the huge challenge of the microbial flora. Genes important in maintaining homeostasis in the innate immune system of the epithelium or antigen presenting cells are shown in the boxes. (IL: interleukin; IFN-γ: Interferon-γ).

combinant proteins randomly derived from the microbiota (see Preliminary Data). Although S-IgA responses were detected to many of these proteins, no serum IgG or systemic T-cell response was identified to any of them in normal C3H mice, nor was there any evidence of immune tolerance to them (*Konrad* et al., 2003), similar to the results of Macpherson. Thus, the concept is emerging that there is a mucosal compartmentation of immune interactions with the microbial flora in normal hosts (*Becker* et al., 2003; *Uhlig* and *Powrie*, 2003), which effectively avoids inflammation or activation of systemic T-cells or B-cells. In colitic mice this innate pathway is subverted and microbial-reactive T-cells and IgG B-cells are activated to a set of immunodominant antigens of the microbiota. Microbial flagellins form a substantial fraction of this small number of immunodominant antigens, perhaps because they act as both adjuvant and antigen. It is well known that the innate immune system activates and thus directs the T-cell response to vaccine antigens (*Bendelac* and *Medzhitov*, 2002) and the same is likely true for endogenous priming to antigens of the microbiota (*Kapsenberg*, 2003).

The epithelium forms an important part of the host innate immune response to the microbiota, and it has been hypothesised that defects in the epithelium might result in IBD. Indeed, there are several mouse models where this appears to be the case, the most compelling of which is the multidrug resistance gene 1 alpha knockout (mdr1a$^{-/-}$) mouse. The mdr1a$^{-/-}$ gene encodes the P-glycoprotein transport protein that is expressed in the epithelium and in some lymphoid cells. Deficiency of this gene in mice results in colitis. Interestingly, bone marrow chimeras have demonstrated that colitis develops in mice that have an mdr1a$^{-/-}$-deficient epithelium but a normal bone

marrow compartment (*Panwala* et al., 1998). Although an abnormality of the epithelium appears to be the primary abnormality, this epithelial abnormality translates somehow into a pathogenic CD4 T-cell effector response to the microbiota and it is these T-cells that directly mediate the colitis. A single nucleotide polymorphism of the *mdr1a* gene has been linked to ulcerative colitis in humans (*Ho* et al., 2003). In addition, two recent reports have identified epithelial related genes causing susceptibility to IBD. The *OCTN1* cation transporter gene on Chr.5 was linked to Crohn's disease (*Peltekova* et al., 2004), and the DLG5 gene on Chr.10, which encodes a scaffolding protein involved in maintenance of epithelial integrity, was linked to IBD (*Stoll* et al., 2004). Interestingly, the *OCTN1* gene appears to interact with the *CARD15/NOD2* gene previously identified as a major susceptibility gene for Crohn's disease (*Hugot* et al., 2001). The epithelium is now recognised as a crucial component of the innate immune system and as such likely directs the adaptive immune response but the detailed mechanisms involved in how this happens remain undefined.

There are certain genes that are important in innate immune interactions with the microbiota, including the STAT3 transcription factor expressed in myeloid cells (*Kobayashi* et al., 2003; *Welte* et al., 2003) and PPARg (*Kelly* et al., 2004) (Figure 1). *Kobayshi* et al. (2003) have shown recently in mice with a myeloid cell-specific deletion of STAT3 that LPS stimulation of IL-12p40 via TLR4 on innate immune cells then induces a vigorous Th1 response and IBD. An important concept that is arising from recent data is that genetic defects that impair the innate immune system's ability to deal with the microbial flora can result in a more vigorous adaptive immune response to them and thus lead to inflammation. Supporting this concept is the discovery that a loss of function mutation in the NOD2/CARD15 PRR in humans results in susceptibility to Crohn's disease (*Bonen* et al., 2003). This mutation is likely to impair the innate immune response to as yet undefined microbes. We have recent data that indicates that the innate immune response to TLR ligands is impaired in colitis-susceptible C3H/HeJBir mice as compared to the more colitis-resistant C57BL/6 strain. Moreover, a colitis susceptibility gene locus on Chr3, Cdcs1 (*Farmer* et al., 2001), appears to regulate the innate immune response to TLR ligands as well as the $CD4^+$ T-cell response to microbial antigens. These data support the notion that impaired innate immune responses to the microbial flora may be a common pathogenic factor in both experimental and human IBD.

CONCLUSION

The inflammatory bowel diseases are complex disorders with genetic, immune, and environmental components that interact to generate disease. In the past decade, many experimental models have been generated that have allowed insights into these various components. Data from these models has shown that a dysregulated immune response to the enteric microbial flora can result in IBD. Studies in humans are converging with what has been learned in mouse models. For example, the CARD15/NOD2 susceptibility gene for Crohn's disease is a bacterial pattern recognition receptor. This report has focused on this 'bacterial connection', particularly the recent identification of flagellins as dominant antigens of the microbial flora that stimulate adaptive immune responses in

multiple mouse models and in patients with Crohn's disease. We expect our knowledge about these bacterial connections to IBD will expand substantially in the near future.

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ATHEROSCLEROSIS AND *CHLAMYDOPHILA (CHLAMYDIA) PNEUMONIAE*

ÅSA LJUNGH

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

SUMMARY

The concept that atherosclerosis was induced by environmental factors such as smoking and high fat intake was perturbated when inflammation was found to be prominent in atherosclerotic lesions. *Chlamydophila pneumoniae* but also other microbes inducing chronic endothelial infections have been implicated, particularly cytomegalovirus (CMV) and the gastric pathogen *Helicobacter pylori*. Large sero-epidemiological studies in different parts of the world have shown seroconversion to *C. pneumoniae* in patients with myocardial infarction, stroke and other forms of cardiovascular disease. The seroprevalence rates vary between 50 and 75%. Older individuals show higher seropositivity rates, suggesting that re-infection may be common. Using polymerase chain reaction (PCR) and immunohistochemistry, several studies have shown *C. pneumoniae* DNA in atherosclerotic lesions but not in normal vessels, although other studies have failed. Animal models with *C. pneumoniae* inducing atherosclerotic lesions have been established. The chlamydia LPS induces foam cell formation of monocytes, and the heat shock protein (Hsp) 60 oxidises low-density lipoproteins. Hsp 60 causes transcription of NF-κB and initiates deleterious immune response. Hsp 60, cross-reacting with human Hsp60, may also be involved in molecular mimicry which is part of the chronicity of *C. pneumoniae* infection. A peptide produced by *C. pneumoniae* mimics human heart muscle protein, which causes immune sentries. A co-infection of *C. pneumoniae* with *H. pylori* increased expression of vascular cell adhesion molecules (VCAM-1) in ApoE knockout mice, which may enhance atherogenesis.

INTRODUCTION

Atherosclerotic heart disease is the leading cause of morbidity in the Western hemisphere with manifestations of coronary artery disease, cerebrovascular disease and renal vascular disease. Contribution of inflammation to the pathogenesis of atherosclerosis was first hypothesised by Virchow in 1859 (*Verkooyen* et al., 1992). The finding of *Chlamydia* particles in damaged heart valves of a fairly great number of bird owners left the cardiologists and infectious disease specialists untouched (*Ward* and *Ward*, 1974). Experimental infection with avian herpesvirus in germ-free chicken produced arterial disease, resembling atherosclerosis (*Fabricant* et al., 1978). Elevated levels of C-reactive protein and fibrinogen, which is typical for infections, were associated with

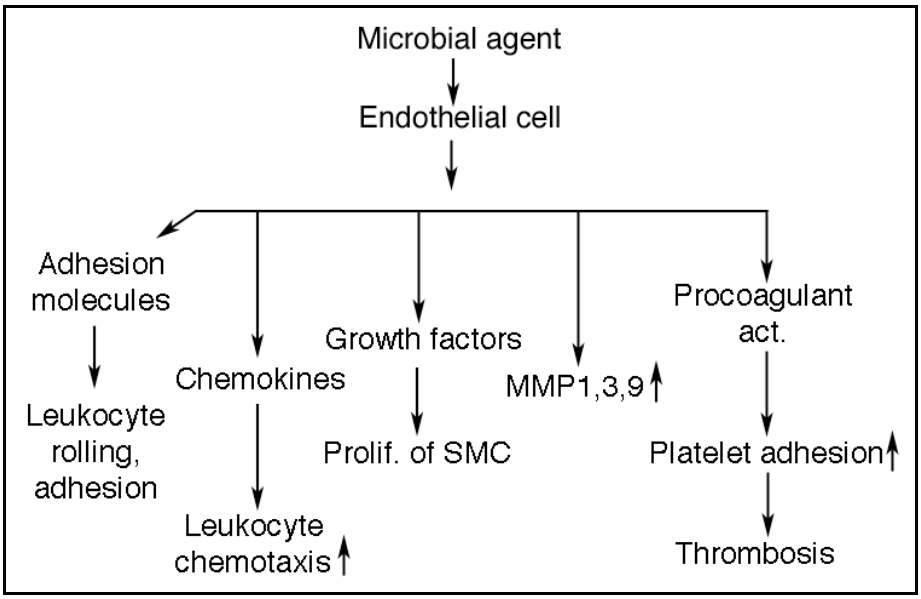

Figure 1: Pathogenesis of atherosclerosis.

coronary artery disease as well as with unstable angina (*Danesh* et al., 2000). Since then, multiple microbes have been investigated as possible aetiological agents of atherosclerosis, such as *C. pneumoniae, H. pylori,* cytomegalovirus, human herpesvirus and periodontogenic bacteria (*Kalayoglu* et al., 2002; *Kusters* and *Kuipers,* 1999; *Nieto,* 1999; *Beck* et al., 2001). No association was found between coronary artery disease and seropositivity to *Bartonella* sp. but a modest association with *Coxiella burnetti* (*Ender* et al., 2001). Before that, the general concept was that atherosclerosis was induced by elevated blood pressure, high caloric (cholesterol) intake, low physical activity and smoking. Gradually, the central role of inflammation as main part of atherosclerosis was accepted.

PATHOGENESIS OF ATHEROSCLEROSIS

Atherosclerosis starts with fatty streaks in the endothelium which, with time, develop into fibrous plaque, i.e. a lipid core with a fibrous cap. Monocytes and T-cells are recruited to the vessel wall across an intact epithelium. This requires expression of leukocyte adhesion molecules (e-selectin, ICAM-1 and VCAM-1) which are transcriptionally regulated by NF-κB. Modified smooth muscle cells (SMC), macrophages, monocytes, T-lymphocytes and several inflammatory cytokines are abundant in the plaques (*Noll,* 1998). This is a result of endothelial dysfunction with accumulation of monocytes, macrophages and lymphocytes in the intima. Macrophages ingest lipid and become foam cells. SMCs proliferate and secrete extracellular matrix (ECM). When sufficient lipid has accumulated the core of the lesion becomes necrotic, and in the

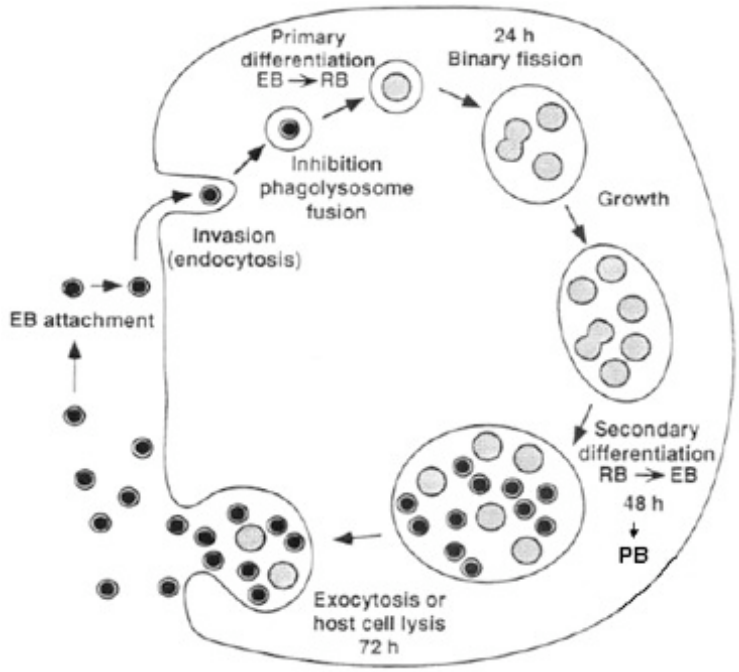

Figure 2: The life cycle of Chlamydia organisms (EB: elementary bodies , RB: reticulate bodies and PB: persistent bodies .

latter stages they become calcified. During atherogenesis, cytokines, growth factors, lipids, nitric oxide (NO) and other small molecules induce and regulate migration and proliferation of cells as well as interfere with lipid and ECM protein synthesis. Of these, TNF-α, often found in atheromatous plaques, enhances production of platelet-derived growth factor, which promotes proliferation of SMCs. These secrete a proteoglycan matrix important for the uptake of low-density lipoproteins (LDL). TNF-α further induces increased expression of cell adhesion molecules and leukocytosis, and inhibit lipoprotein lipase which leads to (i) aggregation of lymphocytes on the endothelium, and (ii) altered lipid metabolism and accumulation of triglycerides in the blood (*Coles* et al., 1998). Matrix metalloprotease (MMP) expression in plaques is also induced by TNF-α whereas NO synthesis can be suppressed. Decreased NO availability is common in early stages of atherosclerosis.

Homocysteine has been shown to have toxic effects on endothelial cells, promote proliferation of vascular SMCs, and enhance monocyte chemotaxis, all factors which can be involved in the pathogenesis of atherosclerosis (*Poddar* et al., 1997). Hyper-homocysteinaemia was further shown to activate NF-κB in endothelial cells as a result of oxidative stress (*Au-Yeung* et al., 2004).

Microbial agents may promote atherogenesis by evoking local inflammation in the arterial wall or by inducing endothelial injury during systemic infection (Figure 1). The human heat shock protein 60 (HSP60) can cross-react with bacterial antigens. Microbes may also promote atherogenesis indirectly by the evoked inflammatory reaction or by inducing changes in lipids, coagulation factors, homocysteine, MMPs or oxidative metabolites.

CHLAMYDOPHILA PNEUMONIAE –THE BACTERIUM

C. pneumoniae was first isolated from the conjunctiva of a child in 1965 in Taiwan, and therefore labelled TW-183. In 1983, it was isolated from the respiratory tract and designated AR-39. During some years it was hence designated "TWAR" but DNA homology studies and ultrastructural analyses defined it as its own species, *C. pneumoniae,* in 1989, beside *C. trachomatis, C. psittaci* and *C. pecorum,* a cattle pathogen (*Grayston* et al., 1989). In 1999, based on DNA homology studies, *C. trachomatis* remained within the genus *Chlamydia* whereas *C. pneumoniae* and *C. psittaci* were transferred to the genus *Chlamydophila* (*Everett,* 1999).

The life cycle of chlamydial organisms has three distinct forms (Figure 2):

- the infectious form, elementary bodies (EB), specialised to survive extracellularly,
- an intracellular form, reticulate bodies (RB), which are metabolically active and capable of reproduction, and
- persistent bodies (PB).

EBs are phagocytosed by endothelial cells and monocytes in the respiratory tract, and differentiate into RBs which localise in inclusion bodies. RBs can revert to EBs which are released by cell lysis or turn into metabolically inactive PBs which may remain dormant for many years (*Ngeh* and *Gupta,* 2000): PBs are unsusceptible to antibiotics as well as to the immune system.

CLINICAL MANIFESTATIONS

C. pneumoniae was first established as a cause of a variety of infections in the upper respiratory tract. Middle-aged adults have a 50-70% prevalence of seropositivity. Industrialised countries encounter epidemics every four to seven years. Re-infection appears to be common. The organism can also cause conjunctivitis and keratoconjunctivitis, acute myocardial infarction and endocarditis. More recently, it has been linked to chronic, inflammatory diseases, viz. atherosclerosis, Alzheimer's disease, multiple sclerosis, arthritis, myocarditis and reactive arthritis (*Gilbert* and *Grayston,* 2000; *Balin* et al., 1998; *Sriram* et al. 1998; *Gdoura* et al., 2002), and lung cancer (Koyi et al., 2001; *Anttila* et al., 2003).

VIRULENCE TRAITS IN *C. PNEUMONIAE*

Binding of heparan sulphate-like glycosaminoglycan was shown to mediate adhesion of the organisms to eukaryotic cells (*Wuppermann* et al., 2001). EBs are known to invade cells, and the role of glycosaminoglycans on the EB as well as on the host cell surface has been debated. *C. pneumoniae* as well as *C. trachomatis* were shown to use glycosaminoglycans on both EBs and host cells for invasion of bronchial epithelial but not of human umbilical vein endothelial cells (*Beswick* et al., 2003). LPS induces foam cell formation by mononuclear phagocytes. *C. pneumoniae* infected monocytes exhibited enhanced heat-resistant adhesion to endothelial cells, suggesting that it was LPS-mediated (*Kalayoglu* et al., 2001). *Chlamydophila* HSP (cHsp) 60 oxidises low-density lipoproteins. Both cHSP60 and human HSP60, which are co-localised in human atheroma, were shown to induce expression of the adhesion molecules e-se-

lectin, VCAM-1 and ICAM-1 which facilitate adhesion of leukocytes to the endothelial wall (*Kol* et al., 1999). They further induced production of IL-6 in endothelial and smooth muscle cells and macrophages similar to that induced by Gram-negative bacterial LPS, effected by transcription of NF-κB to the cell nucleus. *C. pneumoniae* and cHSP were recently shown to stimulate proliferation of human vascular SMCs via TLR4 and protein kinase activation (*Sasu* et al., 2004). Some features of the *C. pneumoniae* atherosclerosis pathogenesis are listed in Table 1. Another outer membrane protein, shown to be immunogenic and to be a pro-inflammatory activator (IL-1, IL-6 and TNF-α), is Outer Membrane 2, OMP2 (*Ciervo* et al., 2002). This protein (of about 60 kDa) is expressed late in the growth cycle and prevalent in EBs. Genus- and species-specific B- and T-cell epitopes have been identified in OMP2 (*Watson* et al., 1994). SMCs infected with *C. pneumoniae* secreted MMP-1 and MMP-3 but not gelatinases (MMP-2 and MMP-9) (*Rödel* et al., 2003). MMP may degrade ECM proteins of the fibrous cap and cause rupture of plaques. Non-immune cells (endothelial and epithelial cells) were reported to respond to *C. pneumoniae* infection by producing pro-inflammatory chemokines, cytokines and growth factors (*Stephens,* 2003). Dendritic cells are the key cells in the initiation and regulation of immune responses and are present in atherosclerotic lesions. The detection of *C. pneumoniae* in dendritic cells obtained from atherosclerotic plaques of 17/60 patients therefore links *C. pneumoniae* stronger to a subset of atherosclerotic patients (*Bobryshev* et al., 2004). Unfortunately, the study did not include diagnostics for other bacterial species.

Hyperhomocysteinaemia and elevated titres to *C. pneumoniae* IgG were correlated in patients with coronary artery disease but a role of *C. pneumoniae* in hyperhomocysteinaemia has not been found (*Stanger* et al., 2002).

In the Apo-E mouse, repeated infection with *C. pneumoniae* resulted in endothelial dysfunction, principally mediated by the NO pathway (*Liuba* et al., 2000).

LABORATORY DIAGNOSTICS

C. pneumoniae is an obligate intracellular bacterium and must be cultured within eukaryotic cells. This is the golden standard of diagnostics. There are some, but few reports on isolation of *C. pneumoniae* from atherosclerotic lesions (*Ramirez*, 1996).

Micro-immunofluorescence was first developed and has become the standard for serology (*Wang* and *Grayston,* 1986). A suspension of EBs with or without LPS are used as antigens. Microscopic reading of the test and lack of standardisation of antigen makes automatisation impossible and evaluation of results between different laboratories difficult. Later, commercial EIAs were introduced with shifting quality. Different antigens are used – some use complete LPS-containing *C. pneumoniae* antigens, some use LPS-free *C. pneumoniae*-specific antigens (*Hermann* et al., 2002). In one study, N-lauroylsarcosine extract of EBs was used, showing 2-5 times higher absorbance values than with native EB as antigen (*Quevedo Diaz* et al., 2002). Recombinant OMP2 was used as antigen in EIA for *C. trachomatis* and *C. pneumoniae* (*Portig* et al., 2003). The sensitivity of the assay was high. The antibody levels of patients infected with *C. pneumoniae* declined

faster in the EIA than with MIF. Chlamydial HSP60 was not suitable for serodiagnosis (*Peeling* et al., 1997). Western blot analyses have identified antigens of 40 and 60 kDa for *C. pneumoniae* (*Ijima* et al., 1994; *Wagels* et al., 1994). *C. pneumoniae* has only one serovar or immunotype while there are numerous serologically distinct strains among other *Chlamydia* species which should simplify establishment of serological diagnostics for this organism. The role of IgA antibodies in serodiagnosis of *C. pneumoniae* is not established but some studies have emphasised IgA antibodies as a good marker of chronic infection (*Paldanius* et al., 2003).

Immunohistochemistry has been employed to demonstrate the presence of *C. pneumoniae* in atherosclerotic tissue. In several studies, problems with high background staining, false-positive results in damaged lesions, and false-negative results because of the small area examined have been encountered (see *Boman* and *Hammerschlag*, 2002).

Nucleic acid amplification techniques caused a break-through in diagnostics of *C. pneumoniae* on tissue samples. Methodological aspects on the different steps of polymerase chain reaction (PCR), including sampling, sample preparation, choice of primers, purification of DNA and RNA to get rid of inhibitors were reviewed by *Boman* and colleagues (1999). A low concentration and patchy distribution of *C. pneumoniae* DNA was shown in carotid artery specimens. This emphasises the need to investigate multiple samples (*Cochrane* et al., 2003). Amplification of *C. pneumoniae* mRNA from atheromas is proof of viable organisms in the lesion (*Gieffers* et al., 2001). Quantitative (real-time) PCR has also been developed which may prove useful to monitor intracellular replication of *C. pneumoniae* (*Bonanomi* et al., 2003).

ANIMAL MODELS

Intranasal infection of New Zealand White rabbits resulted in pneumonia, fatty streaks and atherosclerotic lesions in aortas (*Fong* et al., 1997). When rabbits were re-infected 3 weeks later they developed intimal thickening or fibroid atherosclerotic-like plaques within 4 weeks. Immunohistochemistry was positive for *C. pneumoniae* (*Saikku* et al., 1998). The apo-lipoprotein (apoE)-deficient mouse develops atherosclerosis spontaneously and the C57BL/6J mouse does so when fed an atherosclerotic diet. Following single or repeated intranasal inoculation of *C. pneumoniae* in the apoE mouse *C. pneumoniae* was detected in internal organs and in atherosclerotic lesions after 20 weeks (*Moazed* et al, 1999). In different monkeys, intranasal inoculation of *C. pneumoniae* resulted in systemic spread and persistence but producing mild clinical symptoms (*Holland* et al, 1990).

EPIDEMIOLOGICAL STUDIES

In a case-control study of 250+250 patients and controls, elevated anti cHSP60 antibodies, but no anti-human or anti-*E. coli* homologues were independently associated with coronary artery disease (*Mahdi* et al., 2002).

Helicobacter pylori

Several studies have provided evidence for a causal relation between *H.*

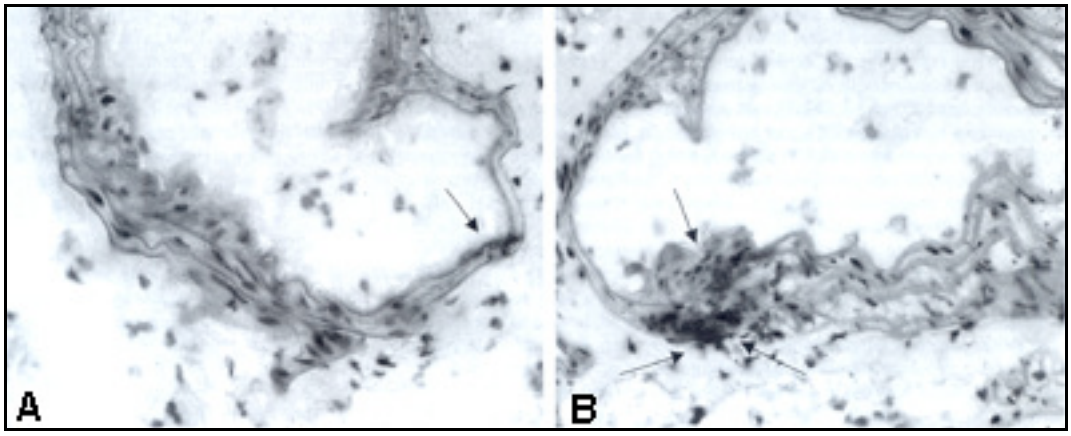

Figure 3: ApoE-knockout mice infected with *C. pneumoniae* (A) and with *C. pneumoniae* and *H. pylori* (B). VCAM-1 staining of branching site of aorta.

pylori and chronic heart disease, and interestingly, the peak in the incidence of coronary artery disease in the U.S. coincided with a peak in duodenal ulcer disease (*Blaser,* 1998). However, meta-analysis of multiple studies failed to show any relation between blood pressure, plasma fibrinogen concentration, blood lipid concentrations, C-reactive protein and other known cardiovascular risk factors and *H. pylori* (*Danesh* and *Peto,* 1998). *Blasi* and colleagues (2000) detected *C. pneumoniae* DNA but not *H. pylori* DNA in atherosclerotic plaques. *H. pylori* transcribes NF-κB, induces formation of pro-inflammatory cytokines and expression of cell adhesion molecules, and has hence the potential of inducing vascular damage (*Ernst*, 1999). Harbouring of the pathogenicity island and production of the toxin CagA was only moderately correlated to stroke, although a higher correlation was found with stroke located in larger vessels (*Cremonini* et al., 2004). The HSP60 of *H. pylori*, a highly conserved protein, was shown to mediate IL-6 production by macrophages via TLR-2 and TLR-4 (*Gobert* et al., 2004). Infection with *H. pylori* was further associated with an atherogenic lipid profile (*Hoffmeister* et al., 2001).

Patients with long-lasting infection and atrophic gastritis were shown to have elevated levels of homocystein, probably as a result of vitamin B12 malabsorption (*Santarelli* et al., 2004).

Synergy

Combined seropositivity for *C. pneumoniae* and *H. pylori* was associated with obesity, low socio-economic factors and age (*Ekesbo* et al., 2000). In the ApoE- mouse model, co-infection with *C. pneumoniae* and *H. pylori* synergistically increased expression of VCAM-1 and leukocyte adhesion (*Liuba* et al., 2003; Figure 3). A group of unselected patients with atrial fibrillation had antibodies to *C. pneumoniae* as well as to *H. pylori* (*Olsson* et al., 2002)

Cytomegalovirus

Cytomegalovirus (CMV) belongs to the herpes group of virusses and indeed other members of this group, like *Herpes simplex* 1, have also been implicated in atherosclerosis development. Between 50 and 80% of adults have antibodies to CMV. Infection is usually acquired in

childhood. Severe infections occur in heart transplantation recipients, and there is strong evidence for a role of CMV in vasculopathy (*Grotton* et al., 1989). CMV appears to directly infect endothelial cells, and can remain latent. Deleterious effects can be mediated by induction of chemokine and other inflammatory compounds (*Streblow* et al., 1999; *Hengel* and *Weber,* 2000). This results in migration of smooth muscle cells, such as monocyte chemotactic protein –1. Because CMV is restricted to the human host related viruses have been used in mouse and rat models of CMV infection. In these models, increased adherence of leukocytes to the aortic intima and accumulation of lipids in the endothelium were found (*Span* et al., 1992).

Periodontal pathogens

Periodontitis is an inflammatory reaction of the tissue surrounding the tooth. It produces few symptoms, progresses slowly and shares a number of features with chronic vascular disease. Some of the recognised species are listed in Table 2. In periodontitis, the lipopolysaccharide → Mϕ seems crucial, and peripheral monocyte (Mϕ) individuals secrete 3-10-fold of cytokine mediators as a response to LPS than normal persons (*Beck* et al., 1999).

CONCLUDING REMARKS

There are substantial reports on the association with chronic infection and development of atherosclerosis. It is likely that chronic infections caused by different microbial agents can induce similar vascular pathology, and that the infectious burden, as revealed by a high CFP is related to peripheral artery disease but not a normal or low CFP value (*Nloemenkamp* et al., 2002.). Hence linkage to one certain agent is hampered. During the last decade efforts have been made to standardise diagnostic methods, viz. serology and molecular microbiology methods. This will help to elucidate the issue of microbial infections as a cause of one of the greatest causes of morbidity and mortality. A clear link between infection and atherosclerosis will direct preventive therapies towards microbial disease and effects thereof. However, *C. pneumoniae* located in lymphocytes as well as in monocytes are refractory to antibiotic treatment (*Yamaguchi* et al., *Gieffers* et al., 2001).

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LITERATURE

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ARTHRITIS ASSOCIATED WITH MUCOSAL INFECTIONS

J.S. HILL GASTON

University of Cambridge School of Clinical Medicine, Department of Medicine, Addenbrooke's Hospital, Cambridge, UK

SUMMARY

Infectious agents which are well tolerated by the majority of those infected can give rise to persistent pathology in predisposed individuals. Inflammatory arthritis complicates certain infections of the gastrointestinal and genitourinary tract in a minority of those infected; this is reactive arthritis, which is in turn related to other forms of inflammatory arthritis - the spondyloarthropathies (e.g. ankylosing spondylitis) - in which host responses to commensal bacteria rather than specific infectious agents may well be important. Reactive arthritis lends itself to clinical investigation, since it is an acute arthritis triggered by known organisms. Thus persistence and distribution of the organism, together with immune responses to it, can be characterised in detail. Since timing of both the triggering infection and the onset of arthritis are well defined, this facilitates studies of the evolution and outcome of disease.

A current view of the immunopathogenesis of reactive arthritis highlights several findings: Firstly, although the triggering organism cannot be cultured from affected joints, there is good evidence that bacterial antigens and, in at least some cases, transcriptionally active bacteria, reach the synovium of the inflamed joints. Secondly, vigorous T-cell mediated responses to the triggering organism are readily detected in affected joints; analysis of the bacterial antigens targeted by the immune response can be used to identify differences in patients with arthritis and uncomplicated infection. Lastly, various genetic and environmental factors influence the likelihood of infected individuals developing reactive arthritis, and its severity or persistence. The best known of these is HLA-B27, but additional genes including those that play a role in other spondyloarthropathies are involved. There is now some evidence that these genes may determine the nature of the immune response mounted to the triggering bacterium in reactive arthritis, or to commensal bacteria in other spondyloarthropathies.

Lessons gained from the study of reactive arthritis and other spondyloarthropathies may be applied to other diseases associated with infectious agents in which host factors, particularly genes, determine outcome, in contrast to diseases whose course primarily reflects the pathogenic properties of the organism.

INTRODUCTION

For most of the 20th century the field of infectious diseases has been concerned with the diagnosis and treatment of those bacterial and, to a lesser extent,

viral infections which pose an immediate challenge to host survival through multiplication of the organism and, in many instances, the induction of inflammatory responses which themselves damage organs and tissues (e.g. septic shock). Whilst septic shock remains a major cause of mortality, the effects of many of the classical pathogens infections have been dealt with very satisfactorily through the development of antibiotics for bacterial infections, and mainly through vaccine strategies for life-threatening viral infections. In the last 2-3 decades attention has shifted to pathogens which have a more subtle interaction with the host. Such infections are typically very common or even ubiquitous in human populations, with the majority of individuals sustaining either brief self-limiting illnesses (e.g. influenza, food poisoning) or no obvious clinical damage from the infectious agent. Indeed, some of these organisms persist indefinitely in the host without any apparent clinical effects. Obvious examples of this would be the herpes viruses such as Epstein-Barr virus and Cytomegalovirus, and in the case of bacteria, organisms such as *Helicobacter pylori* and *Chlamydia pneumoniae*. Nevertheless, whilst these organisms are not associated with any of the kinds of events which are seen in acute sepsis, it has become clear that there are still pathological consequences of the infection, but that these vary in different individuals. Thus, in the case of *Helicobacter pylori*, many patients maintain chronic infection of the gastric mucosa with minimal clinical effects (other than perhaps more dyspepsia than they would otherwise have suffered), but in others the presence of the organism leads to the development of peptic ulcer, and in another subset, to neoplasms such as gastric lymphoma (*Blaser*, 1990). Epstein-Barr virus infection provides an even more striking example: There is near ubiquitous infection of human populations which is normally asymptomatic, unless the virus is acquired in adolescence, when it often causes infectious mononucleosis. In all subjects the virus persists, because it is exquisitely adapted to an almost silent existence in memory B-cells (*Thorley-Lawson* and *Gross*, 2004). However, immunosurveillance is still required to keep it in check since iatrogenic immunosuppression (usually in the context of transplantation) can result in virus-induced B-cell lymphoproliferative disease (*Loren* et al., 2003). Many of these cases can be brought under immunologic control by removing immunosuppressive drugs, but in others outgrowth of malignant B-cells occurs. In addition, natural infection with the virus contributes substantially to the occurrence of Hodgkin's lymphoma and undifferentiated nasopharyngeal carcinoma, although these neoplasms can also occur without viral infection (*Rickinson* et al., 2000). Thus, at different times and in different individuals the host-virus relationship varies from one which is entirely benign as far as the host is concerned to one which is potentially fatal. Much current interest in infectious diseases concerns those situations in which a stable truce between organism and host is replaced by an outbreak of hostilities and the appearance of disease.

For organisms which are able to persist without causing the host's demise, it is important to determine which aspects of the host-pathogen relationship determine whether or not clinical disease occurs. This general paradigm for persistent pathogens can be extended to a consideration of the relationship between host and commensal organisms, particularly bacteria, and the diseases which might result if these relationships are upset. These issues are particularly well illustrated by the association between inflammatory arthritis and infections at various sites, especially the gut.

THE RELATIONSHIP BETWEEN INFECTION OR INFLAMMATION AT 'BARRIER' SITES, AND ARTHRITIS

In addition to the well-recognised ability of particular organisms such as staphylococci or streptococci to disseminate to the joint and cause septic arthritis, there are a number of diseases in which arthritis occurs in relation to either specific infections, or to inflammation at sites where the body encounters commensal bacteria. It is worth noting however that the confidence with which bacteria can be implicated in each of these diseases varies.

Gut inflammation

Examples of arthritis which occurs in the context of gut infection or inflammation include Whipple's Disease, reactive arthritis following enteric infection, spondyloarthropathy complicating ulcerative colitis or Crohn's disease, and arthritis associated with coeliac disease (*Gaston* and *Lillicrap*, 2003). These diseases illustrate a spectrum: Whipple's Disease can almost be regarded as an example of septic arthritis, albeit with a very slow growing organism, *Tropheryma whippelii* (*Relman* et al., 1992), since the organism can be found in joint tissue in an apparently similar state to the gut, i.e. within macrophages (*O'Duffy* et al., 1999). In reactive arthritis, organisms such as *Salmonella* and *Campylobacter* cannot be cultured from the joint but may be readily cultured in stool; nevertheless, there is good evidence that the organism reaches the joint (vide infra). Both ulcerative colitis and Crohn's disease are clearly associated with certain forms of inflammatory arthritis, and both involve inflammation at sites where bacteria are present in large numbers. Furthermore, the normal state of tolerance which is extended towards gut commensals seems to be broken in Crohn's disease (*Duchmann* et al., 1995; *Lodes* et al., 2004). Finally, coeliac disease, due to intolerance for the gliadin fraction of gluten and predominantly involving areas of the gut which are bacteria free, has been associated with arthritis, although this is generally rather mild (*Lubrano* et al., 1996).

Skin

Examples include Lyme disease due to infection with the specific pathogen *Borrelia burgdorferi* or related organisms, which is inoculated into the skin by a tick bite. This produces a characteristic rash which can later be followed by an inflammatory arthritis and other forms of inflammation distant from the skin (*Steere*, 2001). Much more common than Lyme disease is the arthritis which complicates psoriasis, a disease in which specific pathogens are not generally implicated (with the exception of cases of guttate arthritis related to streptococcal infection), but the relationship with the skin flora is disturbed by inflammation.

Urinary Tract

Whilst urinary tract infection is not generally associated with inflammatory arthritis, it is important to note that infection with *Chlamydia trachomatis* produces a clinical syndrome which is indistinguishable from enteric reactive arthritis (*Gaston*, 2000). In addition, an iatrogenic form of reactive arthritis has been repeatedly described when patients have intravesical installation of BCG organisms for the treatment of bladder cancer (*Miossec*, 1996). Thus, particular organisms in the urogenital tract can also predispose to arthritis.

Respiratory tract

The range of associations here also includes specific pathogens such as streptococci, which trigger both the arthralgia of rheumatic fever and a post streptococcal arthritis, and the reactive

arthritis due to the respiratory pathogen, *Chlamydia pneumoniae* (*Deighton*, 1993; *Braun* et al., 1994; *Hannu* et al., 1999), although this infection is a much less common cause of reactive arthritis than *Chlamydia trachomatis* in the urogenital tract. Diseases such as cystic fibrosis, which involve chronic infection and bronchiectasis, have also been associated with inflammatory arthritis (*Merkel*, 1999; *Bradlow* and *Mowat*, 1983).

Whilst it is useful to point out the association between inflammatory arthritis and infection at each of these different sites, the rest of this review will concentrate mainly on infection/inflammation in the gut, together with *Chlamydia*-induced reactive arthritis.

THE PATHOGENESIS OF REACTIVE ARTHRITIS

The term "reactive arthritis" is sometimes used loosely to refer to any form of arthritis which follows infection of any kind. However, it is more accurate to confine its use to a specific clinical syndrome, sometimes erroneously called "Reiter's disease", which follows infection with a relatively small number of organisms: In the gastro-intestinal tract, *Salmonella, Campylobacter*, *Yersinia* and *Shigella*, and in the genito-urinary tract *Chlamydia trachomatis*. A long list of other organisms can occasionally produce the same syndrome, but these five account for the vast majority of cases. The clinical syndrome is classified as a spondyloarthropathy because it share clinical features with other members of this family of arthropathies which comprises: Ankylosing spondylitis, arthritis associated with inflammatory bowel disease and psoriatic arthritis. These include involvement of the spine and entheses (sites of attachment of tendons and ligaments to bone), and extra-articular features such as psoriaform rashes, inflammation of the uveal tract, and gastro-intestinal inflammation. Other forms of arthritis following infection do not show features of spondyloarthropathy and are best termed "post-infectious arthritis".

Progress in our understanding of the pathogenesis of reactive arthritis has occurred in three main areas:

1. Demonstration of the triggering organism or its components in affected joints;
2. Characterisation of the immune response to reactive arthritis triggering organisms, particularly the response within the joints;
3. Exploration of the genes and environmental factors which determine the occurrence of spondyloarthropathies, including reactive arthritis, and their severity.

Bacterial antigens and/or bacteria are present in the reactive arthritis joint

By definition the reactive arthritis joint is sterile, i.e. bacteria cannot be isolated by conventional culture techniques. The first hint that this might not be the end of the story came from electron microscopy studies of synovium in *Chlamydia*-induced arthritis in which *Chlamydia* inclusion bodies, albeit with atypical morphology, were demonstrated (*Ishikawa* et al., 1986). Inevitably, since the organism was not cultivatable the precise status of these electron microscopy findings was much debated. However, it was soon shown that synovial biopsies from reactive arthritis patients stained with *Chlamydia*-specific antibodies (*Keat* et al., 1987). Analogous findings were soon reported by researchers at the University of Turku, Finland, who developed polyclonal and monoclonal antisera specific for enteric organisms known to trigger reactive arthritis. In an important series of studies

they showed the presence of *Yersinia*, *Salmonella* and *Shigella* in synovial fluid and tissue of reactive arthritis patients (*Granfors* et al., 1989, 1990, 1992; *MerilahtiPalo* et al., 1991). In many cases these bacterial components were demonstrable in the joint many weeks after enteric infection, highly suggestive of organism persistence, although stool cultures were often negative at the same time that the organism was detected in the joint. The antisera identified both bacterial proteins and components of the bacterial cell wall such as LPS, but could not determine whether live or dead organisms were present. Even the presence of dead organisms many weeks after initial infection would be suggestive of persistence of bacterium, perhaps at another site such as the gastrointestinal lymphoid system, from whence dead organisms might traffic to the joint. The cells which contain bacterial antigenic material were both neutrophils and macrophage/monocytes and, in addition to demonstrating these findings in the joint, positive staining for bacterial antigens was evident in the same cells in peripheral blood of some patients. The findings based on immunofluoresence were backed up by immunoblotting studies. Together these findings raise the possibility that the disease might at least in its initial stages (1-2 years) be driven by bacterial antigens and reflect unusual persistence with the organism. Additional evidence for persistence came from serological studies showing that patients with reactive arthritis had longer lasting titres of IgA antibodies to the organisms, a finding pointing to persistence since IgA antibodies have a relatively rapid half-life as compared to IgG (*Granfors* and *Toivanen*, 1986).

The final phase in demonstrating organisms at the site of disease utilised PCR technology. Both PCR to demonstrate organisms-specific DNA and, more recently, RT-PCR to demonstrate organism-derived RNA, have been used. Most of the published data concern detection of *Chlamydia trachomatis*, with occasion reports of detection of *Yersinia* (*Gaston* et al., 1999) and possibly *Salmonella* (*Ekman* et al., 1999; Nikkari et al., 1999. Although PCR for detection of bacteria in reactive arthritis is technically demanding and standardised protocols have not yet been agreed, the weight of evidence suggests that *Chlamydia* nucleic acids are indeed present in the joints of affected cases (*Rahman* et al., 1992; *Taylor-Robinson* et al., 1992; *Bas* et al., 1995; *Schnarr* et al., 2001). There is also evidence to suggest that the quantity of specific nucleic acids present is very small. Thus, careful experimental protocols had to be devised for the handling of synovial fluid and synovial tissue, as compared to, for instance, urine or genitourinary swabs and, although this reflects the different tissue, it almost certainly also reflects the increased sensitivity which is required (*Kuipers* et al., 2002). In addition, in longitudinal studies of particular patients, not all samples of synovial fluid are positive, suggesting that the amount of nucleic acid may hover between undetectable and just detectable levels. It has even been suggested that one explanation for the discrepancies found when several experienced laboratories examine the same sample of synovial fluid could relate to the presence of a small quantity of organisms and nucleic acids so that not all aliquots from one sample will be positive. An additional cause of concern and confusion is that when the same PCR techniques have been applied to patients with other forms of arthropathy, the results are not uniformly negative (*Wilkinson* et al., 1998; *Schumacher* et al., 1999a; *Olmez* et al., 2001). However, although at first sight disconcerting, this is in fact what would be expected, if the joint material which is being examined comes from a population in which the prevalence of *Chlamydia* infection is high. There is no mechanism which

could readily be postulated to prevent the access of *Chlamydia* to the inflamed synovium of a patient with, e.g. rheumatoid arthritis, who acquires *Chlamydia* infection. The main study which showed a significant rate of detection of *Chlamydia* nucleic acids in arthropathies other than reactive arthritis still showed a substantially higher prevalence of positive PCR tests for *C. trachomatis* in the reactive arthritis population (*Schumacher* et al., 1999b). Interestingly, the prevalence of *C. pneumoniae* in the same synovial material did not vary much between reactive arthritis patients and those with other forms of arthropathy. This is consistent with *C. pneumoniae* rarely triggering reactive arthritis, and detecting this organism acts as a kind of internal control in the study indicating that dissemination of organisms to inflamed joints can occur (especially organisms which persist long-term), whether or not the organism is causative of the arthritis. In a recent study this dissemination has been emphasized by the observations that, when RT-PCR is performed using universal primers, almost all inflamed synovia contain significant bacterial ribosomal RNA, mainly derived from species known to be commensal in the gut or skin (*Kempsell* et al., 2000; *van der Heijden* et al., 2000; *Cox* et al., 2003). The results from these studies and others are consistent with the idea that there is a continuing traffic of bacteria through joints, most likely within phagocytes. The more phagocytes recruited to the joint, the higher the likelihood of these organisms and their components reaching the joint. Thus, normal synovium which recruits only small numbers of macrophages to the synovial membrane, was found not to contain bacterial rRNA, but all inflamed synovia, including those from osteoarthritic joints (which is often surprisingly inflamed,) were positive for bacterial rRNA. If this notion is correct, then the traffic of reactive arthritis associated bacteria to the joint does not in itself explain the occurrence of reactive arthritis if this traffic would be expected to happen, at least to some extent, under normal circumstances. It might however provide some insight into why reactive arthritis is commoner in large weight bearing joints. One would postulate the rate of macrophage traffic to such joints, which are subject to micro- trauma in every day activities, to be higher than to non-weight bearing joints, with a greater probability that reactive arthritis bacteria will find their way to those sites. Indeed patients occasionally comment that a joint which was recently injured was one of the first to flare in an episode of reactive arthritis.

Immune responses to reactive arthritis associated bacteria

Studies of T-cell mediated immune responses to organisms associated with reactive arthritis have been studied for more than 20 years, beginning with the landmark studies of Denys Ford (*Ford* et al., 1985). It was apparent early on that very marked T-cell proliferative responses to the organisms responsible for triggering disease were readily detectable in synovial fluid (*Gaston* et al., 1989; *Sieper* et al., 1993; *Hermann* et al., 1990). These studies have been extended, and indeed the synovial fluid has proved a useful source of organism-specific T-cells which have been cloned and used to characterise the immune response to organisms such as *Chlamydia trachomatis* and *Yersinia enterocolitica* (*Hassell* et al., 1993; *Deane* et al., 1997; *Mertz* et al., 1998; *Hermann* et al., 1989). There was an initial hope that measurements of responses to bacterial antigens in synovial fluid might be useful diagnostically, but further consideration of the reasons why such responses should be so prominent in synovial fluid and synovium casts doubt on this possibility. First of all, it is clear that there is preferential recruitment of memory T-

cells into sites of inflammation (*Thomas* et al., 1992; *Akbar* et al., 2000), such as the joints affected by reactive arthritis. Virtually all of the cells in the inflamed joint express CD45RO, an isoform which is characteristic of memory T-cells, with little expression of CD45RA which is expressed by naive cells (*Matthews* et al., 1993). Therefore, one would anticipate an enrichment in the joint of T-cells responding to any major antigenic challenge which the patient has previously experienced, and indeed T-cell responses to recall antigens such as PPD and tetanus toxin are also readily recorded. Given that reactive arthritis patients have recently been infected with organisms like *Chlamydia* and *Salmonella* it is not surprising to find prominent responses to these organisms in the affected joints. Furthermore, the antigen presenting cells within the joint are enriched for activated dendritic cells, which again enhances the proliferative responses which can be recorded *in vitro* (*Harding* and *Knight*, 1986; *Viner* et al., 1993; *Stagg* et al., 1991). In the light of these considerations, it can be concluded that a vigorous T-cell proliferative response to a reactive arthritis associated organism will normally be present in reactive arthritis patients, but might also be recorded in patients with arthritis due to other causes, if these patients have also experienced infection in the past by one or other of the reactive arthritis associated organisms.

However, whilst for these reasons measurements of synovial T-cell responses to bacteria are of limited use diagnostically, they may still be relevant to the pathogenesis of inflammation. The responses obtained are generally of the 'Th1' kind, i.e. T-cells which make the pro-inflammatory cytokine interferon-γ, in addition to other pro-inflammatory factors such as TNF-α and IL-17 (*Simon* et al., 1993; *Schlaak* et al., 1992). Indeed, T-cell obtained from synovial fluid make these same cytokines spontaneously *ex vivo*, consistent with their having been activated *in vivo* (*BeacockSharp* et al., 1998). Nevertheless, there are also reports of IL-4 producing T-cells in reactive arthritis synovium and whilst these are in the minority, their presence has led to speculation that the immune response to the organism in the joint is not adequately polarised to Th1, and may therefore allow persistence of the organism (*Simon* et al., 1994). Pathogens such as *Chlamydia* require an interferon-γ producing immune response for clearance of the organism - although paradoxically in certain circumstances interferon-γ may itself drive an organism into a persistent state (*Beatty* et al., 1993). This occurs when interferon-γ induces the enzyme IDO which degrades intracellular tryptophan (*Beatty* et al., 1994). Since Chlamydiae cannot synthesize this amino acid their replication ceases, but there is evidence that they can enter a quiescent state in which they still transcribe a number of genes and can therefore still act as a stimulus to the immune system which could drive persistent joint inflammation (*Gerard* et al., 1996, 2001, 2002).

Much of the research on T-cell responses in reactive arthritis was founded on the hope that bacteria-specific T-cells would be identified which cross-reacted with human proteins expressed at sites of disease (joints or entheses), Thus far this had not been achieved, with the vast majority of T-cells showing specificity for bacterial antigens and no consistent cross-reactivity with self proteins. This general idea- "molecular mimicry" has attracted some criticism recently (*Benoist* and *Mathis*, 2001), though an interesting example involving *Helicobacter pylori* has recently been reported (*Amedei* et al., 2003).

Genetic and environmental influences on the occurrence and severity of reactive arthritis

Three major genetic influences on the occurrence and severity of reactive arthritis can be discussed: (a) HLA-B27; (b) MHC encoded genes other than B27; (c) non-MHC genes; (d) bacterial genes; (e) environmental factors

HLA-B27

The incidence of HLA-B27 in many series of reactive arthritis patients has been reported to be nearly as high as that in ankylosing spondylitis: 60-70% of patients were positive, as compared to approximately 95% of those with ankylosing spondylitis (*Aho* et al., 1973). However, these figures reflect selection of patients with relatively severe reactive arthritis who are referred to secondary care. Other surveys of patients developing reactive arthritis following outbreaks of food poisoning, which include patients with very mild symptoms, have shown much lower incidences of HLA-B27, although the majority have still shown that B27 increases susceptibility to developing the disease. One such survey recently showed a clear effect of HLA-B27 on disease severity, with 75% of the patients who developed definite reactive arthritis being B27+ as compared to around 30% of those who developed any kind of musculo-skeletal symptom following *Salmonella* infection (*Ekman* et al., 2000). It is possible to conclude therefore that B27 positivity is not essential to the development of reactive arthritis, but increases its severity and therefore the likelihood that the disease will present clinically.

The mechanism whereby B27 confers increased susceptibility to reactive arthritis is unknown, and indeed the role of HLA-B27 in the pathogenesis of spondyloarthropathies in general has been much debated for 30 years, with no definitive answer at this time (*Gaston*, 1990). An initial attractive hypothesis built on the paradigm of molecular mimicry as an explanation for autoimmunity. This was first demonstrated by showing that antibodies formed in response to infectious agents could bind to self-antigens because the epitopes recognised on antigens from the pathogen 'mimicked' epitopes on normal cell proteins. This idea could be modified by suggesting that pathogen specific HLA-B27 restricted CD8+ T-cells might also recognise peptides derived from self-proteins presented by HLA-B27. Whilst B27 restricted T-cells which recognise reactive arthritis associated pathogens have been described (*Hermann* et al., 1993; *Matyszak* and *Gaston*, 2004), and autoreactive B27 specific CD8 T-cells have also been seen in spondyloarthropathy patients (*Fiorillo* et al., 2000; *Frauendorf* et al., 2003), there is no clear demonstration of molecular mimicry to confirm that this is the main mechanism underlying the association between HLA-B27 and spondyloarthropathy. In recent years, attention has shifted to some extent to the nature of the B27 molecule itself. This has several properties which are not shared with most other Class I MHC alleles including an ability to be expressed on the cell surface in the absence of tapasin (*Peh* et al., 1998), a relatively slow rate of folding in the endoplasmic reticulum and the formation of haemodymeric structures related to a cysteine molecule at position 67 (*Mear* et al., 1999; *Colbert*, 2000). There are several possible consequences from these abnormalities: The slow rate of folding in the endoplasmic reticulum also leads to the accumulation of misfolded B27 heavy chains which is an instigator of a stress response within cells. This could have consequences for how antigen presenting cells interact with T-cells (and this has recently been shown to be defective (*Hacquard-Bouder* et al., 2004), or how the same cells cope with intracellular infection. Alternatively, the expression of surface

B27 molecules which either contain no antigenic peptide or peptides which have not been edited by the TAP/tapasin mechanism may lead to abnormal presentation of self-peptides, with the possibility of breaking self tolerance. In addition, because these B27 molecules containing sub-optimal peptides are relatively unstable they may acquire exogenously self, or even pathogen related, peptides which would not otherwise be presented. Lastly, the formation of B27 homodimers, or expression of free heavy chains, presents an opportunity both for their recognition by autoreactive T-cells (*Boyle* et al., 2001, 2004) or their interaction with other receptors on T-cells such as the KIR family of receptors (*Allen* et al., 1999; *Allen* and *O'Callaghan*, 2004). Which of any of these mechanisms is involved in susceptibility to reactive arthritis and severity of the disease remains unknown.

MHC molecules other than B27

HLA typing studies have shown a remarkable similarity between patients with reactive arthritis due to enteric infection, patients with inflamed joints related to exacerbations in inflammatory bowel disease, and patients with ankylosing spondylitis in the context of inflammatory bowel disease (*Orchard* et al., 2000). This is not confined to HLA-B27 but has been shown to involve other HLA Class I and Class II alleles. The association between spondyloarthropathies and B27 almost certainly relates to the B27 molecule itself - the best evidence for this is the occurrence of disease in B27 transgenic rodents (*Hammer* et al., 1990). However, the association between spondyloarthropathy and other MHC alleles may well be explained by linkage to equilibrium between these alleles and other genes in the MHC which modulate immune and inflammatory responses. The nature of these genes has yet to be elucidated but since the MHC region has now been entirely sequenced and contains a number of candidate genes, this question should be settled in the near future (*Beck* et al., 1999). Obvious candidates would include the genes for TNF-α which is clearly a predominant cytokine in spondyloarthropathy, judged by the striking clinical efficacy of drugs which inhibit TNF (*Braun* et al., 2002).

Non-MHC genes

The importance of these has been clear for some time in ankylosing spondylitis where the frequency of disease is much higher in B27+ individuals who have a first degree relative with ankylosing spondylitis as compared to B27+ individuals with no such relatives. The nature of the genes involved is under intensive investigation by whole genome surveys in large groups of patients from multicase families. Whilst some of these genes will relate to ankylosing spondylitis specifically, there may be others which play a part in other forms of spondyloarthropathy. It has also been noted that B27 associated diseases 'breed true', i.e. those with B27 and a family history of reactive arthritis have a higher risk of reactive arthritis rather than ankylosing spondylitis, and conversely for those with B27 and relatives with ankylosing spondylitis. It is, however, striking that inflammation in the gut or in the skin can in some ways 'substitute' for HLA-B27 in the development of ankylosing spondylitis, since, in the absence of these factors 95% of patients are B27+, whereas if these diseases co-exist only 50% of the patients are B27+. Therefore, the genes which underlie psoriasis and inflammatory bowel disease are worth attention, and particular interest has been aroused by the recent description of the CARD 15 molecule which is associated with Crohn's Disease and possibly also with psoriatic arthritis (*McGovern* et al., 2001; *Rahman* et al., 2003). Given that this molecule has properties suggesting

that it modulates the response to bacteria products such as LPS, and other molecules detected at the-cell surface by Toll-like receptors (*Pauleau* and *Murray*, 2003), it may be worthwhile looking for similar molecules which would be associated with reactive arthritis and/or ankylosing spondylitis. CARD 15 itself does not appear to be associated with ankylosing spondylitis (*van der Paardt* et al., 2003). Likewise IL-11 has recently been linked to ulcerative colitis (*Klein* et al., 2002); IL-11 can down-regulate the response to LPS which requires signalling through NFκB.

Bacterial genes

Little is known about the specific bacterial genes which influence the occurrence of reactive arthritis, but these much exist since markedly different rates of arthritis are seen after infection with similar organisms. It has already been noted that *Chlamydia pneumoniae* is a much less common trigger than *Chlamydia trachomatis*, and the same applies to *Chlamydia psittaci*. More striking still is the difference between *Shigella sonnei* and *Shigella flexneri,* with the former only rarely reported as a trigger of arthritis (*Lauhio* et al., 1988). Genetically all Shigellae resemble *E. coli* which also does not trigger reactive arthritis.

Environmental factors

These clearly play a role in spondyloarthropathies including reactive arthritis. Thus, in ankylosing spondylitis concordance for disease is not 100% in monozygotic twins (*Brown* et al., 1997; *Jarvinen*, 1995). The role of environmental factors in reactive arthritis is not well defined but two pieces of evidence are worth considering:

The influence of HIV on the incidence of reactive arthritis and other forms of spondyloarthropathy

Prior to the epidemic of HIV in sub-Saharan Africa, reactive arthritis was very rare despite a high incidence of infection with triggering organisms such as *Shigella* and *C. trachomatis*. HLA-B27 is virtually absent form many of these populations, and this might have been taken to be a protective factor. However, since the appearance of HIV patients with reactive arthritis have been commonly reported (*Njobvu* et al., 1998; *Njobvu* and *McGill*, 1999, 2000). This suggests that there is a genetic background in these populations which allows reactive arthritis to occur (or is not protective), but this has only been revealed by the alterations in the immune system brought about by HIV infection.

The "Chlamydia paradox"

The incidence of *Chlamydia* infection in Western countries continues to rise and this is probably not just due to better diagnostic methods. However, no rise in the incidence of *Chlamydia*-triggered reactive arthritis has been seen in these countries, and indeed these cases are somewhat uncommon. This paradox is unexplained. However, investigations of the T-cell-mediated response to *C. trachomatis* have mapped epitopes which are identical in *C. pneumoniae* (*Deane* et al., 1997; *Goodall* et al., 2001). This raises the question of whether prior infection with *C. pneumoniae* might make reactive arthritis more likely by "priming" the immune system to make excessive responses to the infection. This concept has not been proven, but the prevalence of *C. pneumoniae* infection is relatively low in the cohort most likely to acquire *C. trachomatis* (teens and

twenties). The general concept that previous antigenic experience, due to infection with related organisms, might affect the likelihood of post-infectious inflammatory diseases is worthy of further investigation.

CONCLUSIONS

Reactive arthritis illustrates how bacterial infection of the gut or genito-urinary tract can result in severe and occasionally chronic inflammation at distant sites. Although many questions remain about its pathogenesis, host genes which affect the immune response to the organism are implicated, together perhaps with the host's previous antigenic experience.

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MICROORGANISMS AND CANCER

JOSEF BEUTH

Institute of Naturopathy, Medical Faculty, University of Cologne,
Cologne, Germany

SUMMARY

It is well established that defined microorganisms may cause cancer. Basic, molecular mechanisms are known and might contribute to innovative prophylaxis or treatment strategies, e.g. vaccination.

The ability of defined attenuated microorganisms to cause tumour regression is well known, however, the mode of action mostly remained unknown. It is generally believed that e.g. bacteria (or their products, components; probiotics) activate the immune system and that the activated immune system is responsible for cancer regression. However, experiments with *Toxoplasma gondii* have shown that the inhibition of angiogenesis may also contribute to cancer regression, at least in the case of *T. gondii*. Furthermore, the proliferation of anaerobic bacteria in the core of tumours is well established. However, there is a lack of knowledge whether local anti-tumourous anaerobic bacteria secrete soluble factors that could induce cancer cell death.

Administration of bacterial probiotics in defined stages of malignant disease and its treatment is a promising option in complementary oncology and was shown to restore immune functions and to decrease amount and severity of side effects of tumour destructive standard treatments.

MICROORGANISMS: CAUSATIVE AGENTS FOR MALIGNANT DISEASE

Distinct microorganisms are known to cause malignant diseases (cancer), amongst them *Helicobacter pylori* (gastric cancer), human papilloma virus, HPV (cervical cancer), hepatitis B/C virus, HBV/HCV (hepatocellular carcinoma), Epstein-Barr virus, EBV (lymphoproliferative diseases), *Schistosoma haematobium* (urinary bladder cancer) (Table 1). Basic mechanisms of microbial invasion, intracellular uptake and tumourigenicity are well known and confirmed for the microorganisms mentioned (*Pfister*, 2001), however, an array of further microorganisms are suspected to be involved in malignant, metabolic, and autoimmune diseases. Future investigations have to focus on the provoking question: *Are all diseases infectious?* So far, scientific evidence of microorganisms being causative agents for malignant diseases is limited to defined species and warrants further interdisciplinary investigations.

Table 1: Microorganisms: Aetiologic agents for cancer

Aetiology definitely demonstrated:
H. pylori gastric cancer
S. haematobium urinary bladder cancer
EBV nasopharynx cancer; Burkitt's and immunoblastic B-cell lymphoma
HPV (Type 16,18) cervical cancer
HBV/HCV hepatocellular carcinoma
Aetiology supposed:
HPV (Type 5,8,15) non-melanoma skin cancer
HPV (Type 16,18) larynx-, penis-, vulva-cancer/carcinoma
EBV M. Hodgkin
HHV (Type 8) Kaposi sarcoma

MICROORGANISMS: TREATMENT STRATEGY FOR MALIGNANT DISEASE

Administration of microorganisms (mainly bacteria or their products/extracts; sometimes viral or fungal agents or components) in the treatment of cancer is less widely known in the scientific community. It goes back more than 100 years when William B. Coley, physician and surgeon of the Memorial (Sloan-Kettering) Hospital, New York, observed that many of his cancer patients showed tumour regression when they were infected with bacterial pathogens. Treatment to eliminate the infections resulted in cancer relapse (*Coley*, 1893). He developed a treatment modality by making extracts of defined bacteria (e.g. *Streptococcus pyogenes, Serratia marcescens*) called *Coley's Toxin* which he administered to shrink tumours in his patients (*Coley*, 1893; *Nauts* et al., 1946).

Subsequently, other bacteria have been investigated in an effort to reduce the growth or the size of tumours. The most prominent example would be the use of *Mycobacterium bovis* BCG (Bacillus Calmette-Guerin) the vaccine strain in the treatment of defined stages

Table 2: Evidence-based medicine: Basis for clinical evaluation (Centre of Evidence-Based Medicine, University of Oxford, UK)

Levels of evidence:	
Ia:	Metane alysis of RCTs
Ib:	RCT
IIa:	Metane alysis of epidemiological/cohort studies
IIb:	Epidemiological/cohort study
III:	Non-randomised/experimental study
IV:	Case report
V:	Expert opinion/consensus

Table 3: EBM-Evaluation of microorganisms for cancer treatment

M. bovis BCG urinary bladder cancer EBM level Ib
P. avidum KP-40 colorectal carcinoma EBM level Ib
Coley's Toxin diverse cancers, sarcomas EBM level III
Newcastle Disease Virus lymphoproliferative diseases EBM level III
S. pyogenes OK 432 diverse cancers EBM level III
C. parvum diverse cancers EBM level III

of urinary bladder cancer (*Lamm* et al., 1980). Several good clinical practice (GCP) performed clinical studies (Randomised Controlled Trials; RCTs) have shown a clear relationship between the use of *M. bovis* BCG immunoprophylaxis after surgical removal of the tumour and the decreased recurrence rate or the prolonged relapse-free interval (*Lamm* et al., 1980; *Morales* et al., 1976).

Currently, *M. bovis* BCG immunoprophylaxis is evidence-based in agreement with proposals of the Centre of Evidence-Based Medicine (EBM), University of Oxford, UK (Table 2), since EBM-levels Ib and IIb studies are available (*Agarwala* and *Kirkwood*, 1998; *Sheperd*, 1997). However, long-term administration of BCG might induce problems, e.g. lack of predictability of its effectiveness and serious side effects like sepsis leading to the death of patients (*O'Donnell* and *DeWolf*, 1995).

The mode of action of BCG to induce its antineoplastic effect (see Table 3) is suggested to result from its effects on the (local, mucosal) immune system, with mononuclear cells (T-lymphocytes, monocytes) playing a major role (*Ratlift* et al., 1993). Thus, intravesical instillation of BCG induces a non-specific cystitis, which is accompanied by local production of cytokines and accumulation of inflammatory cells being able to damage malignant cells (*Alexandroff* et al., 1999). The requirement of live cells of BCG for its anticancer activity is reflected in the fact that monocytes and helper T-lymphocytes type 1 (TH_1) are most important for its effectiveness (*Thanhauser* et al., 1995) and that high doses of defined vitamins have shown a positive effect on the treatment of bladder cancer in human clinical trials (*Lamm* et al., 1994).

The ability of bacteria to modulate the immune response to non-related antigens is well documented. *Propionibacterium* species are amongst the most potent immunomodulators stimulating cell populations involved in non-specific

Table 4: Hypotheses on basic mechanisms:
Microorganisms as treatment strategies for malignant diseases

Local application: → Coley's Toxin; *S. pyogenes* OK 432; *M. bovis* BCG
• Microbial toxin lyses cancer cells
• Inflammation activates cytokines, immune cells
• Immunoactivation (cytokines, immune cells)
Systemic application: → Coley's Toxin; *P. avidum* KP-40; *C. parvum*; *S. pyogenes* OK 432
• Inflammation
• Immunoactivation
• Fever induction
→ all induce cytokine release; immune cell activation

Table 5: History of immunomodulating *Propionibacterium* species

Basis: Promising data on bacterial immunomodulators
→ Coley's Toxin; *M. bovis* BCG
1980: Selection of *P. avidum* KP-40
→ optimum immunomodulator from about 200 strains
1981: Basic investigations on immunomodulating effects
1982: Clinical studies in oncology, infectiology
1997: Research on active components
→ LTA, glycopeptides
2001: Research on oral application of *P. avidum* KP-40

resistance (*Jeljaszewicz* et al., 1982; *Isenberg* et al., 1995). Three species (*Propionibacterium acnes, P. granulosum, P. avidum*) appeared to be of special medical interest and after evaluating the immunoactive potential of a great number of strains (*Ko* et al., 1981) *P. 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 immunoactive capacity is absolutely identical to *P. granulosum* KP-45.

The obvious therapeutical benefit of *P. avidum* KP-40 treatment in neoplastic disease induced a great amount of experimental studies (*Isenberg* et al., 1995; *Pulverer* et al., 1985). During these investigations we were able to determine the effects of *P. 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 *P. 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 *P. avidum* KP-40 injection (*Isenberg* et al., 1995; *Beuth* et al., 1990).

Table 6: Preclinical evaluation of P. avidum KP-40

• Proliferation/activation of RES cells
→ spleen cells, macrophages, monocytes
• Proliferation, maturation and emigration of thymocytes
• Activation of peripheral blood cells
→ granulocytes, monocytes, lymphocytes, NK-cells
• Anti-infective activities
→ against bacteria, viruses, parasites
• Antineoplastic/antimetastatic activities
→ in various murine models

To evaluate antitumour/antimetastatic effects of *P. avidum* KP-40 induced immunomodulation, BALB/-c mice were intravenously challenged with RAW 117-H lymphosarcoma cells and checked for liver tumour colonisation as described elsewhere (*Beuth* et al., 1987). Compared to a control group the number of liver colonies was significantly lower in *P. avidum* KP-40 treated mice (*Isenberg* et al., 1995; *Beuth* et al., 1987).

Clinical investigations proved that surgical treatment of malignant diseases (e.g. colorectal carcinoma) induced an evident decrease of peripheral blood lymphocyte counts and activity, as compared to pre-operative values. However, single pre-operative administration of *P. avidum* KP-40 induced a considerable increase of peripheral blood cell counts and activities, especially of lymphocytes. Clinical effects of pre-operative immunostimulation by propionibacteria were investigated in prospectively randomised clinical studies in colorectal carcinoma patients. Beneficial effects of survival time, local tumour recurrence and distant metastasis could be demonstrated in stages I and II, whereas no advantage of immunotherapy was found in advanced stages III and IV (14).

Another prospectively randomised clinical study was initiated on the quality of life of colorectal carcinoma patients. Three months after surgical treatment negative effects could not be determined after immunotherapy. Quality of life even proved to be better in patients with abdomino-perineal resection as compared to non *P. avidum* KP-40 treated control patients (*Isenberg* et al., 1995).

Generally, the activated immune system provides protection from infectious pathogens and growth/spread of malignant cells through mechanisms of recognition and elimination. Accordingly, Coley's Toxin, *M. bovis* and *P. avidum* KP-40 could be shown to be effective in the treatment of neoplastic disease in human medicine. Further clinical studies are warranted to confirm and enlarge these promising data.

MICROORGANISMS/PROBIOTICS: INNOVATIVE COMPLEMENTARY TREATMENT IN ONCOLOGY

New approaches to curative cancer therapy are being explored and evaluated around the world. Great hopes have been placed in the *Human Genome Project* as well as in advances in the fields of molecular biology, molecular genetics and immunology, without leading to the validation of a new beneficial concept of treatment.

Searching for new substances of therapeutic importance (e.g. in rain forests, oceans or by developing new technologies) seems promising. However, it is time-consuming and expensive because scientific evaluation is an obligate prerequisite before clinical application. Currently, the optimisation of curative cancer therapy seems to be possible especially through the development of interdisciplinary concepts.

In the United States, the use of tumour-destructive standard therapies (surgery, chemotherapy, radiotherapy) did not significantly lower cancer mortality over the last 20 years. Despite extensive efforts in both research and therapy in response to *President Nixon's declaration of war against cancer* at the beginning of the 1970's, age-adjusted cancer mortality even increased about 6% (*Bailar* and *Gornik*, 1997). Therapeutically beneficial results were achieved for relatively rare types of tumours such as lymphoma, leukaemia, testicular tumours. This outcome demanded innovative concepts of treatment and ushered in the scientific, experimental, and clinical efficacy testing of

Table 7: Scientifically-based complementary cancer treatment

- ***No alternative*** to standard treatment, but ***optimisation***
- Evaluated in RCTs, thus ***integrated in EBM***
- Integrated in Disease Management Program; DMP in Germany
- Accepted by German Medical Association and Health Insurance Companies
- Integrated into educational curricula for German physicians
- ***Rigorously demanded by patients***

therapeutic approaches used in complementary oncology (*Abel*, 1995).

About 80% of all cancer patients in Germany use complementary medicine, often without knowledge of the attending oncologists (*Beuth*, 2002). Their main motives are

- to actively participate in fighting against the disease or promoting recovery,
- to activate the immune system,
- to optimise standard therapy.

These understandable wishes need to be addressed with critical openness, and therapists should be aware that active patients profit from the activation of their psycho-neuro-immunological system.

As per definition, therapeutic approaches of complementary oncology do not replace the approved standard therapies. Hence, they are not *alternative therapies*. Complementary approaches in oncology proved to be beneficial additions to the tumour destructive standard therapies to optimise them (*Beuth*, 2002).

Preliminary data from scientifically-based studies have demonstrated the importance of various approaches. The benefits to the patients included improvement of the quality of life, reduction of symptoms and side effects due to standard therapy, and improvement of the immunological state (*Beuth*, 2002).

Indication-based administration of probiotics (live/attenuated/killed micro-organisms, their components or metabolic products) is part of the scientifically-based complementary oncology. Indications include peri-/post-tumour-destructive immunocompromisation; after care-/regeneration period and its accompanying weakness/fatigue; disease-/treatment-induced (metabolic) disorders, e.g. in the gastro-intestinal (GI)-tract and regeneration of physiological microflora (*Beuth*, 2002). So far, EBM-level III/IV studies are available and show beneficial effects (safety and efficacy) of the indication-based administration of *medical probiotics*.

Table 8: Scientifically-based complementary medicine

- Recommended EBM-level I/II evaluated therapies:
  - → Nutrition optimisation/guidance
  - → Moderate sportive activities
  - → Psycho-oncological guidance
  - → Sodium selenite (on indication)
  - → Standardised proteolytic enzymes (on indication)
  - → Immunoactivating standardised mistletoe extract (on indication)
- Extended, EBM-level III/IV evaluated therapies:
  - → E.g. probiotic/microbiological treatment

Table 9: Beneficial effects of medical probiotics in cancer treatment

- Lactic acid producing bacteria/probiotics
  → e.g. *Lactobacillus* species; *Bifidobacterium* species
- Bacteria/probiotics of GI-tract origin
  → e.g. *E. coli; E. faecalis*

⇒ Regulate metabolism (→ e.g. GI-tract)
⇒ Optimise physiological microflora
⇒ Modulate immune functions (→ e.g. MALT)

However, further studies (EBM-level I or II) are warranted as to integrate this complementary medication into standard treatment concepts.

The lack of predictability of efficacy, the non-specific (non-controlled) immune response and the associated side-effects and toxicity have so far limited the use of live/attenuated/killed microorganisms and their components/products in the treatment of cancer. Innovative approaches to tailor specific molecules to target defined cells and their metabolism might lead to a specific treatment modality for malignant diseases. Current investigations are promising.

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GENES, ENVIRONMENT AND PATHOGENS: AN EVOLUTIONARY PERSPECTIVE ON THE CAUSES OF CHRONIC DISEASES

PAUL W. EWALD

Department of Biology, University of Louisville, Louisville, Kentucky, USA

SUMMARY

Chronic diseases are commonly studied through quantification and accumulation of risk factors. To reliably lead to an understanding of disease causation, however, the role of risk factors in the process of pathogenesis must be evaluated. This evaluation must consider in an integrated way the full range of possible causes of chronic disease, which can be categorised as genetic, infectious and non-infectious environmental. To understand how to prevent disease causal risk factors need to be distinguished from risk factors that are spurious correlates of causal processes, and primary causes need to be distinguished from exacerbating causes. Insights from evolutionary biology provide a foundation for this process by distinguishing feasible causal hypotheses from infeasible ones. This paper applies this approach to the epsilon-4-associated diseases as a paradigm for chronic diseases, and then considers atherosclerosis in more detailed as an illustration of epsilon-4-associated diseases. The most parsimonious conclusion is that the epsilon-4-associated diseases in general and atherosclerosis in particular are for the most part infectious diseases that are exacerbated by the documented environmental and genetic risk factors.

INTRODUCTION: CATEGORIES OF CAUSATION

Diseases can be attributable to genetic, infectious, and environmental causes. Although diseases are often referred to as though they belong to one of these categories, it is generally recognised that more than one of these categories of causal factors generally contribute to each disease (Figure 1). Cystic fibrosis, for example, is referred to as a genetic disease but life-threatening crises result from respiratory infections with pathogens such as *Streptococcus pneumoniae* or *Pseudomonas aeruginosa*. All three categories of causal factors generally contribute to infectious diseases (defined broadly in this paper to include all examples of internal parasitism); infections with *Mycobacterium tuberculosis*, for example, can range from asymptomatic to lethal depending on genetic susceptibility and a person's nutritional status. The corollary of this generalisation is that the identification of genetic or non-infectious environmental influences on disease cannot be used as evidence against infectious causation, because such influences are expected among infectious diseases. Nevertheless, researchers often commit this logical error when they dismiss infectious causation on the basis of the evidence consistent with genetic causes or non-infectious environmental causes. This error is especially counterproductive when the evidence that is consistent with genetic causation is also consistent with infec-

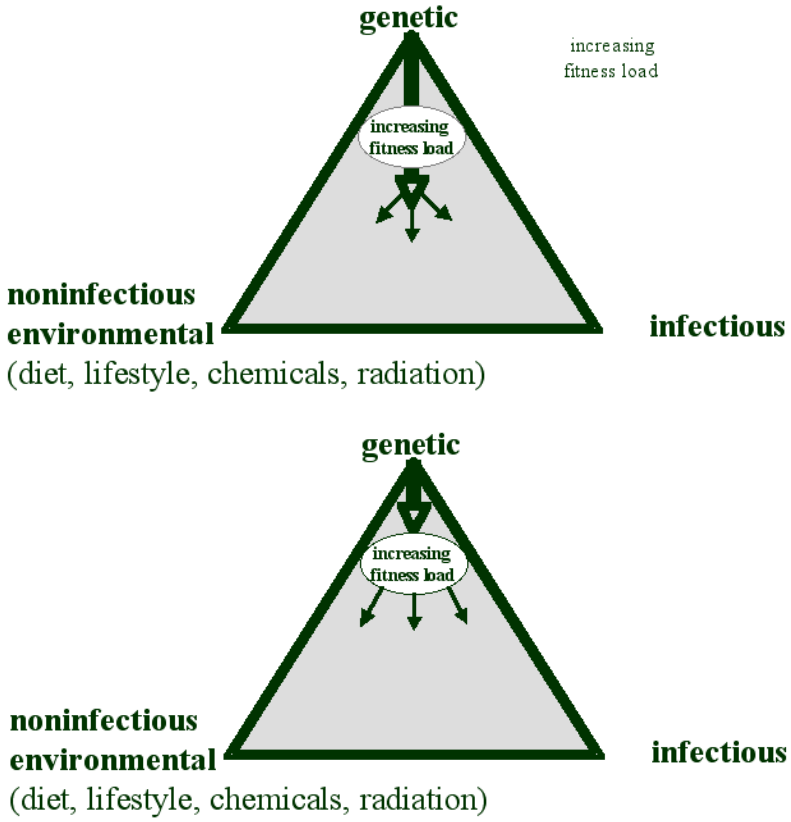

Figure 1: The triad of disease causation. The diagram emphasises that more than one category of disease causation is in operation for any particular disease. The location of a disease within the triangle corresponds the relative importance of the three categories. The further from the apex the less the relative importance of the designated category of causation. The arrow thus signifies decreased importance of genetic causation relative to the influences in the other categories. Increased fitness load (defined in text) implies reduced importance of genetic causation, because mutation alone can maintain a genetic basis only for diseases with very low fitness loads.

tious causation, as is the case for example, for schizophrenia (*Ledgerwood* et al., 2003).

The tendency for candidate causes of disease to be correlated with other variables and the difficulty of distinguishing correlation from causation has contributed to the tendency for researchers to couch discussions of chronic diseases in terms of risk factors rather than causes. This tendency can be counterproductive, however, by clouding the different roles of different risk factors. Some risk factors will be primary causes, which initiate the disease process, whereas others will be secondary causes, which exacerbate the disease process. Other risk factors may not play a causal role at all, but may simply be correlated with primary or secondary causes.

The identification of primary causes is critical for the eventual control of disease, because blocking of a primary cause eliminates the disease, whereas blocking of a secondary cause does not. *M. tuberculosis* is the primary cause of tuberculosis because tuberculosis cannot occur without *M. tuberculosis* infection.

The same claim cannot be made for host genetic or non-infectious environmental factors that exacerbate *M. tuberculosis* infections. Tuberculosis can occur among people who are not particularly genetically susceptible to *M. tuberculosis* infection or who have not been exposed to particular environmental factors that exacerbate tuberculosis infection, if other factors such as high dosage or temporary immunosuppression allow *M. tuberculosis* infection to progress to tuberculosis. *M. tuberculosis* is therefore considered the primary cause of tuberculosis, and tuberculosis is considered an infectious disease, even though human genetic variation and non-infectious environmental factors may influence the manifestations of *M. tuberculosis* infections. The problem of tuberculosis can be eliminated if *M. tuberculosis* infection is blocked. Categorising tuberculosis as an infectious disease emphasises this point. Analogously, mutations in the cystic fibrosis trans-membrane conductance regulator gene are primary causes of cystic fibrosis, whereas *S. pneumoniae* is a secondary causes, because eradication of *S. pneumoniae* would not eradicate cystic fibrosis.

MOVING FROM RISK FACTORS TO CAUSATION

Consideration of risk factors is safe, because the term "risk factor" implies only correlation and not causation, and correlations can generally be demonstrated more easily and definitively than causation. This generalisation is especially valid for chronic diseases. But risk factors are studied with hopes of intervening to control disease; the most important risk factors are therefore those that play a causal role. Careful study and logic are required to assess which risk factors are parts of a causal process and which are not. Unfortunately, although reference to risk factors is often made with sufficient care to avoid jumping from correlation to causation, the repeated identification of risk factors without assessment of the feasibility of a causal mechanism has created a vacuum that has led the popular media and the medical literature to blur the distinction between risk factors and causes of illnesses. This blurring then influences the hypotheses that are evaluated, with some risk factors being favoured targets of research effort because their causal role is presumed. When the feasibility of causal hypotheses is directly addressed, however, some of these risk factors are often found to be inadequate as primary causes. When considered against the full spectrum of possible causes, such risk factors may be exacerbating or ameliorating influences or merely correlates.

These considerations provide an important framework for understanding infectious disease because causation of disease is not well understood for about half of all human diseases. The importance of this framework is evidenced by the track record in identifying infectious causes of disease over the past three decades. The recognition of new examples of infectious causation has not slowed during this time and evidence suggests that infection may be a primary cause of a large proportion of the most important diseases of unknown cause (*Cochran* et al., 2000).

Assessment of the relative importance of infectious, genetic, and environmental causes requires the integrated application of insights from several different disciplines of biology. The concept of evolutionary fitness is particularly useful in distinguishing genetic primary causes from infectious and non-infectious primary causes. Evolutionary fitness is a measure of genetic contribution into the succeeding generations. When applied

to genes, the fitness of an allele refers to the change in its representation over time relative to alternative alleles. The negative effects of a disease on the passing on of the presumptive genetic basis for the disease are referred to as the fitness load of the disease (*Cochran* et al., 2000). If the negative effects of a disease are so great that genetic instructions for the disease could not be maintained through time by mutation, then genetic factors are not feasible as primary causes.

The main caveat is that compensating advantages of disease-causing alleles could allow the maintenance of severe genetic diseases at moderately high frequencies. Sickle cell anaemia provides the classic example. Where falciparum malaria is common, sickle-cell anaemia can be maintained at a frequency that is over two orders of magnitude greater than the frequency that could be maintained simply by new mutations, because individuals who are heterozygous for the sickle-cell allele are protected against falciparum malaria (*Vogel* and *Motulsky*, 1997).

Such genetic diseases are maintained at high frequency because their genetic basis provides a "self-destructive defence" against an infectious disease. All such diseases recognised to present have distinctive characteristics: they result from mutations that cause a protein product to lose normal function, they are inherited by simple Mendelian ratios, they confer protection against infection in heterozygous form, and they occur in high frequency in restricted geographic regions or in certain ethnic groups, and in low frequency in other populations. Several diseases besides sickle cell anaemia share such characteristics category, for example, thalassemia, cystic fibrosis, haemochromatosis, glucose-6-phosphate dehydrogenase deficiency, Tay Sachs, Gauchier's disease (*Cochran* et al., 2000). These diseases probably all represent rapid, "quick-and-dirty" evolutionary response to recent threats or environmental challenges. Although it is not known for most of these diseases whether this challenge is posed by an infectious disease, infectious diseases are prime candidates for generating the selective pressure that favours many of them. The responsible infectious challenges may new in the sense that the infectious agents recently entered the human population or because they represent ever-changing threats as they co-evolve in response to human defences.

Although the known self-destructive defences share these characteristics in common, the model of self-destructive defences has been applied indiscriminately to explain how damaging genetic diseases could be maintained at relatively high frequencies even when the diseases do not share these characteristics. As a consequence, considerations of disease causation generally fail to appreciate the severe restrictions that the fitness load of a disease imposes on the feasibility of genetic causation of disease. For most of the common and damaging chronic diseases of unknown cause, the fitness load is too high to allow the maintenance of the disease simply by mutation (*Cochran* et al., 2000). Hypotheses of genetic causation for these diseases must therefore be evaluated critically to assess how they could be feasible in the context of the observed fitness load. In practice, however, hypotheses of genetic causation are generally accepted uncritically on the basis of family studies, particularly when the concordance for the illness among identical twins is high. By themselves these family studies do not demonstrate genetic causation because other non-genetic causes of disease may correlate with genetic causes. The *in utero* environment for monozygotic twins, for example, is more similar than is the in utero environment for dizygotic twins because monozygotic twins share a common gestational sac and placenta more often than do dizygotic twins.

Monozygotic twin may therefore not only share more genes in common but more infectious agents *in utero*. High monozygotic twin concordances and lower dizygotic twin concordances are therefore consistent with genetic causation but are not sufficient to demonstrate genetic causation. Rather than revealing the degree of genetic causation they provide a ceiling on the extent of genetic causation--low-to-moderate concordance indicates that some environmental cause, either infectious or non-infectious, is playing a major role. Such considerations call into question, for example, the commonly held belief that schizophrenia is largely a genetic disease (*Ledgerwood* et al., 2003).

GENETIC PREDISPOSITIONS TO INFECTION: THE EPSILON 4 ALLELE

This framework of inquiry can be applied to every disease of unknown cause. For illustrative purposes this paper will consider the diseases that have been associated with the epsilon 4 allele of the Apolipoprotein E gene, namely atherosclerosis, stroke, Alzheimer's disease, and severe cases of multiple sclerosis (*Hardy*, 1995; *Ji* et al., 1998; *Urakami* et al., 1998; *Evangelou* et al., 1999; *Ilveskoski* et al., 1999; *Mahley* and *Huang*, 1999; *Love* et al., 2003). The epsilon 4 allele is a provide a particularly informative example, because the discovery of its association with these diseases is considered one of the great medical advancements of human genetics and the rich body of knowledge that is now available for the epsilon-4-associated diseases. A balanced approach to consideration of the causes of these epsilon-4-associated diseases may therefore provide a general model for evaluation of genetic associations with human disease as well as a better understanding of the contribution of human genetics to the health sciences.

The epsilon-4-associated diseases have their greatest negative impacts on health after direct reproduction; they therefore probably have had much of their effect on fitness through reductions in resources available to children and other relatives such as grandchildren. Still a substantial portion of the negative effects of the epsilon-4-associated diseases on fitness load occur during reproductive periods for especially for males, because males continue to have children into sixth and seventh decades of life and because debilitating and lethal cases of epsilon-4-associated diseases (particularly heart attacks, strokes, and multiple sclerosis) often occur in the fifth decade of life or even earlier.

The fitness load of the epsilon 4 allele resulting from the negative effects of Alzheimer's disease, multiple sclerosis, atherosclerosis, and stroke is probably well over 1% (*Cochran* et al., 2000). Although this 1% figure is a rough estimate, it is about two orders of magnitude above the percentage that could be maintained by mutation. This assessment indicates that there must be some compensating benefit or that this disadvantage of the epsilon 4 allele has not always been present throughout the existence of *Homo sapiens*. It seems doubtful, however, that the negative effects of the epsilon 4 associated diseases could have been negligible if the risk of developing them at a given age had been present human history and pre-history. Less death and incapacitation from these illnesses due to early death from other causes was probably substantially compensated for by more severe consequences on survival of offspring and reduced birth control in older people in previous centuries.

The distribution of the epsilon 4 allele among human populations and primate species indicates that the epsilon 4 allele itself could not be intrinsically bad or defective. Epsilon 4 is maintained in all human populations at frequencies from about five to 40% (*Corbo* and *Scacchi*, 1999; *Fullerton* et al., 2000). If epsilon 4 were an inherently inferior allele it could not be maintained at such frequencies over long periods of time. Phylogenetic analyses indicate, however, that it has been maintained over long periods of time and that epsilon 4 is the ancestral allele, with the epsilon 4 being more similar to the epsilon alleles of chimpanzees and other primates than are the other epsilon alleles of humans (*Fullerton*, 2000); it could not have predominated in primates for millions of years, if it were defective. In humans its frequency is about 5-15% in populations that have had been living in agricultural or urban setting for thousands of years but at frequencies of about 20-40% in populations that have been living as hunter/gatherers until the 20th century. This difference indicates that the allele has been declining as humans shifted away from hunting and gathering over the past 10,000 years.

One commonly accepted explanation for the persistence of epsilon 4 is that humans have only recently lived long enough to experience the negative effects of the epsilon 4-associated diseases, which often occur after the third decade of life. But this argument does not apply to multiple sclerosis, which typically occur during or before the third decade of life. With a prevalence of up to 0.3% in regions, it alone could have exerted a substantial selective pressure against epsilon 4. Another problem with this short-life argument is that its fundamental assumption does not hold up to the evidence. Studies of hunter/gatherer societies have found a high probability of survival into older age groups, contradicting the widely held but poorly supported belief that hunter-gatherers rarely lived past 40. The probability of surviving from the onset of reproduction to age 65 was about 70% among the San (a.k.a. "Bushmen" of southern Africa) (H.C. Harpending, unpublished data). Even among the more violent Ache of South America survival over this interval was about 45% (*Hill* and *Hurtado*, 1996). Evidence from tooth wear also suggest that humans have regularly lived into and beyond their fourth decade of life during the 20,000 years prior to the onset of agriculture (*Caspari* and *Lee*, 2004).

Another hypothesis to explain the current presence of the epsilon 4 allele has been derived from the function of the epsilon 4 protein: transport of cholesterol and lipids. Application of the "thrifty genotype" hypothesis for diabetes (*Neel*, 1962) to the epsilon 4 diseases suggests that the epsilon 4 allele may be too good rather than too bad (*Corbo* and *Scacchi*, 1999). That is, its high efficiency of transport of lipids and cholesterol was beneficial during times when nutrients were scarce, but epsilon 4 is transporting too much lipid and cholesterol now that diets are so much richer than they were in the past. This hypothesis, however, has two problems. The first is that even hunter-gatherer populations do not have epsilon 4 frequencies that approach 100%. If the frequency of epsilon 4 dropped as a result of the rich diets in agricultural settings then we would expect that hunter/gatherer should have virtually 100% epsilon 4. But epsilon 4 among hunter-gatherers generally lies between 20% and 40%. The shift away from epsilon 4 therefore must have begun well before the shift to rich agricultural diets.

Phylogenetic analyses indicate the other two major epsilon alleles, epsilon 3 and epsilon 2, were derived evolutionarily from the epsilon 4 allele in humans and increased in frequency over the past 200,000 years (*Fullerton* et al., 2000);

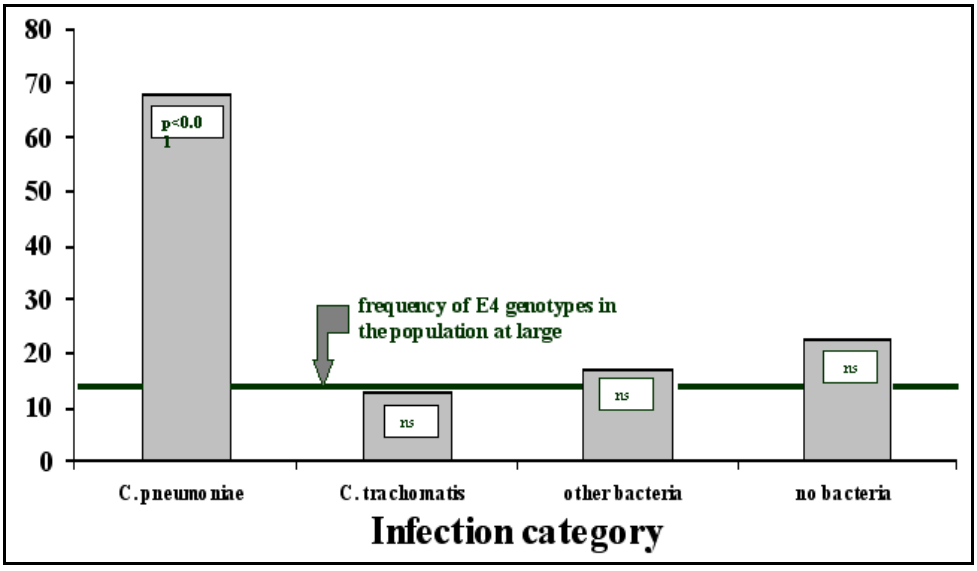

Figure 2: Association of *Chlamydia pneumoniae* infection with epsilon 4 genotype in the synovial tissue of arthritis patients. Patients who were positive for *C. pneumoniae* DNA were much more likely to have the epsilon 4 allele than the general population, but epsilon 4 was not significantly more common among patients who were positive for DNA from *Chlamydia trachomatis* or other bacteria, nor among those who were negative for all of the tested pathogens. The abbreviation, ns, indicates that the frequency of epsilon 4 genotypes was not significantly greater than the frequency in the general population from which the arthritis patients belonged. Data are from *Gérard* et al. (1999).

that is, epsilon 2 and epsilon 3 have increased at the expense of epsilon 4 during the time interval over which *Homo sapiens* is seen as a distinct from *H. erectus*. Although the timing of this increase cannot be determined with accuracy, the phylogenetic analyses together with the substantial presence of epsilon 2 and epsilon 3 in hunter gatherers indicates that the shift began long before the shift from hunting and gathering to agriculture.

The frequencies of epsilon 4 among the few hunter-gatherers with rich diets also argue against the thrifty allele hypothesis as an explanation of epsilon 4's detrimental effects. If rich modern diets were responsible, one would expect that hunter-gatherer populations with rich diets should have low epsilon 4 frequencies. Inuits, however, who have eaten a rich for many thousands of years have a relatively high frequency of epsilon 4, one that is comparable to other populations of humans whose ancestors lived indigenously, in low densities in North and South America and (*Corbo* and *Scacchi*, 1999).

Another problem with the thrifty allele hypothesis is that different defective functions must be envisioned for each epsilon-4-associated disease. It can offer a hypothetical explanation for the association of rich diets with atherosclerosis and stroke, but it does not offer a mechanism for the association between epsilon 4 and Alzheimer's and multiple sclerosis. The association between epsilon 4 and these other diseases has been explained by generating hypotheses based on mechanistic aberrancies other than lipid deposition (*Henderson*, 2004). Such post-hoc hypothesis, still have the same weaknesses mentioned for the

association between epsilon 4 and fat deposition in atherosclerosis; for example, a variant on the thrifty allele hypothesis proposes that the negative effects of epsilon 4 on Alzheimer's disease arise in response to the carbohydrate rich diets associated with agriculture (*Henderson*, 2004). But, as argued above, the negative effects of epsilon 4 must have been in action many thousands of years before the beginning of agriculture to explain the epsilon 4 allele frequencies of hunter-gatherers.

These considerations lead to the conclusion that epsilon 4 must have conferred vulnerability to some other cause of the epsilon-4 associated diseases that was present when humans were still hunter-gatherers, but became more important for humans in agricultural settings. Genetic vulnerability to infectious agents, for example, would tend to be more problematic as human populations become larger and denser and hence could maintain higher levels and intensities of infection. Genetic vulnerabilities to infectious agents appear to be a pervasive cause of allelic associations with disease (*Abel* and *Dessein*, 1997; *Cochran* et al., 2000). This genetic-vulnerability-to-infection hypothesis is consistent with the overall trends, because it suggests that the pathogen pressure would increase in agricultural societies but would have existed before the onset of agriculture.

GENETIC PREDISPOSITIONS TO INFECTION

Chlamydia pneumoniae is one of the most plausible candidates for such a process because it is the only infectious organism that is implicated as a cause of each of the epsilon 4-associated diseases. Accordingly, research on arthritis patients has demonstrated the predicted association between the epsilon 4 allele and *C. pneumoniae* infection (*Gérard* et al., 1999; see Figure 2). This finding suggests that epsilon 4 somehow increases the vulnerability to *C. pneumoniae* infection. *In vitro* studies of growth in macrophages of different genotypes provide further support: *C. pneumoniae* grow most prolifically in cells that are homozygous for epsilon 4, moderately well in epsilon 4 heterozygotes and much less well in macrophages of other epsilon genotypes (A.P. Hudson, unpublished data). These findings provide a theoretical basis for integrating the entire spectrum of epsilon 4-associated diseases. In contrast to the inadequacy of the "thrifty genotype" hypothesis as an explanation of the full array of epsilon 4 associated disease, the hypothesis that epsilon increases vulnerability to *C. pneumoniae* readily explains the entire spectrum of available evidence, because a given pathogen can cause very different pathologies in different tissues. The different epsilon-4-associated diseases may therefore result from the different effects of the increased vulnerability to epsilon 4 infection in different tissues. By this argument, *C. pneumoniae* in the brain causes damage that manifests itself as Alzheimer's disease (*Balin* et al., 1998). If brain damage occurs through an autoimmune mechanism triggered by *C. pneumoniae* (*Lenz* et al., 2001), it may be manifested as multiple sclerosis. Invasion of the endothelial lining of arteries and subsequent accumulation and oxidation of lipid is manifested as atherosclerosis (*Byrne* and *Kalayoglu*, 1999; *Kalayoglu* et al., 1999)

The association between epsilon-4-diseases and *C. pneumoniae* offers a specific hypothesis to explain the pattern of epsilon 4 allele frequency in human populations. Because *C. pneumoniae* is spread as a respiratory tract pathogen by coughing, the exposure should increase with increasing population density.

Those peoples who have been living in high densities and close quarters for more millennia should have had the greatest exposure to high frequencies and dosages of infection and thus should have experienced the strongest selective pressure against epsilon 4 thus explaining why Inuits have high epsilon 4 frequencies like other populations who until recently have been hunter-gatherers, and why people with a long ancestry in the Mediteranean and China have low frequencies of epsilon 4 (*Corbo* and *Scacchi*, 2000).

This line of logic also helps explain seemingly discordant findings about suites of related diseases. Different forms of Alzheimer's disease, for example, are associated with different risk factors. Early-onset Alzheimer's disease (also known as "familial Alzheimer's disease") is a genetic disease in the traditional sense. Presently mutant alleles of three different genes code are responsible for three variants of early-onset Alzheimer's disease: the beta amyloid precursor protein gene on chromosome 21, the presenilin-1 gene on chromosome 14, and the presenilin-2 gene on chromosome 1 (*Clark* et al., 1996; *Pastor* et al., 2003). The epsilon 4 allele exacerbates the variants of early onset Alzheimer's Disease that are attributable to mutations in the amyloid precursor protein. The evidence from individuals with presenilin mutations is mixed with one large study indicating exacerbation whereas smaller studies have documented no significant effect (*Haan* et al., 1994; *van Broeckhoven* et al., 1994; *Sorbi* et al., 1995; *Lendon* et al., 1997; *Romero* et al., 1999; *Pastor* et al., 2003). In the large study (*Pastor* et al., 2003), rural residence was associated with later onset of Alzheimer's disease. This result is consistent with an influence of infection on development of early-onset Alzheimer's disease, because the incidence of infections with respiratory pathogens such as *C. pneumoniae* are typically greater in urban environments where populations are more congested and tend to spend more time indoors. The clarification of this different causal mechanism is thus suggesting that Alzheimer's disease is actually a spectrum of related but distinct diseases. Late-onset Alzheimer's disease, which is much more common the early-onset Alzheimer's disease, appears to be one or more infectious diseases for which *C. pneumoniae* is a primary cause and epsilon 4 an exacerbating cause (*Balin* et al., 1998). Early-onset Alzheimer's disease is thus a collection of genetic diseases for which epsilon 4 is sometimes an exacerbating cause, particularly when they co-occur in the same individuals. Although *C. pneumoniae* has never been studies as an exacerbating cause of early onset Alzheimer's disease, the association between epsilon 4 and *C. pneumoniae* raises this possibility as a hypothesis for future study.

The emphasis on infectious causation and the genetic variability in susceptibility to infection may also help clarify some confusion over infectious causation of other members of the suite of epsilon 4-associated diseases. Epidemiological patterns, fitness costs, and the low monozygotic twin concordance associated with multiple sclerosis, for example, implicate infectious causation (*Gilden*, 1999). Although the initial report (*Sriram* et al., 1998) of an association between *C. pneumoniae* and multiple sclerosis was roundly dismissed by experts, a recent independent confirmation of this finding lends much strength to the hypothesis (*Munger* et al., 2003). This association is bolstered by the finding that a *C. pneumoniae* specific peptide cross reacts serologically with a portion of myelin basic protein (an antigen that stimulates the autoimmune response of multiple sclerosis) and causes an MS-like disease in rats (*Lenz* et al., 2001). *C. pneumoniae* is most strongly associated with severely progressive

multiple sclerosis (*Munger* et al., 2003), as is epsilon 4 (*Evangelou* et al., 1999; *Hogh*, 2000; *Fazekas* et al., 2001; *Enzinger* et al., 2004); this parallel accords with the evidence that epsilon 4 increases vulnerability to *Chlamydia pneumoniae*. Indeed this association between severe multiple sclerosis and *C. pneumoniae* was predicted on the basis of the association between severe multiple sclerosis and the epsilon 4 allele (*Ewald* and *Cochran*, 2000).

These arguments offer a broad causal perspective on epsilon 4-associated chronic diseases. Rather than viewing epsilon 4 as a deleterious allele that damages cardiovascular and neuronal tissue by disregulating the transport and reactivity of lipids and cholesterol, this new perspective considers the epsilon 4 allele to be an Achilles heel that makes the person vulnerable to *C. pneumoniae* infection. This argument thus casts *C. pneumoniae* infection, but not epsilon 4, as a primary cause of the epsilon-4-associated diseases.

Unlike the thrifty allele hypothesis, this genetic-vulnerability-to-infection hypothesis is consistent with the available information on distribution of apolipoprotein E alleles in different populations. Whereas the thrifty allele hypothesis links the onset of a disadvantage associated with the epsilon 4 allele with the onset of rich agricultural food supplies, the genetic-vulnerability-to-infection hypothesis links the disadvantage with events that that favour pathogen transmission and could have occurred long before the onset of agriculture. This disadvantage of epsilon 4 could have arisen before the onset of agriculture if human populations were increasing, people were living increasingly in interior dwellings, or lifespan was increasing.

The epidemiology of *C. pneumoniae* fits this scenario especially well. It is a respiratory tract pathogen that appears to be present in all human populations; it therefore can persist across a broad range of population densities, technologies, and social structures. The persistence in small populations probably is attributable in part to its ability to cause persistent infections in humans. The intensity of exposure to *C. pneumoniae*, however, surely must have been less in hunter-gatherer societies where *Chlamydia* coughed into the outside air tend to be quickly diluted and destroyed by solar radiation. Conversely as population density increases it is probably transmitted to individuals more frequently or in higher doses. As is generally the case for pathogens it would undoubtedly persist more continuously in larger human populations because the chance of local extinctions would decrease. These transitions should tend to increase the negative effects of *C. pneumoniae* per vulnerable individual in the population and hence the relative fitness disadvantage associated with epsilon 4. Any increase in human lifespan would also tend to increase the negative fitness effects of *C. pneumoniae* on vulnerable people, because most of the life-threatening disease for which *C. pneumoniae* is a suspect tend to occur in the later decades of life, typically after the fourth decade. With longer lifespans, negative events that would occur in these later decades of life would have a greater negative impact on the fitness of diseased individuals. In accordance with this hypothesis, recent evidence from tooth wear suggests that around 30,000 years ago--about 20,000 years before the onset of agriculture--human population size and longevity increased substantially, with a five-fold increase in the proportion of people living past age 30 (*Caspari* and *Lee*, 2004). Perhaps this change contributed to the disfavouring the epsilon 4 allele by increasing the negative effects of *C. pneumoniae* or other epsilon 4-associated pathogens. During the agricultural period this disfavouring of epsilon 4 may have increased

further as a result of further increases population size and interior dwelling, thus explaining the differences in allele frequencies between modern hunter-gatherers and populations with a long agricultural tradition.

INTERPLAY OF ENVIRONMENTAL RISK FACTORS AND INFECTION

The preceding overview illustrates the importance of considering genetic associations with disease in the context of infectious causes. Non-infectious environmental risk factors may similarly influence the expression of diseases in ways that only make sense if underlying infectious causes are recognised. Atherosclerosis provides a particularly important example of this problem, because atherosclerosis is so damaging and because so much is known about risk factors associated with atherosclerosis. As discussed above, the association between epsilon 4 and *C. pneumoniae* resolves some of the questions raised by the geographic patterns of the epsilon alleles. Integrating hypotheses of infectious causation similarly resolves questions that are raised by analyzing each of the major non-infectious environmental risk factors for atherosclerosis. As is the case with epsilon 4 allele, non-infectious environmental risk factors for atherosclerosis may be not only consistent with infectious aetiologies but difficult to explain without invoking infectious aetiologies (see also: *Saikku*, 1995; *Leinonen* and *Saikku*, 1999).

Smoking

Tobacco smoking is a risk factor for atherosclerosis (*Berenson* et al., 1998; *Zieske* et al., 1999). But is smoking is a primary cause of cardiovascular disease or an exacerbating influence? It is reasonable to propose that harmful components of tobacco smoke directly damage the linings of arteries causing them to accumulate fat and cholesterol. But smoking also contributes to pulmonary infection; it is, for example, associated with *C. pneumoniae* infection which infect macrophages in the lungs that subsequently spread systemically (*Saikku*, 1995; *von Herzen*, 1998; *Mizooka* et al., 2003). Either hypothesis--direct damage from smoke or indirect damage through exacerbation of respiratory tract infections--could explain the evidence from studies of smokers.

The direct-damage-from-smoke hypothesis, however, seems problematic as an explanation for the increased risk for cardiovascular disease associated with exposure of non-smokers to smoke from smokers. This exposure, termed "passive smoking," has been implicated as a risk factor for atherosclerosis (*He* et al., 1999) and atherosclerosis-associated diseases such as stroke (*Bonita* et al., 1999). The increased risk associated passive smoking is about one-third of the increased risk associated with smoking (*He* et al., 1999) even though passive smokers inhale only about 1% of the amount of smoke that is inhaled people who smoke 20 cigarettes per day (*Pechacek* and *Babb*, 2004). The risk associated with passive smoking seems way out of proportion to the small amount of smoke inhaled by passive smokers relative to smokers (*Bailar*, 1999). Lab tests have documented negative effects of small amounts of smoke on the functioning of platelets, vascular endothelium, myocardial exercise tolerance, antioxidants and lipid metabolism (*Valkonen* and *Kuusi*, 1998; *Howard* and *Thun*, 1999). These findings lend some credence to the hypothesis that second-hand smoke could contribute directly to atherosclerosis, but these effects do not negate the difficulties inherent in interpretations that presume that the negative effect per unit of smoke is

much greater at very low doses than at high doses. If the effect of smoke were largely from generation of interactive intermediates one would expect a more liner relationship as has been found between tobacco smoke and lung cancer (*Pechacek* and *Babb*, 2004). An exponentially increasing dose-response curve would also be reasonable if the defences that are effective at low exposures to smoke become overwhelmed at high exposures. From an evolutionary perspective the hypothesised effects of extremely small amounts of inhaled smoke on life-threatening disease seem especially unlikely for humans, who have spent most of their evolutionary history in smoky environments (*Ewald* and *Cochran*, 2000). Although seemingly illogical, it is possible that cigarette smoke contains some particular compounds for which humans have not evolved the ability to detoxify at low levels and for which increasing doses are progressively much less damaging, but the disproportionately large effects of second hand smoke relative to the smoke inhaled by smokers remains a paradox if the effects on passive smokers are direct effects of smoke.

But this paradox is resolved if the relevant effects of smoke are indirect, occurring through exacerbation of infectious causes of atherosclerosis. The risk to passive smokers may thus arise because of exposure to the more florid or more frequent infections of smokers rather than to the second-hand smoke itself (*Ewald* and *Cochran*, 2000). This hypotheses seems especially feasible because it assumes only that the infection-proneness of smokers would increase the transmission of *C. pneumoniae* or some other pathogen sufficiently to elevate the risk among those exposed to second hand smoke by one-third. Smoking suppresses immune function and is associated with elevated rates and intensities of a variety of infectious diseases as well as diseases suspected of being caused by infection (*Sopori*, 2002). Associations between exposures to second-hand smoke and increased frequencies of respiratory tract infections have been documented (*Takala* and *Clements*, 1992; *Vadheim* et al., 1992; *Arnold* et al., 1993; *Sorpori*, 2002). A similar argument applies to *Porphyromonas gingivalis*, which is a cause gingivitis and periodontal disease and is present in atherosclerotic lesions (*Stoltenberg* et al., 1993; *Haraszthy* et al., 2000; *Eggert* et al., 2001; *Kuroe* et al., 2004). Smoking is strongly associated with periodontal disease, which in turn is strongly associated with stroke and myocardial infarction. Evaluations of associations between smoking and *P. gingivalis* have given mixed results, though overall it appears that the prevalence of *P. gingivalis* at different sites in the mouth is greater among smokers than non-smokers, even though the presence or absence of *P. gingivalis* differs little if at all between smokers and non-smokers (*Haffajee* and *Socransky*, 2001). Non-smoking partners of smokers are also more likely to have exacerbated periodontal disease (R.J. Genco, unpublished data), presumably because *P. gingivalis* and other causal organisms are transmitted by kissing or other salivary contact to the non-smoking contacts of smokers. A recent study of the relationship between smoking, infection, and the development of atherosclerosis showed that the atherosclerosis was significantly associated with smoking only when an indicator of chronic infection (particularly chronic obstructive pulmonary disease, chronic bronchitis, or periodontitis) was present (*Kiechel* et al., 2002). This finding accords with the idea that it is the second hand pathogens are the culprit rather than the second hand smoke, because these illnesses are associated with pathogens that are candidate causes of atherosclerosis: the pulmonary diseases are associated with *C. pneumoniae* and periodontitis is as-

sociated with oral bacteria, particularly *P. gingivalis, Actinobacillus actinomycetemcomitans,* and *Bacillus forsythus.*

Lipids

Lipid accumulation in atherosclerotic lesions has long been recognised as a hallmark of atherosclerosis. This association has led to the conclusion that high fat diets contribute directly to atherosclerosis. Although this hypothesis is intuitive--too much input of fat leads to too much accumulation of fat in the arteries--it has been readily accepted over the past 30 years with scanty evidence (*Taubes*, 2001) and almost no consideration of alternative hypothesis that could explain the development of atherosclerotic lesions in the absence of high fat diets. Nor has the evidence allowed the parsing of the lipid hypothesis to distinguish exacerbating effects of lipids from initiating effects. Initiating effects have been hypothesised to involve reactive lipid compounds that may damage to the endothelium, but this idea raises other concerns about causality: If reactive lipids are important initiators of damage, why is there so much variability from person to person in the atherosclerosis that is uncorrelated with lipid intake? And if this variation is due to variation in the degree of disregulation and reactivity of lipids, why is vulnerability so variable from person to person?

An evolutionary perspective places considerable weight on these questions because people who are genetically vulnerable to such disregulation and damage should have had this vulnerability weeded out by natural selection. The resolution is that some environmental factor must be generating the disregulation. *C. pneumoniae* is a candidate because it induces cellular oxidation of low-density lipoproteins and lipid accumulation in macrophages, which are thus transformed into foam cells (*Kalayoglu* et al., 1999; *Muller* et al., 2003). This finding is important because the presence of foam cells is a hallmark of the early stages of atherosclerosis. Because *C. pneumoniae* is lipophilic, hyperlipidaemia and obesity may contribute to atherosclerosis as an exacerbating response to *C. pneumoniae* as a primary cause. This hypothesis also applies to *P. gingivalis*, which can similarly stimulate lipid aggregation in macrophages and their transformation into foam cells (*Miyakawa* et al., 2004).

Alcohol

Another problem with the lipid hypothesis is that high fat diets are not associated with high rates of cardiovascular disease in some geographic areas. In France, for example, diets are high in fat but cardiovascular disease is only approximately one-third the rate in other western countries with comparable fat intake (*Gorinstein* and *Thrakhtenberg*, 2003). This anomaly in the association between high fat diets and atherosclerosis is attributed to relatively high intake of wine in France. Although it was originally thought that wine might uniquely suppress development of atherosclerosis, it is now clear that alcoholic beverages generally provides protection. The emerging view is that alcohol and some phenolic compounds found in wine and beer may have beneficial effects; the evidence mustered in defence of these mechanisms, however, is mostly indirect, being documentations of effects on lipid characteristics that are presumed to be causes of atherosclerotic damage. Lipids researchers are generally presuming that any direct effects of alcohol are on lipid metabolism (*Gorinstein* and *Thrakhtenberg*, 2003) even though infectious agents, such as *C. pneumoniae* are known to alter lipid sequestration and metabolism. Because alcohol has antimicrobial effects, research into the effects of alcohol on atherosclerosis must consider indirect effects of alcohol on atherosclerosis via effects on candi-

date microorganisms. It is important to assess for example whether alcohol levels that occur in the blood could inhibit the growth of *C. pneumoniae* in macrophages and the transformation of *C. pneumoniae*-infected macrophages into foam cells.

Garlic

Garlic is another dietary component that has attracted interest because of its apparent beneficial effects on cardiovascular disease and other chronic ailments. Evidence of beneficial effects has led to studies that have attempted to determine the particular biochemical and physiological influences of the components in garlic. Effects on serum lipids, blood pressure and platelet aggregation have been studied (*Rahman*, 2001; *Brace*, 2002). Though some studies have reported beneficial effects on these manifestations of disease, results from different investigators have been contradictory (*Brace*, 2002). Most randomised, placebo-controlled studies, for example, have not support the proposed suppressive effect of garlic on serum lipids (*Brace*, 2002). Evidence for a suppressive effect on blood pressure and platelet aggregation are also inconclusive (*Brace*, 2002). Like alcohol, garlic is known to have powerful antibacterial effects (*Billing* and *Sherman*, 1998; *Ankri* and *Mirilman*, 1999; *Harris* et al., 2001; *Lee* et al., 2003). Assessments of the value of garlic in protecting against atherosclerosis depend on the extent to which a mechanism of action can be demonstrated. The evidence referred to above suggests that the failure to demonstrate such a mechanism of action to date may result from an inappropriate focus. Research has investigated direct effects of garlic on correlates of atherosclerosis but not on infectious agents that may be the primary cause of atherosclerosis. Studies of effects of garlic on the growth and survival of the pathogens that have been implicated in atherosclerosis are needed. As is the case with studies of alcohol, such studies need to address whether even slight inhibition of pathogens *in vivo* could result in protection by allowing immune responses to better suppress the pathogens. Such studies also need to consider whether any physiological effects of garlic (e.g., on lipid levels) represent direct effects of garlic on human physiology or indirect effects that are brought about by suppressing the effects of lipid-altering pathogens.

Iron

High iron levels have been associated with atherosclerosis (*deValk* and *Marx*, 1999). As is the case with garlic, most of the research on this association has investigated whether iron directly influences some biochemical interaction with cells. One hypothesis proposes that iron ions oxidise fat, which in turn damages the arteries. Evolutionary considerations cast doubt on this sort of explanation. Humans have probably had substantial iron and fat in their diet throughout our evolutionary history, because humans hunted animals for food. Adaptations for protecting against damage from iron-induced reactivity of fats should be well in place. These mechanisms could not have evolved in response to iron supplements found in vitamin pills, but comparisons of dietary iron (e.g., from red meat) with iron supplements indicate that regulatory mechanisms control dietary iron less effectively. So it is dietary iron--the form that humans should have evolved to cope with--that is the bigger problem.

Iron may influence the progression of atherosclerosis indirectly by enhancing the growth of pathogens (*Sullivan* and *Weinberg*, 1999). Bacteria, like human cells, need iron. If iron levels are not too high within the body, our iron sequestering proteins can bind the free iron, keeping it from the pathogens. Bacteria also produce iron-sequestering

proteins to usurp it for their own use before host sequestration mechanisms make the iron unavailable. If iron levels rise through excess iron in the diet, the ability of the body to sequester iron may be compromised, allowing the bacteria to acquire it, reproduce and consequently cause more damage. This argument leads to the hypothesis: the association between increased iron intake and exacerbations of atherosclerosis results from an indirect enhancing effect of iron on the pathogens that cause atherosclerosis. This hypothesis is related to the lipid hypotheses mentioned above. Unless the full range of alternative hypothesis is considered an association between atherosclerosis and red meat diets might be accepted as evidence of lipid induced disease. A broader consideration emphasises not only the feasibility but also the parsimony of hypotheses that consider interaction of pathogens with lipids and iron as a mechanism by which red meats and lipids may exacerbate atherosclerosis.

Inflammation

Associations between atherosclerosis and indicators of inflammation, such as C-reactive protein (CRP) have led to an emphasis by some on the role of inflammation in the pathogenesis of atherosclerosis. Although this association clarifies the pathogenesis of atherosclerosis, one of the most important parts of a full causal explanation is the mechanism by which the inflammatory process is switched on. This important aspect is generally glossed over in descriptions of the role of inflammation in atherosclerosis, but it is central to an understanding of the primary causes of the disease. The initiating event is now often ascribed to collections of factors such as the oxidised lipoprotein-cholesterol complex, injury, and infection (*Willerson* and *Ridker*, 2004). Reference to "injury" does not resolve the problem of primary causation but rather raises the question, "What causes the injury?" Similarly, reference to oxidised lipoprotein-cholesterol complexes raises the question, "What causes the oxidation, and why is it so variable from person to person?" In contrast, reference to infection does point toward a hypothesis of primary causation, because infectious processes may cause injury and production of reactive intermediates that contribute to oxidation of compounds such as lipoprotein-cholesterol complexes. Accordingly, CRP is elevated in persistent *C. pneumoniae* and *P. gingivalis* infections (*Huittinen* et al., 2003; *Craig* et al. 2003; *Kuroe* et al., 2004).

One source of confusion pertains to the tightness of the correlation between a risk factor and measurements of disease. The tightness of such correlations is particularly important in identifying clinical markers of progression to damaging illness. But it is less relevant to discussions about primary causation, because exacerbating causes that are downstream in the process of causation may be more tightly correlated with resulting damage than the primary causes that set the entire process in motion. This generalisation is especially relevant to chronic diseases for which the process of causation may take place over years or decades. CRP, for example, is a stronger predictor of severe cardiovascular events than in *C. pneumoniae* infection. CRP may therefore be a better indicator of risk, even if the elevated CRP is initiated by *C. pneumoniae* infection, but the tight correlation between CRP and cardiovascular damage tells us little about the primary causes of atherosclerosis. Linking CRP with infection offers a more complete explanation. *C. pneumoniae* infection has been associated with elevated CRP in early and late phases of cardiovascular disease, and risk of coronary events is greater when *C. pneumoniae* positivity is associated with elevated CRP (*Huittinen* et al., 2003; *Tasaki* et al., 2004). These find-

ings are consistent with a primary causal role for *C. pneumoniae*. Elevated CRP may serve as an indicator for those *C. pneumoniae* infections that are more likely to cause damaging effects much as elevated histamine serves as an indicator for bee stings that are more likely to cause damaging effects. The fact that elevated histamines is more strongly associated with life-threatening anaphylactic shock than the presence of a bee-sting does not argue against bee stings being the primary cause of anaphylactic shock. In the same way, a stronger association between elevated CRP and atherosclerosis does not logically lead to the conclusion that CRP should be considered a cause of atherosclerosis to the exclusion of *C. pneumoniae* (or any other pathogen) as the primary cause.

This argument also bears on interpretation of protective effects of non-steroidal anti-inflammatory drugs such as aspirin. Use of these drugs has been associated with protection against several chronic illnesses including cardiovascular events and Alzheimer's (*Etminan* et al., 2003; *Nilsson* et al., 2003). Although some attribute the beneficial effect of aspirin on cardiovascular events to blood thinning and beneficial effects of aspirin on other chronic diseases to other mechanisms, it is more parsimonious to consider that aspirin alters some common pathological process. The inflammatory process is the obvious choice. If the anti-inflammatory effect is the important commonality, bringing infectious causation into the picture alters interpretations by implicating an alteration of the infectious process, namely the inflammatory response to it. Because chronic diseases are those that are not controlled by the immunological defence, it is reasonable to expect that the immunological defence will be particularly damaging in chronic infections as compared with acute infections because the inflammatory response evolved largely as a defence against infection. If it is successful the infection will tend to be short-lived and any disease it causes will be acute. Accordingly effects of aspirin are generally beneficial for chronic diseases but generally detrimental for acute infectious disease (*Ewald*, 1994).

IMPLICATIONS FOR THE FUTURE

If effects of all the non-infectious risk factors for coronary artery disease are combined, only about half of the overall risk can be explained (*Muhlestein*, 2002). This finding indicates that research needs to look beyond the current list of non-infectious risk factors. The association with epsilon 4 has led some researchers to believe that a search for more genetic determinants is warranted. Current evidence indicates, however, that epsilon 4 creates a genetic vulnerability to infectious causes of atherosclerosis and the other epsilon-4-associated diseases rather than directly causing the damage that characterises these diseases. Evolutionary considerations of fitness load similarly indicate that genetic causes of these diseases--particularly atherosclerosis and multiple sclerosis--will be of minor importance relative to other causes. Detailed consideration of non-infectious environmental risk factors for atherosclerosis illustrates how these risk factors are better explained in the context of infection. This integrative perspective offers several lines of inquiry that can improve our understanding of the primary and exacerbating causes of disease through the testing of hypotheses that specify environmental and genetic influences on infectious processes.

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18th OLD HERBORN UNIVERSITY SEMINAR: SUMMARY AND OVERVIEW OF THE DISCUSSIONS

GLEN D. MITHOE AND WILLEM L. MANSON

Department of Medical Microbiology, University Medical Centre Groningen, Groningen, The Netherlands

FROM FRIENDS TO FOES: SPECIFIC EXAMPLES

Helicobacter pylori is the first named species of the Helicobacter/Wolinella family. This genus consists of more than 20 species and about 10 candidate species. With a few exceptions all these organisms are micro-aerophilic "mucinophiles". *Helicobacter pylori* is the prototype for a number of bile-sensitive organisms able to colonise the stomach of most animals, including dolphins and whales. The link between gastritis and peptic ulcer disease and the presence of this spiral shaped organism on the surface of gastric mucosa was discovered in 1982 by Warren & Marshall. *Helicobacter pylori* is a common widespread organism with a worldwide spread of 30-90%. It has a higher prevalence in developing countries with an increase up to 60 years of age. *Helicobacter pylori* is found almost entirely in humans, and oral-oral transmission between humans in crowded institutions, parents and children seems to be common.

Although *Helicobacter pylori* is strongly associated with the development of pathological conditions of the stomach, most of the infected patients never develop *Helicobacter* related diseases. This indicates that some of the bacterial strains might be more pathogenic than others or that certain persons are more susceptible to develop *Helicobacter pylori* related disease. *Helicobacter pylori* has several ways to persist in colonising the mucus of human stomach. One of these mechanisms to facilitate persistence is to increase diversity. Within a large population a small proportion of cells arise that have heightened mutation rates. Most strains of *Helicobacter pylori* are considered to be such a hypermutator phenotype; this favours the emergence of selective variants. A good example is the point mutation that leads to high-level resistance to commonly used antibiotics such as the macrolide clarithromycin.

The ability to cause life-long infections depends on the low toxicity/biological activity of the lipopolysaccharide and several other unique factors like host protein binding and protein shedding. Other factors that are supposed to play a role in evading the host defences are the location in the gastric lumen, beyond the reach of most host immune recognition and effector mechanisms. TLR stimulation triggers pro-inflammatory signalling through NF-κB activation, and *Helicobacter pylori* has evolved to minimise such stimulation, the TLR5 is not stimulated by *Helicobacter pylori* flagella. The highly methylated DNA of *Helicobacter pylori* is barely recognised by the TLR9, which recognises largely unmethylated DNA of most bacteria. *Helicobacter pylori* LPS is anergic compared with that of other Gram-negative enteric bacteria. This is caused by lipid A modifications, nevertheless it stimulates macrophage TLR4, but it does not stimulate gastric epithelial TLR4. Although the cloaking abilities of this CagA positive strains do stimulate NF-κB activation in epithelial cells, apparently through recognition by NOD, an intracellular pathogen-recognition

molecule that detects soluble components of bacterial peptidoglycans, the resultant NF-κB induced pro-inflammatory cytokine expression is an important and continuing stimulus to inflammatory cell infiltration and thus to pathogenesis. Another way to evade the host response is by mimicry of the gastric epithelial fucosylated antigens, and by antigenic variation of surface proteins including a critical pilus molecule, CagY. Other mechanisms that are proposed in the improvement of *Helicobacter pylori* survival in the stomach are superoxide dismutase, catalase and phospholipases. It is unknown if extracellular and immunoglobulin proteases play a role in the survival of *Helicobacter pylori*. The pathogenesis of *Helicobacter pylori* has been linked to the expression of two proteins, the CagA (cytotoxin associated protein A) and the VacA (vacuolating cytotoxin). No homologues for the CagA gene have been found suggesting that it reflects a human gastric specific gene of Helicobacter pylori. The CagA protein contains tyrosine phosphorylation sites that are recognised by the host cell Src kinase. Once phosphorylated, CagA interacts with the SHP-2, a tyrosine phosphatase, which affects spreading, migration, and adhesion of epithelial cells. The helicobacter CagA protein interacts with several major signal-transduction pathways present in epithelial cells. The Cag apparatus promotes anti-apoptotic pathways, which may aid persistence by slowing turnover of the epithelial cells to which they are attached.

VacA is a high molecular weight multimeric pore-forming protein that causes massive vacuolation in epithelial cell lines, which lead to egress of anions and urea. This is important for *Helicobacter pylori* since urea hydrolysis catalysed by *Helicobacter pylori* urease protects against gastric acidity. VacA also induces loosening of epithelial tight junctions, potentially allowing nutrients to cross the mucosal barrier, which can favour the organism in the gastric lumen. VacA also blocks phagosome maturation in macrophages, selectively inhibits antigen presentation in T-cells, blocks T-cell proliferation and downregulates Th1 effects by interacting with calcineurin to block signalling. Peptic ulcer disease is considered to be associated with CagA+ strains; there is also strong evidence that CagA+ strains are associated with the development of atrophic gastritis and gastric cancer.

Another important factor in the severity of pathogenesis is the heterogeneity in immune response among human populations. This leads to the presence of cytokine polymorphism.

Polymorphisms that increase the IL-1β response to *Helicobacter pylori* are associated with an increased risk of developing gastric atrophy, hypochlorhydria and adenocarcinoma. Polymorphisms in TNF-α and IL-10 genes have a similar, butt less pronounced association.

Thus *Helicobacter pylori* has several ways of adaptation in order to facilitate its persistence on the human gastric mucus layer. It does this so by mutation, evasion of the immune response, the presence of proteins that can interact with several major signal-transduction pathways and which have a direct influence of the epithelial tissue. But this host-pathogen relation seems to be pathogenic for the host, as indicated by the extensively studied relation between the presence of certain *Helicobacter pylori* strains in the stomach and gastric ulcer disease and gastric carcinoma.

Another example of chronic disease related to infection, given by dr. Ljung, is the growing evidence that an infectious agent plays a role in atherosclerosis. In 1921 the hypothesis that infections could lead to atherosclerosis was proposed by Ophus. This hypothesis was based on pathologic specimens of blood vessel, which showed macrophage

infiltrates and foam cells. But this theory was overshadowed by the common conception that atherosclerosis was caused by factors such as smoking, hypercholesterolaemia, diabetes and hypertension. It was in the late 70's that the hypothesis of infection related pathogenesis of atherosclerosis re-emerged. The progress in diagnostic techniques contributed to the acceptance of the concept that microorganisms could be involved in the multi-factorial process of atherogenesis. In this regard, sero-epidemiological evidence of varying designs as well as experimental evidence has focused primarily on three pathogens: CMV, *Helicobacter pylori* and *Chlamydophila pneumoniae*. Anti-*Chlamydophila pneumoniae* antibodies are unusual in children younger than 5 years and can be found in about 50% of individuals by the age of 20. The prevalence of antibodies continues to increase with age, reaching a peak seropositivity of 80% in men and 70% in women by age of 65 years. There are several pathogenic mechanisms by which microbial infection could directly or indirectly induce atherogenesis, thrombosis and plaque rupture. Chlamydia LPS has been shown to enhance LDL uptake en to down-regulate cholesterol efflux in monocytes or macrophages. LPS can induce not only LDL oxidation but also transforms human mononuclear phagocytes into foam cells, which is a key atherogenic event. Chlamydial LPS can directly alter atheroma cell function by inducing the production of TNF-α, IL-1β and IL-6 which lead to the to the propagation of the inflammatory response with the atherosclerotic tissue.

Another important substance in atherogenesis by *Chlamydophila pneumoniae* is HSP60. This 60 kDa protein is considered to have a low biological activity based on structure-to-function studies. The HSPs form a group of highly preserved proteins among species. This can lead to significant cross-reactivity and immunopathology from anti-body response to HSP60. Human and Chlamydial HSP60 activate human peripheral blood mononuclear cells and monocyte-derived macrophages by a CD14-dependent mechanism. Signalling through this pathway resembles LPS mediated cell activation and LPS and HSP60 may share signal transduction machinery to activate a cell. Thus HSP60 may contribute to atherogenesis by antigenic stimulation and subsequent cross-reactivity to self-proteins, as well as direct modulation of atheroma cell function. HSP60 also induces inflammatory cytokines such as TNF-α by endothelial cells, macrophages and smooth muscle cells. LPS and HSP60 both induce the formation of metalloproteinases which can destabilise the atheromatous plaque. All these events contribute to the development of cardiovascular disease.

Further evidence of the causal relationship of bacterial infections and chronic disease states can be found in inflammatory bowel disease. It is evident that there is a life-long counteraction between the enteric bacterial flora and the host immune system. This relationship starts immediately after birth. Nevertheless this overwhelming chronic immune stimulation does not lead to illness in healthy people; in fact it is believed that this leads to a better development of the intestinal immune system. Inflammatory bowel disease results in the chronic inflammation of the intestine. The current working hypothesis is that inflammatory bowel disease is due to a dysregulated mucosal immune (CD4+ T-cell) response to enteric bacterial antigens, in a genetically susceptible host. This postulates that bacterial flora drives the disease, and since the lower intestine harbours the largest concentration and diversity of resident microbial antigens in the body this should be an overwhelming chronic stimulation. So the question arises how the commensal

bacteria of the gut are involved in the pathogenesis of Crohn's disease. It is suggested that inhibitory cytokines produced by mucosal cells after antigen specific recognition may play a role in maintaining hyporesponsiveness to gut bacterial antigens and may be responsible for the lamina propria phenotype of activated yet hyporesponsive cell. In fact, data from several animal models suggest that there are 2 functionally different populations of CD4+ cells. One is capable of inhibiting disease and the other is able to mediate disease. Dr. Elson and his team showed in an animal model with C3H/HeJBir mouse that the inflammation in the intestine is due to unrestrained effector T-cell responses to the enteric bacteria, and that a small subset of antigens out of the thousands of bacterial proteins is responsible for an inflammatory response (CD4+). The possible antigen proposed by dr. Elson is the Flax-flagellin. This structure was found after sequence homologies of Cbir proteins cloned from caecal bacteria. These flagella show a significant positive colitis score in animal models that have Cbir1-reactive CD4+ T-cells. They also showed that the colitis was driven by bacterial specific T-cells and that effector T-cells reactive to commensal bacterial antigens are pathogenic unless their activity is properly regulated. Dr. Elson proposed 2 subsets of regulatory cells. The first one is the TR1 cell which inhibit both IL-12 and IFN-γ production in cultures of bacterial-reactive TH1 cells in animal models. Another subset of regulatory cells termed T helper 3 which are characterised by their high TGF-ß production but lower levels of IL-4 and IL-10. Mice deficient in TGF-ß develop spontaneous inflammation. So these regulatory mechanisms form a working frame for the possible role of bacteria in inflammatory bowel disease.

Even in the field of psychiatry relations between bacteria and chronic disease can be found. The role of bacteria in chronic psychiatric disease was presented by dr. S. Rosseneu. She discussed the possible relationship of abnormal aerobic gut flora and the presence of autism in children. Autism is a life-long developmental disorder which affects 1 in 500 children. The diagnosis is conferred after extensive evaluation according to the DSM IV criteria. Usually the child is 2 to 5 years old. The symptoms appear after some time of normal development. They gradually suffer a loss of newly acquired skills (language, eye to eye contact, and sociability). The cause for this disease is yet unknown but it is well accepted that there are multiple causes for this disorder. Studies with monozygotic twins show a 60% concordance in suggesting that there is a genetic basis for autism. The need to understand the cause(s) of autism and the underlying pathogenesis has become more acute since the number of diagnosed cases has risen markedly in recent years. There is growing evidence that there is a relationship between intestinal pathology and autism. In several studies with children suffering from autism and gastrointestinal symptoms showed significant more ileal and colonic lymphoid nodular hyperplasia. An active acute inflammation and chronic inflammation could also be seen in respectively 8% and 88% of the patients. Another study showed altered function of the upper gastrointestinal tract in children with autism. The results of these different studies suggest a widespread gastrointestinal pathology in patients with autism. Earlier studies have shown that children with autism and celiac disease have worsened symptoms after gluten consumption. Another piece of evidence is provided by the measurement of TDC (transcephalic direct current) in children with autism and celiac disease. After taking gliadin the TDC showed a significant inhibition of frontal voltage. In two separate studies children

with autism showed an improvement in social skill, cognitive function and communication after eradication of gluten and cow's milk in their diet. Recent research has also shown that there is a difference in microflora between normal children and children with late-onset autism. Rosseneu and her team showed that there is a significant difference in the amount of aerobic Gram-negative bacilli between autistic children and normal children. Overgrowth was determined as $\geq 10^5$ CFU AGNB per ml of saliva and/or g of faeces. Rosseneu showed that in a population of children with autistic spectrum disorder with 95% regressive or late-onset autism, 95% of GI symptoms and 72% with gluten, dairy-free diet have an abnormal amount of AGNB. About 61% of the subjects have abnormal AGNB in overgrowth, 95% have *E. coli* overgrowth, and 55% have *S. aureus* in overgrowth and *Candida spp.* showed no difference. The consequences of AGNB overgrowth are AGNB translocation and/or endotoxin absorption; liver macrophages (Kupffer cells) detoxify endotoxin and release cytokines which leads to inflammation of the intestine and eventually to systemic effects and influence distant organs. She also found differences in glycocalyx expression in patients with ulcerative colitis and patients with autism but not Crohn's disease, decreased colonic sialylation and α-1,2 fucosylation in UC colon, decreased α-1,4 fucosylation in both ileum and colon in autism. The question remains whether this is the cause or result of this abnormality. Rosseneu attempted to answer this question by trying to reduce the abnormal carrier state to a normal carrier state. This was realised by administering polymyxin E and tobramycin for three months to patients with autism and GI symptoms. Improvement was measured by using the global behavioural scale. This therapy led to decrease of abdominal pain score, decrease of laxative intake, improvement in overall behaviour/cognition and overgrowth concentrations were significantly reduced. Another important fact is that the improvements were transient, after stopping the antibiotic treatment the bacterial overgrowth rose to the pre-treatment levels and symptoms gradually reappeared. This suggests that there is a relationship between the state of the enteric flora and the pathogenesis of autistic disease.

EVOLUTIONARY PERSPECTIVE

Over the past centuries, disease has been separated into three categories: Infectious disease, genetic disease and disease caused by too much or too little of some non-infectious environmental constituent. These three categories offer a conceptual framework for understanding diseases, but they pose a danger of canalising thinking. They have for example, contributed to the rejection of infectious causation when evidence in favour of non-infectious causes has been acquired but evidence against infectious cause is lacking. This tendency to dismiss infectious causation has occurred in spite of the recognition that for infectious diseases host genetic and non-infectious environmental influences are of importance.

In the 1970's and 1980's medical texts typically attributed peptic ulcer to gastric acidity, stress, smoking, alcohol consumption and genetic predisposition. Infectious causes were not mentioned, even though evidence of infectious causation had been accumulated from the 19th century: A spiral bacterium was associated with ulcers at the end of the 19th century, ulcers had been experimentally transmitted in laboratory animals during

the second decade of the 20th century, and peptic ulcers had been successfully treated with antibiotics in New York City hospitals during the late 1940s. In spite of the accumulated evidence, several attributes of ulcers made infectious causation cryptic: The loose correlation between infection and ulcers, the internal site of infection, and variable delays between onset of infection and the onset of overt disease. The net effect is a chain of transmission that is so cryptic that the transmission of *Helicobacter pylori* is still not totally resolved today. The same crypticity can be seen in the relationship between an infectious cause of atherosclerosis, which was proposed over a century ago. Despite the growing evidence of *Chlamydophila pneumoniae* as the leading suspect in the infectious cause of atherosclerosis it is only until recently that medical doctors are accepting the possible role of *Chlamydophila pneumoniae* in the formation of atherosclerosis besides the role of cholesterol, high fat diets, stress, smoking and genetic predisposition in the formation of atherosclerosis.

Because genetics can alter the course of infection, we expect to find that genetic determinants of disease may sometimes be best-explained genetic influences on infection. The epsilon 4 allele of the human apolipoprotein E gene has been identified as a genetic risk factor for atherosclerosis, stroke, and Alzheimer disease. It also appears to increase susceptibility to *Chlamydophila pneumoniae* infection. The genetic risk imposed by the epsilon-4 allele may therefore result from a genetic vulnerability to infection rather than a direct influence of epsilon-4 on disease progression. In other words the evolutionary maintenance of "bad" alleles is problematic and not desirable in an evolutionary sense. A resolution to this problem is to make the host more vulnerable to infectious causes in order to eliminate the persistence of the supposed "bad" allele in the evolutionary path.

The high allelic variability of genes that are involved in resistance pathogens (e.g. HLA variability) suggests that this situation may be common.

Examples as given above generate a growing sense that more chronic diseases will prove to be caused by pathogens that may be familiar causes of acute infection, identified but not yet associated with disease, or not yet identified. The key problem is how to facilitate recognition of infectious causation among these diseases. One step toward resolution of this problem involves increased awareness of the sources of crypticity that can likely be encountered in ascribing infectious causation. One source of crypticity is the increasing difficulty in obtaining suitable animal models. Few mammals live as long as humans, it is therefore difficult to find experimental animals that can be infected by an organism thought to cause long-delayed chronic disease and that is able to survive long enough to demonstrate the same chronic disease found in humans. Nevertheless the evolutionary relationship between chronic disease and bacterial infection provides a challenging concept and this provides an interesting working frame for further research.

TARGETS FOR MANIPULATION OF HOST DEFENCES AGAINST CHRONIC DISEASES

Mast cells

Until recently mast cells were considered as primarily harmful which was based on the facts that mast cells play a key role as effector cells of allergic and potential lethal anaphylactic reactions and that their contribution was limited to the elimination of parasites. However

there is growing evidence that the mast cells could be involved in other processes. Recently, mast cells have been shown to exert beneficial functions such as in tissue repair and in acquired and innate immune responses against foreign molecules and infectious agents. Mast cells fulfil all basic prerequisites to be classified as Antigen Presenting Cell. These basic functional prerequisites are phagocytosis, antigen uptake, expression of adhesion molecules, attraction and activation of lymphocytes, amplification via cytokine production and endothelial activation. To further clarify this dr. Maurer presented data from his own research with animal models using Kit+/+ and Kit w/Kitw-v mice, of which the latter is deficient in mast cells. He showed that mice deficient in mast cells which develop bacterial sepsis in a caecal ligation and puncture model of acute peritonitis have significantly larger chance of dying in an early stage of disease. In other words mast cells protect from mortality in septic peritonitis. In another example with the same type of mice he showed that skin lesions after infection with *Pseudomonas aeruginosa* are increased in the absence of mast cells and that mast cells control the skin lesion size. He also showed in this model that neutrophil recruitment in *Pseudomonas aeruginosa* infections in the skin is mast cell dependent. To show the role of mast cell in parasitic infections dr. Maurer conducted the same experiment with *Leishmanias major*. These results show similar effects of mast cells on the lesion size. The mice deficient in mast cells show larger skin lesions, exhibit markedly increased parasite numbers, lower IFN-γ levels, higher IL-4 levels after injection with *Leishmanias major*, impaired recruitment of dendritic cells, macrophages and CD8+ T-cells. These data show strong support that mast cells are key players in innate host responses. Skin mast cells protect from bacteria and parasites and bridge innate and adaptive immunity.

Infectious diseases may cause frank infections in the host, like pneumoniae and/or septicaemia, but infectious agents may also cause more chronic diseases due to the failure of the host to eliminate the pathogen or by a breakdown in normal truce. Infectious agents have been for a long time connected with arthritis. Microorganisms can be involved in the aetiology of arthritis in three different ways: There are septic forms of arthritis in which microorganisms are directly involved in the arthritis and reactive arthritis and post-infectious arthritis. Reactive arthritis mostly is triggered by specific microorganisms in for instance causing infections in the GI tract or genito-urinary tract, the joint symptoms appear 1 to 3 week after the infection. Moreover a role for microorganisms in the development of idiopathic arthritis and autoimmune arthritis (RA and AS) has been supposed.

Several mechanisms may play a role in the development of arthritis after mucosal infection. This may be the result of mimicry of bacterial substances with human antigens, as has been supposed for *Campylobacter*. But also the way the intestinal immune system reacts with intestinal microorganisms may play a role in the aetiology of autoimmune arthritis

Although there is a long history of the relationship of mucosal infections and rheumatism, real aetiological agents have not been found. The theory is that within a certain genetic condition commensal microorganisms may trigger an immune response leading to inflammatory joint disorders. So, arthritis maybe the result of an immunological response to microorganisms. On the other hand different syndromes in which joints may be involved may be caused by different mechanisms. So in AS and RA different structures and microorganisms eliciting different immunological responses may be involved.

Especially attention has been focused on the role of Gram-positive microorganisms in the aetiology. Gram-negatives although important in reactive arthritis do not seem to play a major role in autoimmune arthritis.

Special attention has been paid to the involvement of peptidoglycans in the immune response. Several mechanisms have been suggested; peptidoglycans may have cross reactivity with joint tissue and it also may activate an autoimmune response. In the past a role for EBV has been suggested. Although EBV may trigger B-lymphocytes and stimulate antibody synthesis, there is no evidence for a role for EBV in the pathogenesis of RA.

There are some studies that show those microbial products – peptidoglycans and bacterial DNA – to react with lymphocytes in the joint, which can be traced in joint fluid.

In conclusion, several microorganisms may be involved in several diseases and several mechanisms may trigger an inflammation of the joints.

Microbial immunomodulation against cancer

Different factors are involved in the aetiology of malignant diseases. Toxins and metabolites produced by microorganisms are thought to play a role in the aetiology of cancer. Examples of such microorganisms are *Helicobacter pylori* (gastric carcinoma), *hepatitis B virus* (hepatocellulair carcinoma), *HPV* (cervix carcinoma) and *EBV* (Burkitt lymphoma, B-cell lymphoma), and *Schistosomiasis haemotobium* (urinary bladder cancer). So, although microorganisms may play a role in the aetiology of malignant diseases, they may also play a therapeutic role in cancer treatment. Already at the end of the 19th century the therapeutic potential of microorganisms in cancer treatment has been supposed.

Microorganisms may have anti-tumour capacity by different mechanisms: Toxicity for the cancer cells by microbial toxins, immuno-activation and inflammation including fever induction. Although several microorganisms have been used in cancer therapy - *Corynebacterium parvum, Newcastle Disease Virus, Streptococcus pyogenes* - the best-documented results have been reached with BCG in bladder carcinoma and *Propionobacterium avidum* in colon carcinoma.

P. avidum possesses anti-tumour capacity by its immunomodulating properties. It proliferates and activates the RES-cells and thymocytes. It activates granulocytes, macrophages, lymphocytes and NK-cells. In mice it reduces significantly the number of metastases in experimental cancer models. In human experiments lyophilised *P. avidum* has been shown to enhance immunity and increase survival in patients with colorectal cancer

Beside their anti-tumour capacity, microorganisms may also play a complementary role in cancer treatment. Probiotics are not an alternative for standard treatment but may play a role in optimisation of the standard therapy.

Possible prophylactic/therapeutic approaches to controlling infection-induced chronic diseases

For new approaches to treat infections, there are different organisations involved, such as the pharmaceutical industries (of which there are only 5 major companies involved), the biotech industry which comprise about 3000 small companies whit very small breakthroughs, regulatory agencies, academics and professional societies. To achieve a major breakthrough in agents against infectious microorganisms it is important that the above-mentioned institutions collaborate. In the USA this is already the case, in Europe this collaboration is planned for the near future.

The ongoing struggle to battle infectious diseases requires continuous de-

velopment of antimicrobial agents. The development of newer vaccines against opportunistic pathogens is promising. Research in the field of immunomodulators provided many new compounds and clinical trials but so far this has not lead to any significant breakthrough. Another area which is of great interest nowadays is the field of probiotics. As stated before it is of great importance that the collaboration between the different industries, regulatory agencies and academic institutions is realised to develop a better understanding and the development of therapeutic or prophylactic measures against the possible effect of chronic infections.