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TITLE:
99th Dahlem Conference on Infection, Inflammation and Chronic Inflammatory Disorders: Darwinian medicine and the ‘hygiene’ or ‘old friends’ hypothesis

ABSTRACT:
The current synthesis of the ‘hygiene hypothesis’ suggests that the recent increase in chronic inflammatory disorders is at least partly attributable to immunodysregulation resulting from lack of exposure to microorganisms that have evolved an essential role in the establishment of the immune system. This document provides a background for discussion of the following propositions. 1. The essential role of these organisms is an example of ‘evolved dependence’. 2. The most relevant organisms are those that co-evolved with mammals, and already accompanied early hominids in the Paleolithic. 3. More recently evolved ‘childhood infections’ are not likely to have evolved this role, and recent epidemiology supports this contention. 4. This mechanism is interacting with other modern environmental changes that also lead to enhanced inflammatory responses [inappropriate diet, obesity, psychological stress, vitamin D deficiency, pollution (dioxins), etc.]. 5. The range of chronic inflammatory disorders that is affected is potentially larger than usually assumed [allergies, autoimmunity, inflammatory bowel disease, but also vascular disease, some cancers, depression/anxiety (when accompanied by raised inflammatory cytokines), and perhaps neurodegenerative disorders and type 2 diabetes].

Introduction:
The year 2009 is the 150th anniversary of the publication of On the Origin of Species (24 November 1859) and the 200th anniversary of Darwin's birth (12 February 1809). Darwin's insights increasingly underpin our attempts to understand human diseases, and the hygiene hypothesis is becoming a major component of ‘Darwinian medicine’. This therefore is a good moment to wonder at an astonishing paradox: Google searches for ‘Darwinian medicine’ or ‘evolutionary medicine’ on 18 March 2009 revealed 22 200 and 32 100 hits, respectively. However, these terms did not exist in the MeSH database, so PUBMED searches yielded only a handful of papers. This might reflect a real failure of the medical establishment to value evolutionary medicine, and there is certainly a failure to recognize the ‘hygiene hypothesis’ as one of its most obvious and important components.

Evolution turns the inevitable into a necessity:
The scientific establishment is slowly beginning to accept evidence for the involvement of specific organisms (for example, hepatitis A virus, gut microbiota and helminths) in the regulation of the immune system. However, if we are thinking in a Darwinian way, we should be starting from the hypothesis that any organism that has been consistently present for a significant part of mammalian evolution might have been ‘written into’ the mammalian genome, because ‘Evolution turns the inevitable into a necessity’; or, to put it more simply, anything that was always there must continue to be there (an extreme example is oxygen: when it started to appear on planet earth, some organisms adapted to its presence, and now cannot do without).
Bacteria were among the first life forms, and the most recent common ancestor of modern bacteria existed 2–3 billion years ago. The first vertebrates [~500 Ma (million years ago)] and mammals (~200 Ma) evolved in a world where there will already have been at least a million bacteria in every ml of water, tens of millions in every gram of soil, and no doubt similar numbers of all other groups of microorganism, both in the environment, on their skins and in their guts. Mammalian cells are, of course, derived from a series of endosymbioses.

Initial, evolved or exploitative dependence?:
Because all complex multi-cellular animals evolved from, and in the continuous presence of, microorganisms, there must be some organisms that perform functions for the immune system that were never encoded in the mammalian genome, because they did not need to be. This we can call ‘initial dependence’, because these organisms were present as the immune system evolved, and became essential components of that system. For example, background levels of microbial polysaccharides, lipopolysaccharides, phospholipids and peptidoglycans are likely to be normal signals for establishment of the immune system.

In other cases, there will be ‘evolved dependence’. This term often refers to situations where an organism has become adapted to the presence of a partner through loss of genetic material, and can no longer function without that partner [1]. A classical example was seen in the laboratory environment when a strain of Amoeba discoides became infected with a bacterium [2]. Initially this infection compromised the growth of both species, so it was not a case of mutualism. However, after 5 years neither organism could survive without the other. This indicates genetic changes leading to dependence. For instance, an enzyme that is encoded in the genome of both species might be dropped from the genome of one of them. Access to that gene is now ‘entrusted’ to the other species. This idea is at first somewhat alien to immunologists, but is in fact rather commonplace. For instance, most mammals can synthesize vitamin C, but large primates and guinea pigs have lost the relevant pathways. Man and guinea pig are now in a state of evolved dependence on fruit and vegetables; we had the genes in the past, but we do not any more. Perhaps the best example of evolved dependence is the mitochondrion. The mitochondrial genome is closest to that of the Rickettsiae, but has lost much genetic material.
Logically, there might also have been situations where a newly evolved (or newly encountered) microorganism offered a function or molecule that allowed the immune system to evolve a new capability, on which animals came to rely. One might call this ‘exploitative dependence’. This is not likely to be common, but we should try to identify examples.
These three types of dependence must be distinguished from situations where an infection kills a susceptible subpopulation, as has clearly happened in the past. This is obviously not dependence but in theory, if the same subpopulation were particularly susceptible to a chronic inflammatory disorder occurring later in life, this mechanism could lead, over the short term, to changing patterns of disease. However, the frequency of the genes leading to susceptibility to the infection will decrease rapidly. Moreover, the changes in mortality in recent decades have been small, whereas the increases in chronic inflammatory disorders have been so large that this mechanism cannot be important to the recent changes that have given rise to the hygiene hypothesis.
We now need to identify the organisms upon which the immune system is likely to be dependent.

Environment of evolutionary adaptedness (EEA):
The term EEA was first used in 1969 by John Bowlby, who was concerned that those aspects of human behaviour that are genetically determined (such as instincts) might be adapted to the hunter-gatherer existence rather than to modern city life [3]. Since the start of agriculture and pastoralism about 10 000 years ago, human adaptation to new environments has been cultural and technological rather than genetic. (Interestingly, human genetic diversity appears to be increasing more rapidly than ever before, but this is due to the population explosion rather than to adaptation to specific environments [4].) For example, we have not adapted genetically to living in cold places: we have learnt to make fur coats. Humans detect gene–environment misfit easily within the physical environment and invent appropriate technological adaptations. However, the immune system does not provide us with conscious awareness that it is receiving inadequate microbial stimuli, so we have been unaware of the problem and we have not sought solutions. Only since the hygiene hypothesis appeared have we been wondering if the immune systems of people living in clean modern cities are receiving appropriate inputs.
The human EEA is the hunter-gatherer environment of the Paleolithic (Fig. 1). Does this allow us to define the microbial inputs that our immune systems have evolved to ‘expect’? The hunter-gatherer lifestyle was in fact many different lifestyles, in many different environments, so this is a complex issue, and the EEA concept is often criticized for this reason [5,6]. Nevertheless one can identify types of organism that will inevitably have been abundant in all manifestations of the human EEA, but are diminished or absent from the modern city environment.

Ways in which microbial exposures can change ::: Epidemiological transitions:
These changes can involve elimination of the microorganism, or a change in the dose of the organism or its components, or a delayed infection. Elimination of most helminths, and of HAV, is well established [15]. A change in dose of microbial components can switch the lymphocyte types that are driven by their adjuvant effects. For instance, doses of lipopolysaccharide (LPS), dsRNA or Chlamydia pneumoniae determine whether T helper type 2 (Th2) is increased or decreased [16]. Delayed infection can be crucial because it can occur after levels of antibody acquired from the mother have waned. For instance, HAV is harmless in small babies, but often fatal in the over-50s [10]. In the present context the important point is that the timing of virus infections is relevant to whether they enhance or inhibit type 1 diabetes [17,18]. Similarly, the epidemiology of the recent

rapid increase in childhood acute lymphatic leukaemia (ALL) is similar to that of type 1 diabetes and delayed infection with an unidentified organism is also a suggested mechanism in ALL [19].

‘Old friends’, pseudocommensals and orofaecal transmission ::: The organisms implicated in the hygiene hypothesis:
Only a few illustrative examples are discussed here. The orofaecally transmitted organisms, implicated in recent epidemiological studies of the hygiene hypothesis, are particularly illuminating [15,22–25]. The genetic diversity in H. pylori decreases with geographic distance from East Africa, where modern humans evolved, and like humans, H. pylori seems to have spread from East Africa around 58 000 years ago [26]. Similarly, Salmonella is an ancient organism (~50 000 years), readily giving rise to carrier states [8,11]. Enteroviruses and HAV are picornaviruses that, like most of this group, probably co-speciated with Old World apes and humans [9]. HAV is stable and resistant to inactivation by environmental conditions [10], and its involvement in the hygiene hypothesis is explained in detail in this issue [15]. In developing countries the virus is ubiquitous, and babies are infected by the age of 3. Toxoplasma gondii is a protozoan parasite with a complex life cycle involving sexual replication in members of the cat family (Felidae) and asexual propagation in nearly all orders of placental mammals (except baleen whales and insectivorous bats), as well as marsupials and birds. However, cysts can transmit the infection to carnivores, omnivores and scavengers, without any need for the sexual phase in the Felidae. Thus transmission to man is likely to have been extremely common long before the Neolithic, although perhaps increased after the start of husbandry and the domestication of the cat [27]. Other protozoa not yet considered in this context, to my knowledge, include the very ancient Entamoeba, Giardia and Trichomonas, all of which have lost their mitochondria and have a close association with humans.

Commensal microbiota of gut ::: The organisms implicated in the hygiene hypothesis:
The immunological roles of gut microbiota and the presence of changes in the microbiota due to diet, hygiene and antibiotics are already accepted, and need not be discussed here [28], although the argument needs to be extended. A recent study indicated that manipulations of the immune system sometimes operate indirectly via changes to the microbiota. Non-obese diabetic (NOD) mice develop spontaneous autoimmune destruction of the pancreatic β cells. This did not occur in NOD mice, in which the gene-encoding myeloid differentiation primary response gene 88 (MyD88) had been inactivated, suggesting a direct role for MyD88 in T cell-mediated responses to β cells. However, this turned out to be an incorrect interpretation. Inactivation of MyD88 led to major changes in the gut microbiota, and this altered microbiota was responsible for the inhibition of the autoimmunity, which still occurred in MyD88–/– germ-free NOD mice [29]. If this is correct, much conventional gene knock-out immunology might need to be re-interpreted. How many of the changed phenotypes seen have been secondary to changes in the microbiota? At the very least, this experiment emphasizes the very close relationship between the microbiota and the immune system, so gross changes caused by lifestyle, diet and antibiotics must be important. Some roles of the gut in immunoregulation are discussed elsewhere in this issue [30].

Commensal microbiota of skin, lung and breast milk ::: The organisms implicated in the hygiene hypothesis:
The role of recent changes to the microbial flora of skin, lung and breast has received almost no attention. Before the invention of modern soaps and detergents skin was probably colonized by ammonia-oxidizing bacteria (AOB). These are ubiquitous in soil, but they are exquisitely sensitive to alkylbenzene sulphonate detergents [21]. AOB can convert the high concentrations of urea and ammonia found in human sweat into nitrite and nitric oxide (NO). These molecules are absorbed rapidly and efficiently via the skin, so this source of nitrite will have been biologically significant [21], and able to supplement the well-established blood–saliva–stomach–blood cycle of nitrate/nitrite/NO. Feelisch believes that without AOB in the skin flora modern man is nitropenic ([21] and M. Feelisch, personal communication). Because NO is fundamental to immunoregulation, this might be another way in which modern hygiene is affecting our immune systems. It needs to be investigated.
The lung cannot be sterile, and several groups are now starting to look at the flora of the lower, as well as the upper airways.
Recently there has been a suggestion that breast milk is not sterile [31]. There appears to be an ‘entero-mammary’ circulation. During lactation, translocation of bacteria from gut to Peyer's patches is increased, and bacterial nucleic acid signatures can be detected in peripheral blood mononuclear cells. Bacteria can also be seen in the glandular tissue of healthy breast, and in

mononuclear cells in breast milk which, when ‘sterile’, contains small numbers of cultivable organisms (<103). The mononuclear cells seem to be partially matured dendritic cells that might promote tolerogenic responses in the neonate [31]. It remains to be seen whether this is an important part of the colonization and education of the neonatal gut and immune system but if it is, it must be severely disrupted in modern humans.

Compatibility of the hygiene hypothesis with infection-based views of the aetiology of chronic inflammatory disorders:
As discussed elsewhere in this issue, there is some evidence that certain virus infections can trigger autoimmune disorders [32], and there is also the possibility that some such disorders are caused in fact by cryptic infections [33]. These views are not incompatible with the hygiene hypothesis. The organisms in question clearly did not cause these diseases in the past, when most of them (allergic disorders, inflammatory bowel disease, multiple sclerosis, type 1 diabetes, etc.) were rare. The immunoregulatory problem caused by the lack of ‘old friends’ may be facilitating the triggering of autoimmunity and also permitting infections that did not cause disease before the second epidemiological transition.

Compensatory genetic variants:
In parts of the world where there was a heavy load of organisms causing immunoregulation there has been selection for single nucleotide polymorphisms (SNP) or other variants that compensate partially for the immunoregulation. This is seen for several proinflammatory cytokines [34] and immunoglobulin E (IgE) [35]. There is an increased frequency of a truncated form of the serotonin transporter that also has a marked proinflammatory effect [36]. The problem here is clear. As soon as the immunoregulation-inducing organisms are withdrawn by the modern lifestyle, these genetic variants lead to excessive inflammation and become risk factors for chronic inflammatory disorders [34–36]. This constitutes a second layer of evolved dependence on the continuing presence of the ‘old friends’.

Interactions with other changes in modern lifestyles:
It would be foolish to assume that decreased exposure to these ‘old friends’ is the only reason for the increasing frequency of chronic inflammatory disorders in developed countries. Other aspects of modern life must contribute, and are likely to interact with and amplify the immunoregulatory deficit resulting from the changes to our microbial environment. Diet and obesity are associated with modified gut flora (Fig. 2) [37]. Psychological stress also modulates gut flora and gut permeability, while both obesity and stress result in greater release of proinflammatory cytokines (referenced and reviewed in [38]). Similarly, vitamin D is involved in driving regulatory cells [39]. Deficiency of vitamin D is extremely common, and increasingly implicated in the increases in chronic inflammatory disorders. Finally, pollution, particularly dioxins, which drive Th17 cells via the aryl hydrocarbon receptor [40], will also encourage inflammatory responses.

Cancer ::: The broader clinical implications:
The view that delayed infection with an unidentified organism might explain the rapid recent increase in acute lymphatic leukaemia of childhood was mentioned earlier [19].
There is another way in which the hygiene hypothesis might be relevant to some cancers. A failure to terminate inappropriate inflammation could favour oncogenesis. Chronic inflammation is associated with increased cancer risk [43]. Inflammatory mediators are involved in control of cell replication, angiogenesis and cell migration, and reactive oxygen intermediates can cause DNA damage. Many of these functions of inflammation are regulated by the transcription nuclear factor kappaB (NF-κB), and manipulating the activity of NF-κB has profound effects on tumorigenesis. Interestingly, tumour necrosis factor (TNF)-α–/– or TNF receptor 1 (TNFR1)–/– mice are more resistant to chemically induced carcinogenesis. Similarly, there are several SNPs of chemokines and cytokines that are associated with malignancy [43]. A regular input of non-steroidal anti-inflammatory drugs such as aspirin is associated with reduced risk of colorectal cancer [44].

Depression ::: The broader clinical implications:
The incidence of depression is increasing. Recent studies have been able to eliminate the possibility of changing diagnostic criteria, and to control for substance abuse [45]. Many people suffering from depression have raised markers of ongoing inflammation, including raised levels of proinflammatory cytokines, and low ratios of anti-/proinflammatory mediators, in the absence of any localized inflammatory disorder [38]. Similarly, when there is an identifiable inflammatory

illness, depression correlates with the levels of circulating inflammatory cytokines, rather than with the symptoms of the illness itself. Administration of inflammatory cytokines for immunotherapy of cancer or hepatitis [IFN-α or interleukin (IL)-2] causes depression, while anti-depressant drugs cause a relative increase in anti-inflammatory cytokines (reviewed and referenced in [38]). Some ways in which the microbiota can influence CNS function are discussed elsewhere in this issue [46]. Similarly, the recent loss of regulatory pathways that shut off inflammation, as suggested by the hygiene hypothesis, can provide further mechanisms [38] and contribute to the recent increases [45].

Atherosclerosis ::: The broader clinical implications:
Atherosclerosis is a Th1-mediated inflammatory lesion. In animal models it is exquisitely sensitive to inhibition by IL-10 or by regulatory T cells (Tregs), and there is accumulating evidence that IL-10 is also beneficial in human atherosclerosis [47]. Atherosclerosis is more common among people with chronically raised C-reactive protein (CRP) and, like depression, it is more common in the presence of other chronic inflammatory disorders [48].

Neurodegenerative disorders ::: The broader clinical implications:
The neurodegenerative disorders, Alzheimer's (AD) and Parkinson's disease (PD), appear to be mediated by inflammation. There is also some evidence that SNP of genes encoding pro- or anti-inflammatory cytokines influence susceptibility, and a large meta-analysis concluded that prolonged intake of non-steroidal anti-inflammatory drugs can give some protection against AD (discussed in [49]). Meanwhile, transforming growth factor (TGF)-β might have anti-Alzheimer effects both because of its immunoregulatory and anti-inflammatory properties and because it enhances clearance of amyloid-β[49].

Type 2 diabetes ::: The broader clinical implications:
Traditionally, type 1 diabetes has been regarded as an inflammatory disorder, whereas type 2 diabetes has been regarded as metabolic and hormonal. New findings cast doubt on this distinction. Inflammation can be detected in the damaged islets of type 2 diabetes [50]. This might be due entirely to metabolic stresses associated with poor diet and obesity, or it might also involve an immunoregulatory deficit. As outlined above (Fig. 2), metabolic stress might interact with, and enhance, the effects of reduced exposure to immunoregulation-inducing microorganisms.

The future:
The progressive identification of the organisms that are relevant to the hygiene hypothesis is already leading to exciting clinical trials of new potential therapies for inflammatory bowel disease, allergies and multiple sclerosis. Similarly, trials of a bacterial Treg-inducer are now planned for the type of depression that is accompanied by raised cytokine levels. Pritchard and colleagues in the United Kingdom have determined the maximum load of hookworm (Necator americanus) that can be tolerated without adverse effects [51]. A Phase 1 trial in allergic rhinoconjunctivitis has been completed [52], and further studies are in progress in allergies, multiple sclerosis and IBD. Similarly, Trichuris suis has been tested in inflammatory bowel disease [53], and is now entering trials in other disorders. Meanwhile, the exponents of probiotics are beginning to see that strains used for clinical trials in chronic inflammatory disorders need to be selected for their ability to induce immunoregulation. There is little point in trials of unsuitable strains imposed by companies that happen to hold intellectual property rights.
However, there are several crucial questions that we cannot yet address, and that make it difficult to predict the longer-term progression of this aspect of our increasing ‘gene–environment mismatch’, or whether the problem will be susceptible to simple solutions:

Conclusions:
In this discussion document I have assumed that the current view of the hygiene hypothesis as an immunoregulatory issue is fundamentally correct, and that detailed mechanisms will be discussed in other papers in this issue [30,32,54]. I have tried to explore the underlying principles at the environmental and evolutionary level. The views expressed here help to explain the consistent failure of epidemiology that targets the identifiable, clinically apparent infections of childhood. Such studies fail to find any relevant associations with protection from allergic disorders [55]. The bias in these studies was dictated by hasty over-interpretation of the original findings of Strachan and colleagues [56] that had not undergone Darwinian scrutiny. Now we need to concentrate on

organisms that have very long associations with the mammalian immune system, usually traceable back to the Paleolithic or earlier. Often these organisms will have been present as commensals, environmental ‘pseudocommensals’, subclinical infections or asymptomatic carrier states. This type of reasoning can potentially sharpen the focus of future epidemiology, simplify the quest for clinical solutions to the problem posed by the increasing incidence of inflammatory disorders, and broaden the range of microorganisms going into clinical trials.