Metchnikoff and the Intestinal Microbiome

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Introduction

Written at a time when microbiology was in its infancy and few intestinal diseases had been ascribed to a specific bacterial infection, Metchnikoff's essay contains some perceptive ideas that have subsequently been proven by modern science (Supplement 1). Amongst these were the concepts that many, if not most, bacteria found in the intestine could be beneficial to health, that these "good" bacteria may inhibit pathogenic species and that disruption of the balance between these different kinds of organisms may underlie susceptibility to disease. Most interestingly, the paper refers to several studies that had already begun to explore how this balance might be altered *in vivo* by administering beneficial bacteria or by favouring their growth via modifying the diet. All of these concepts are central to our current understanding of the intestinal microbiome and how it influences health, now one of the most heavily investigated areas in immunology and medicine.

The Intestinal Microbiome and its Physiological Functions

We now know that the normal intestine contains around 10^{14} bacteria, together with as yet unknown numbers of viruses, fungi and archaea [1, 2]. Together, these organisms make crucial contributions to health, with bacteria providing essential metabolic functions such as production of vitamins, deconjugation of bile acids and production of the short chain fatty acids (SCFA) that are required for epithelial cell proliferation and differentiation in the large intestine [3, 4]. These commensal bacteria are also important for preventing invasion by pathogens, partly by competing for space, nutrients and attachment receptors, as well as by "tuning" the local immune system into a state of readiness for generating protective immune responses and preserving memory [5, 6]. In recent years, it has become clear that the effects of the microbiome extend far beyond the intestine, influencing susceptibility to an enormous variety of diseases, particularly those with an immunological and/or metabolic basis, but also other conditions such as cancer and infectious disease [4, 6, 7]. Even more remarkably, there is growing evidence that the development and normal function of many organs, including the central nervous system, are heavily dependent on the bacterial composition of the intestine [8].

Germ free animals have brain abnormalities, are severely immunocompromised and have altered susceptibility to many diseases and infections [5, 9]. While this is clearly a highly artificial situation, dramatic consequences are also associated with more subtle imbalances in the different species of bacteria that make up the microbiome [10]. Metchnikoff would probably not have been surprised by this, although he could have had no idea of how extensive

the effects of imbalance would turn out to be. This "dysbiosis" has been implicated particularly in the modern epidemic of allergic and chronic inflammatory diseases and is a fundamental aspect of the hygiene hypothesis [11].

Composition and Establishment of the Microbiota – the Weaning Reaction

Much remains to be understood about which specific organisms might be associated with individual diseases. Indeed, despite the enormous strides in technology and taxonomy made in the 110+ years since Metchnikoff, we still have limited knowledge of exactly how many bacteria are present in the healthy intestine, what species they comprise and how they interact with the host. The vast majority of the microbiota comprises obligate anaerobes that cannot be cultured using conventional methods and identification has only become possible by exploiting the recent advances in 'omics techniques and bioinformatics [7]. This has revealed that the normal human microbiome is extremely diverse, with dominant contributions from the *Firmicutes* and *Bacteroidetes* phylae, together with smaller numbers of *Proteobacteria, Verrumicrobia, Actinobacteria, Fusobacteria and Cyanobacteria* [2, 12].

The exact composition of the microbiome varies considerably from individual to individual, being dependent on age, diet and geographical location. Genetics also play a role, but this is much less than that of environmental factors [13]. The newborn animal becomes colonised by maternal bacteria during delivery and the human neonate acquires an almost adult level density of total organisms within a few months of life. However the full diversity of species does not become established until 2-3 years old, then being relatively stable for most of adult life, before reducing again in old age [7, 14]. After being defined initially by the mother's microbiome and following a mostly stochastic process thereafter, a number of specific factors can influence the subsequent development of the adult microbiota. Unsurprisingly, diet plays a substantial role in this, with a "Western" diet rich in fat and processed sugars and low in complex polysaccharides being associated with a low diversity of species. In turn, this is believed to be the mechanism that explains the increased susceptibility to autoimmune, allergic, inflammatory and metabolic disease seen in industrialised countries. Other external factors which can reduce the final diversity of the microbiome include a Caesarean compared with vaginal delivery, while there is increasing evidence that disruption of the establishing microbiome by antibiotic use early in life can have significant consequences for subsequent disease predisposition [14]. Conversely, exposure to farm animals, family pets and multiple siblings favours the development of a diverse microbiota that appears to protect against disease [15-17].

There is a crucial window early in life in which external factors have their most important effects in shaping the developing microbiota [15, 18, 19]. This appears to be associated with the onset of weaning on to a mixed diet, a period that is associated with highly dynamic changes in the composition of the microbiota, as well in the anatomy and functions of the intestine, endocrine and immune systems. One elegant example of this "weaning reaction" comes from recent studies by Al Nabhani et al, which showed that recolonisation of germ free mice at weaning with a limited number of commensal bacteria could reverse the increased susceptibility of such animals to allergic lung disease, colitis and intestinal cancer [18]. Recolonisation of mice before or after weaning did not have the same effects. Several studies in both animals and humans are also consistent with the idea that the microbiome that becomes established around the time of weaning has long-lasting consequences for later life.

At the other end of the age spectrum, elderly humans and animals show marked changes in the microbiome, with decreased populations of *Firmicutes*, *Akkermansia* and *Bifidobacteria* and increases in *Proteobacteria*, *Bacteroidetes* and *Enterobacteria* compared with younger individuals. As discussed below, this kind of dysbiosis is can lead to a pro-inflammatory environment in the intestine and it is associated with increased leakiness of the aging intestine to bacterial products, leading to a systemic inflammatory state. This process may account for the increased susceptibility of the elderly to chronic inflammatory disease [20], a phenomenon that has become of current importance in understanding the age-related effects of severe Covid infection. However to date there have been no studies of the microbiota in patients with Covid.

Host-Microbiome Mutualism

A) Immune Adaptation to the Microbiota Promotes Local Tolerance

Although it has been known for many decades that animals and the microbiota have co-evolved over millennia and that the microbiome provides several crucial health benefits to host physiology, it is only in recent years that we have come to appreciate how intimate are the connections between commensal bacteria and the immune system [21]. Indeed, both the host and the microbiota expend enormous amounts of energy in recognising and shaping each other, establishing a phenomenon of mutualism [15, 22, 23]. As noted above, germ free animals are severely immunocompromised, while the healthy animal contains large numbers of T and B cells that are specific to constituents of the microbiome. The normal human produces 3-4g IgA/day in the intestine, most of which is directed at local bacteria. Furthermore, large numbers of microbiota-specific Rorγt^+ Th17 and Tbet$^+$ Th1 resident/effector memory cells are present

in the healthy mucosa. Products of these $CD4^+$ effector memory cells such as IL17, IL22 and γIFN play important roles in maintaining the integrity and function of the normal epithelial barrier. However their full effector functions are normally held in check by local $FoxP3^+$ regulatory T cells and IL10 producing Tr1 cells, whose generation and maintenance are promoted by several bacteria-derived factors including SCFAs, bile acid metabolites and TLR ligands. Thus, the intestinal immune system is not "ignorant" of the microbiome, but generates a form of tolerance in which non-inflammatory IgA production is paralleled by balanced populations of effector and regulatory T cells [24]. Together, this ensures that the host prevents invasion by commensal organisms without producing damaging pathology. Importantly, this tolerance is anatomically confined to the intestine and its draining lymphoid tissues, leaving the systemic immune system essentially ignorant of the microbiota and fully competent to respond appropriately if bacteria invade through the intestinal wall.

B) Shaping of Innate Immunity by the Microbiome

The microbiota drives the development and activation of many forms of innate immune cells in the intestine and elsewhere [25, 26]. Macrophages are the most abundant leukocyte in the normal intestine, where they contribute to homeostasis by expressing scavenger receptors that allow them to engulf and destroy invading organisms, as well as dying epithelial cells. They produce metalloproteinase enzymes that are involved in tissue remodelling and importantly, they are an important source of the IL10 which is needed to maintain the local anti-inflammatory environment. Unlike most other tissue resident macrophages, those in the intestine are replenished continuously by circulating monocytes, a process that is dependent on the presence of the microbiota, as is their production of IL10 and many of their other homeostatic properties [27]. Although conventional dendritic cell numbers are not consistently affected by the presence of an intact microbiota, their full maturation and ability to prime T cells under steady state conditions is dependent on epigenetic and metabolic changes promoted by type 1 interferon (type 1 IFN) produced by plasmacytoid DC in response to commensal bacteria [28]. Recent evidence shows that the effects of microbial products such as SCFAs on monocytes and DC may operate via selective induction of myelopoiesis in bone marrow precursors [29].

Innate lymphoid cells (ILC), particularly the ILC3 subset expressing Rorγt, also contribute to mucosal homeostasis by the production of IL22 which underpins epithelial barrier function, and by production of cytokines such as GM-CSF that maintains local myeloid cells. ILC3

respond to the microbiota via their expression of PRR and the aryl hydrocarbon receptor (AhR) that can respond to bacterial tryptophan metabolites, or by responding to cytokines produced by other cells present in the mucosa. Invariant NKT cells and mucosal associated invariant T cells (MAIT cells) are T lymphocytes with wide-ranging innate functions, whose role is to recognise microbiota-derived lipids and vitamin B2 metabolites respectively, adding further layers to the host-microbiome mutualism [6, 23, 30].

C) Immunomodulatory Effects of Individual Bacteria

Metchnikoff's idea that individual species of bacteria might have distinct effects on the host remains one of the most intensively researched topics in modern immunology, but few specific examples have been proved [7, 31]. One of the best known is the ability of Segmented Filamentous Bacteria (SFB) to stimulate IgA production and Th17 cells in mouse intestine, a phenomenon believed to involve activation of local DC via SFB-induced production of serum amyloid A protein (SAA) by epithelial cells [32]. Whether SFB are present in human intestine remains uncertain, although analogous IL17-inducing organisms have been described [33]. A further organism that favours local IgA production in steady state is the *Verrucomicrobia* member *Akkermansia muciniphila*, which acts by inducing the generation of $CD4^+$ follicular helper T (T_{FH}) cells [34]. More recent work indicates that a consortium of 11 bacterial strains from healthy human faeces including *Parabacteroides* and *Bacteroides spp,* can drive the appearance of IFNγ-producing $CD8^+$ T cells in GF mouse intestine via the stimulation of $CD103^+$ DC [35].

Conversely, a cluster of human *Clostridial* species have been shown to drive the selective generation of $FoxP3^+$ Treg in the intestine, by producing the SCFA butyrate and by stimulating the production of TGFβ from epithelial cells [6, 23, 36]. A consortium of *Bacteroides spp* can also favour the generation of peripheral $Ror\gamma t^+FoxP3^+$ Treg by producing bile acid metabolites that inhibit farnesoid X receptor (FXR) signalling in DC [37]. A number of other, less well-defined species have been implicated in the generation of intestinal $FoxP3^+$ Treg, including *Escherichia*, *Akkermansia*, *Bacteroides*, *Clostridium*, *Lactobacillus* and *Streptococcus* strains, while the capsular polysaccharide A (PSA) from *Bacteroides fragilis* induces the development of IL10 producing $FoxP3^-$ $CD4^+$ T cells with regulatory function (Tr1) [6, 23, 38]. Much remains to be discovered in this area and indeed, wide-ranging screening studies show that many different individual bacterial strains can have multiple and diverse effects on the immune system after mono-colonisation of germ free mice [31].

Dysbiosis and Disease

Imbalances in the overall composition of the intestinal microbiota (dysbiosis) have been associated with the susceptibility to and pathogenesis of many human diseases, ranging from inflammatory bowel disease (IBD) to autoimmune disease, atopy, metabolic syndrome, obesity, cardiovascular disease, neurodegenerative conditions and behavioural disorders such as autism. However, a variety of different patterns have been observed, even within the same disease, and consistent cause and effect links to individual organisms have been difficult to prove [6, 10, 15]. A common finding is that chronic inflammatory conditions are accompanied by reduced diversity in the microbiome, often reflecting a shift from the normal predominance of *Firmicutes* and *Bacteroidetes* towards the *Proteobacteria* phylum that contains Gram negative aerobes/facultative anaerobes such as *E coli* and other *Enterobactericeae*. Of particular note, reduced populations of SCFA-producing anaerobes such as *Faecalibacterium prausnitzii*, *Akkermansia* and *Lachnospira spp* have been found in IBD, asthma and other conditions, implying that the susceptibility to inflammation is due to the loss of bacteria that sustain Treg. In contrast, expansion of adherent invasive *E coli* (AEIC) has been associated with active lesions of Crohn's disease, suggesting that preferential expansion of pro-inflammatory organisms may also be important [39]. In all these cases, it remains controversial whether the dysbiosis is a cause or consequence of the disease, although faecal transplants and mono-colonisation experiments have indicated that susceptibility or resistance to disease can be transferred in appropriate animal models. Furthermore, dysbiosis has been detected in infants who go on to develop asthma several months before clinical disease occurs, indicating this may be a causal relationship, as well as being consistent with the crucial window when a healthy microbiota is establishing early in life (see above). One clear example in which the composition of the microbiota is of direct clinical relevance comes from the recent findings that it determines the success of checkpoint blockade in the treatment of cancer, an aspect which could not have been imagined in Metchnikoff's time [40].

Therapeutic Potential of the Microbiota

Metchnikoff discussed in some detail the idea that beneficial bacteria (which we would now call "probiotics") might be administered with the aim of restoring a healthy balance within the microbiome. However this has not yet proved widely successful in man [41]. Although transplant of faeces from healthy individuals is an effective treatment for *Clostridium difficile* enterocolitis [42] and there are occasional reports that chronic diseases can be ameliorated by administration of probiotic bacteria or nutrients that favour their outgrowth (prebiotics), such

effects have been difficult to prove reproducibly in well-conducted clinical trials [42, 43]. It is also important to note that many probiotic products contain mixtures of organisms whose properties have not been defined in any detail. Interestingly however, *Lactobacillus spp* have remained a particular focus of attention for promoting health, as they were in Metchnikoff's day, where the potential benefits of fermented milk products containing such organisms were of great interest. With the ever increasing knowledge of the constituents of the microbiota and better understanding of their biological roles, this is an area where it is easy to see progress in the future.

Conclusions

Much has been learnt about the microbiota since Metchnikoff's perceptive review, but reading it more than a century later not only underlines how many of our current ideas were already being discussed, but also highlights how much we still have to learn. It is has become increasingly clear that the microbiome plays a central role in determining health and there has been an explosion of information generated by modern immunology and molecular biology. However we remain some way away from knowing exactly which bacteria are present in the normal intestine, how they interact with the immune system and how they might be manipulated for the prevention and/or treatment of human disease. This is one of the greatest challenges to modern biology and will form a focus for research for many years to come.

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