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Intracellular pathogens: living in an extreme environment

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Introduction

The phylogenetic tree segregates into three major groups, which probably descended from a common ancestor: the bacteria, the archaea, and the eucarya (1–3). While bacteria and archaea lack a true nucleus and intracellular organelles, eucarya possess these. The origin of the ancestor remains elusive, but it emerged about 4 billion years ago. The bacteria probably started life 3.5 billion years ago and eukaryotes emerged 1.75 billion years thereafter. Sometime in between archaea appeared. Eukaryotes began as unicellular organisms but soon also developed multicellular forms.

Bacteria and archaea had sufficient time to exploit all niches of the abiotic environment, ranging from ice cold glacial lakes to fiery geysers – from the highest mountain peaks to the deepest sea beds. They also quickly developed forms of mutualism by forming colonies composed of mixed populations. With the evolution of eucarya, new environments could be exploited.

Today prokaryotes are the most prevalent form of life on earth, making up the majority of our biomass. It has been estimated that more than 10^{30} prokaryotic organisms live on our globe. Thus, archaea and bacteria are far more prevalent than eucarya. Even human beings comprise more prokaryotic cells than mammalian cells, which range on the order of 10^{12} human cells and 10^{14} prokaryotes mostly concentrated in our intestinal system (4–7).

Archaea, bacteria, and eucarya: friend and foe

It is surprising how well archaea have adapted to extreme environments, yet remained focused on abiotic habitats. They survive in highly salty environments as the halobacteria do, or at extremely high temperatures as the thermophils do. One of the few biotic environments populated by archaea is the gut of mammalians (6, 8). Methanobacteria intriguingly have chosen the digestive tract of ruminants, such as cattle and are responsible for the enormous amount of methane released

into the atmosphere due to excessive beef consumption by the human population (9). Archaea are also a common component of the normal human microbiota (6). Notably, *Methanobrevibacter* spp. produce methane by biodegradation of complex polysaccharides and remain a major population of our normal intestinal flora. Intriguingly, however, archaea never became a threat to humankind, they never developed a parasitic lifestyle, and never exploited the intracellular compartment of cells. Hence, archaea remained mutualistic and are friends that do not cause infectious diseases. In contrast, bacteria heavily capitalized on biotic environments and have become foes of humankind as major causes of infectious diseases (10).

Exploitation of other living organisms as habitat is not restricted to bacteria; they have also been heavily exploited by eucarya. Notably, the unicellular apicomplexa (malaria plasmodia and *Toxoplasma* spp.) and the kinetoplastida (*Trypanosoma* spp. and *Leishmania* spp.) followed convergent evolutionary traits toward living within eukaryotic host cells. They even went one step further and learned to exploit other species, notably insects as vectors, for the purpose of dissemination.

Exploiting humans as habitat

When human beings evolved some million years ago, microbes were well equipped for conquering this new habitat. Even more so, human beings created novel opportunities for microbes by their rapidly changing cultural behavior (10). Thus, ca. 20 000 years ago, people started to live in more settled patterns. During nomadic times, humans were already inhabited by microbes, some of them harmful to the individual and their family companions. Yet, they often failed to spread to larger populations. Bacteria living on carrion found entry into humans, who consumed raw meat. They quickly adapted to their new host as did numerous helminths, viruses, and protozoa. When humans changed to settlements over nomadic lifestyles, they came together closer, first in small hamlets and later in towns; they domesticated animals and crops in their vicinity. This provided attractive new opportunities for microbes, which had colonized cattle, dogs, or cats earlier and now had ample opportunity to exploit humans as a novel host. Opportunities also arose for fecal microbes, which were transmitted from human-to-human directly or through contaminated food or water. Protozoan parasites learned to capitalize on insects as vectors for efficacious human-to-human transmission and helminths benefited from close contact between humans and domesticated animals by transmission from their definitive host to an intermediate host and back again.

The first towns arising 1 or 2 millennia before Christ facilitated further spreading and global migrations – such as the Crusades in the first millennium after Christ – favoring microbial exchange between populations living in different regions of the earth. Importantly, spreading of pathogens to larger populations had become possible, and plagues became commonplace.

Vertebrates and humans fight back

In the case of synergistic coexistence to the benefit of both host and intruder, defense mechanisms do not make sense. In the case of peaceful coexistence, defense strategies were not really needed since no harm was encountered. A different picture arises when microbial colonization results in stable infection followed by disease as a frequent, albeit not essential, consequence. Defense strategies are needed to protect the host from damage or even death. Innate defense mechanisms are common among multicellular eurcarya. Even unicellular eucarya know how to engulf and kill microbes, probably mostly for the purposes of food consumption. Based on particulate nutrient uptake, defense strategies evolved and it was Elie Metchnikoff (1845–1916), the discoverer of innate immunity, who described the existence of phagocytosis in various eucarya for the first time (11). Similarities of numerous innate immune mechanisms in invertebrates and vertebrates offer testimony to the fact that multicellular organisms were consistently attacked by microbial intruders and urgently needed appropriate defense stratagems to survive.

In contrast, the specific adaptive immune system is a privilege of vertebrates. Vertebrates emerged on our globe 525 million years ago and 300 million years later, true mammals emerged. Only 5 million years ago, apes appeared on earth from which human beings are descended. Vertebrates must have been highly vulnerable to microbial intruders, which caused marked evolutionary pressure toward development of an adaptive immune system with unique specificity to avoid excessive collateral damage. As a consequence, they all developed such an immune system, albeit through two distinct modes of evolution. Both systems are characterized by enormous specificity due to highly variable receptors expressed by specialized cells, the lymphocytes. Antibodies are secreted into the surrounding milieu, whereas the T-cell receptors remain cell-bound. A quantum leap occurred 450 million years ago in jawed vertebrates, which was made possible by the appearance of recombination activation genes as central directors of gene rearrangement and recombination (12). Jawless vertebrates did not acquire this capacity. Yet, they have at hand an

alternative immune system that provides variability and thus unique specificity for antigens, as well. This system is based on usage of variable lymphocyte receptors comprised of leucine-rich repeat segments. Hence, adaptive immunity was so essential for survival it was twice independently discovered by vertebrates (13, 14).

Since the 19th century, a series of profound changes in human lifestyle occurred. Cities became less muddy, stones and later concrete became dominant building materials – unfavorable habitats for microbes. Clean water was provided, sanitary and sewage equipment installed, and hygienic measures became more efficacious. De-worming methods, insecticides to kill vectors, vaccines to prevent infectious disease, and finally, antibiotics to treat and cure infectious disease were developed as countermeasures against microbial pathogens – at least in industrialized countries. In developing countries, overcrowding and poor hygiene in slums and townships fostered diseases of poverty, such as acquired immuno deficiency syndrome (AIDS), tuberculosis (TB), and malaria. On top of this, increasing migratory behavior, uncontrolled exploitation of our last natural reservoirs bringing wild animals into close contact with humans, as well as large-scale industrialized animal farming to satisfy enormous meat consumption demands have caused novel challenges to humans and novel opportunities for newly emerging and re-emerging pathogens (9).

The intracellular habitat

Microbes needed to develop not only invasion strategies to enter the host but also strategies to evade host immunity. Based on this knowledge, it may come as a surprise that numerous microbes – bacteria, fungi, and parasites – chose one of the most efficacious effector cells of antimicrobial defense as habitat: the mononuclear phagocyte. Beyond doubt, this cell type provides an extreme environment raising the question as to the advantages of this habitat for the predator (15).

It has often been stated that the inside of a cell is a rich source of nutrients. This may be questioned. First, most intracellular pathogens reside in the phagosome, which they have to manipulate to reduce the aggressive environment that is formed after fusion with lysosomes. Few pathogens have designed egression strategies from the phagosome into the cytosol, with *Listeria monocytogenes* being the best-studied example. Probably one major advantage of the intracellular lifestyle is the low risk of mixed intracellular infection by different pathogens on a single-cell level. (One rare occasion may be macrophage coinfection by human immunodeficiency virus and *Mycobacterium tuberculosis*.) Hence, in the intracellular milieu, competition between microbes, viruses, bacteria, fungi, and protozoa seems to be rare: a clear advantage for the intruder managing to live there.

Probably the capacity of mononuclear phagocytes to actively engulf microbes was a critical step to pave the way for acquisition of an intracellular lifestyle, and this may be the reason for the predilection of numerous pathogens for these cells programmed for host defense. Moreover, macrophages are long-lived, and this distinguishes them from the neutrophils, which are also professional phagocytes but with a much shorter lifespan. Many mononuclear phagocytes live for weeks, whereas neutrophils die within less than a day and hence cannot provide a stable niche for intracellular predators.

Once entry into the host cell has been accomplished, host cell function and integrity had to be maintained to allow for prolonged persistence within the host cell. As a final step, microbes need to be released from their cellular habitat so that they can transit to other cells and accomplish the ultimate goal – transmission to the next host. *L. monocytogenes*, malaria plasmodia, and *Toxoplasma gondii* are well-studied examples of how pathogens pass from cell to cell without entering the extracellular milieu. Indeed, malaria plasmodia and *T. gondii* are obligate intracellular pathogens, which strictly depend on host cells in the human host. In contrast, *L. monocytogenes* as well as *M. tuberculosis*, *Brucella* spp., and *Salmonella enterica* have retained their capacity to live in the extracellular milieu prior to entering new host cells. These pathogens can even live in the abiotic environment for quite some time. Even though *M. tuberculosis* is often seen as the ultimate intracellular bacterium, it flourishes in the extracellular detritus of caseous granulomas where it reaches numbers of up to 10^{13} organisms.

Transmission to the next host can be a major obstacle. *T. gondii* and *L. monocytogenes* are transmitted both vertically and horizontally. Hence, they are major causes of miscarriage and abortion. *Leishmania* spp. and malaria plasmodia use insects as vectors for spreading – sand flies and mosquitoes, respectively. *M. tuberculosis* is typically transmitted through aerosols, and *Brucella* spp., *T. gondii*, and *S. enterica* through smear infections or uptake of contaminated food and water.

In consideration of host defense, the intracellular habitat offers another advantage: it shields the microbial invader from attack by antibodies. T lymphocytes, therefore, are critical mediators of protection. They monitor host cells for infection and mobilize appropriate effector functions. On the molecular level, T cells produce and induce cytokines and effector molecules. On the cellular level, they attract professional

phagocytes to the site of microbial deposition and activate their antimicrobial capacities. On the organ level, granulomatous lesions frequently develop which help to contain the pathogen and in some instances even achieve their eradication.

Intracellular infection ranges from short to long term. Listeriosis is the paragon example for a short-lived bacterial infection since the pathogen is eradicated once T lymphocytes have reached full power. In contrast, *M. tuberculosis*, the paragon of a long-lived bacterial infection, can persist within macrophages over the lifespan of the human host, often in a stage of dormancy. Frequently this pathogen is contained at such a low level that clinical disease does not develop, and asymptomatic latent infection continues.

In contrast to intuition, intracellular life in mononuclear phagocytes must have an evolutionary attractiveness, since microbes of distant relationship have chosen processes of convergent evolution toward this goal. Thus, *Brucella* spp., *M. tuberculosis*, and *Leishmania* spp. have selected this cell type as habitat and established measures to avoid attack by aggressive effector molecules.

Immunity to intracellular pathogens

This volume of *Immunological Reviews* comprises 19 excellent reviews, which give an exciting though necessarily selective overview of the crosstalk between intracellular pathogens and the host immune response. The field is too diverse to cover all aspects and selection was needed. Importantly, the volume attempts to be bilingual. Several chapters emphasize the language of the host, analyzing the immune response from different angles. Others use the language of microbial pathogens, analyzing their invasion and evasion strategies in the face of an active immune response.

Views of immunology focus on pathogen sensing and activation of cellular and humoral host factors. These determine the outcome of infection with an emphasis on the central host cell, the mononuclear phagocyte, which frequently serves as habitat and effector cell, depending on its activation status (16). The mononuclear phagocyte is not only an effector cell but also has the capacity of monitoring invasion by microbial intruders. By means of pattern recognition receptors, deviations from homeostasis are recognized and signals transmitted to other members of the host defense armamentarium, notably, the T lymphocytes (17). The T cells as central mediators of acquired protection receive attention in two articles: effector T cells, the first line in the acquired defense (18); memory T cells, the long-lived cells that guarantee a better control of repeated infection (19). Despite the original conception that $CD4^+$ T cells expressing T-helper 1 (Th1) functions are responsible for defense against intracellular microbial pathogens, we now know that in addition, $CD8^+$ T cells perform effector functions against many intracellular pathogens (20). In addition to major histocompatibility complex molecules, which serve as reference structures for $CD4^+$ and $CD8^+$ T cells, CD1-restricted T cells have received increasing interest in infections with intracellular bacteria (21). Memory is generally defined as a persisting adaptive immune response in the absence of nominal antigen (22). Thus, by definition, during chronic infection true memory should not develop. Yet, the value of immune memory for host defense is beyond any doubt and amply illustrated by the success of vaccination against numerous infectious diseases – albeit against diseases conquered by antibodies rather than T cells.

Even though B cells are considered to make a more peripheral contribution to adaptive protection against intracellular microbes, recent findings have revised our thinking. B lymphocytes play a role in intracellular infections (23), not only as antibody producers but also as regulators of immunity independent from antibodies.

Intracellular crosstalk between host and pathogen

A significant number of reviews focus on the intimate crosstalk between host and pathogen. Invasion strategies are being elucidated on the molecular level to identify the key bottlenecks of entry into and exit from host cells (24). Although it was originally thought that microbes, once they have entered their host cells, would leave their habitat biologically intact, more recent findings have revealed that autophagy is a frequent consequence of the interplay between intracellular pathogens and their host cells, highlighting that this crosstalk is much more dynamic and complex (25). Autophagy may be viewed as a defense mechanism that allows microbial clearance. Yet, it is also part of the survival strategies of pathogens. The complexity of intracellular microbial infections is well reflected by the multigenicity underlying susceptibility to respective diseases. Without doubt, certain monogenic disorders predispose to intracellular infectious diseases such as mutations or deletions of cytokine genes critical for Th1 cell function and development. Beyond this, however, recent findings have begun to reveal the more complex interplay of multiple genes that modulate human susceptibility to infection and disease progression (26).

The high complexity of intercommunication between pathogen and multicellular host in a matrix-like arrangement

demands a systems biology approach, which is just starting to be explored (27). Additional layers of complexity are added by the inclusion of insect vectors as third partner. Insects do not serve as passive transport vehicles. Rather they actively interact with pathogens of human hosts. The review by Steinert and Levashina (28) deals with host defense mechanisms of insects.

Even so, this is not the end of the story. Increasing evidence suggests that intracellular pathogens can serve as cofactors for chronic inflammation and autoimmune disease and may also participate in malignant processes. Reciprocally, declining incidences of infectious diseases in industrialized countries are paralleled by increasing incidences of allergic and chronic inflammatory diseases as well as autoimmune diseases, suggesting negative feedback between these two categories (29). The hygiene hypothesis was a first attempt to provide an explanation for these findings. Obviously this field is far more complex and has to extend to different types of lifestyle as driving forces (30). No doubt, we can expect exciting news as this area unfolds.

The culprits

The second part of this volume is purposefully highly restrictive and covers four bacterial and three protozoan pathogens. This selection focuses first on studies of pathogens, which provide insights of general relevance into mechanisms underlying the host–pathogen relationship. Second, selection is based on importance of pathogens as health threats, such as TB and malaria, which together with AIDS rank highest on the infamous mortality list of infectious diseases (31). *L. monocytogenes* is a pathogen of minor threat to humans, although it can endanger the fetus and cause abortion. *L. monocytogenes*, however, has provided the most powerful model to study the molecular biology of bacterial virulence factors and host immune responses (32, 33). *S. enterica* comprises numerous serotypes, which can cause acute or chronic infections, either locally (e.g. gastroenteritis) or systemically (e.g. typhoid) (34). This pathogen is highly versatile living within and outside of host cells and hence defense requires both humoral and cellular immunity (35). TB is a paragon among the causative agents of chronic infections. This pathogen resists immune attack, either in a dormant stage without causing clinical disease or in a metabolically active stage, causing highly lethal disease (36). The chronicity of latent infection is highly demanding for the immune system and requires continuous fine-tuning in response to coinfections, which can impair protective immunity (37, 38). Even though *Brucella* spp. has some similarity to *M. tuberculosis*, it has developed specific strategies to escape immune attack and survive in the host, ultimately leading to chronic infection sometimes with relapsing disease (39).

The protozoan agents of malaria (40), toxoplasmosis (41), and leishmaniasis (42) all undergo complex life cycles in different hosts. Malaria plasmodia and *T. gondii* can infect a variety of host cells, which they enter and exit by gliding motility. *T. gondii* infects muscle cells and cells of the central nervous system, while malaria plasmodia prefer erythrocytes and hepatocytes. Hence, both pathogens favor non-professional phagocytes as habitat with the extreme example of erythrocytes lacking the antigen-presentation machinery which are misused by malaria plasmodia. In contrast, the intracellular lifestyle of *Leishmania* spp. bears significant similarities to that of *M. tuberculosis* and *Brucella* spp., in that they prefer to reside in mononuclear phagocytes.

Concluding remarks

Although the chapters compiled in this volume of *Immunological Reviews* intentionally focus on basic mechanisms underlying infections with intracellular pathogens, it is obvious that they can provide guidelines for novel intervention measures, notably, rational vaccination strategies. Vaccine candidates are currently undergoing different stages of clinical trials for malaria and TB, and the next decade will reveal whether we are successful in tipping the balance of this long-standing combat in our favor (43, 44).

Microbial pathogens have exploited the human species from its beginning. Neither have microbes succeeded in eliminating humans, nor have humans succeeded in eradicating pathogens, with the one example of smallpox (9). Apparently they are equally strong opponents with very different survival strategies. On the one hand, microbial pathogens place their trust solely in the Darwinian principle of random mutation followed by selection of the fittest (45). Although humans have to obey these rules as well, they are significantly hampered by slow replication time. Higher organisms have therefore added specialization as a complementary strategy. To defend us from microbial intruders, a highly efficacious immune system has evolved which rapidly senses the invaders and mobilizes the most appropriate defense mechanisms specifically targeting the culprit. It is this specialized system rather than mutation and selection that has allowed us to withstand constant invasion by microbes. Humans have the opportunity to add one more layer to this repertoire of defense, that is, the use of another specialized organ to create novel defense

strategies – the brain. With our ingenuity, we can devise novel tactics, notably vaccines and antibiotics. Using our intelligence, we have already developed numerous vaccines against pathogens. Yet, vaccines against intracellular microbial pathogens have thus far evaded successful implementation. Better understanding of the host–pathogen relationship can provide the blueprint for rational vaccine design against these major threats.

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