Evolution of Intracellular Pathogens

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Abstract

The evolution of intracellular pathogens is considered in the context of ambiguities in basic definitions and the diversity of host-microbe interactions. Intracellular pathogenesis is a subset of a larger world of host-microbe interactions that includes amoeboid predation and endosymbiotic existence. Intracellular pathogens often reveal genome reduction. Despite the uniqueness of each host-microbe interaction, there are only a few general solutions to the problem of intracellular survival, especially in phagocytic cells. Similarities in intracellular pathogenic strategies between phylogenetically distant microbes suggest convergent evolution. For discerning such patterns, it is useful to consider whether the microbe is acquired from another host or directly from the environment. For environmentally acquired microbes, biotic pressures, such as amoeboid predators, may select for the capacity for virulence. Although often viewed as a specialized adaptation, the capacity for intracellular survival may be widespread among microbes, thus questioning whether the intracellular lifestyle warrants a category of special distinctiveness.

Contents

INTRODUCTION

The topic of evolution of intracellular pathogens cannot be considered without first grappling with the uncertainties and ambiguities in the meaning of the terms evolution, intracellular, and pathogen. Unfortunately, none of these terms lends itself to a straightforward definition. In combination, they present a vexing problem in semantics that must be addressed before delving into this complex subject. The word evolution connotes change, yet in the context of this essay the word is used to refer to an origin, as the goal is to explore how certain microbes that reside inside cells came to acquire that lifestyle. Because intracellular pathogens are extremely varied, and because most if not all have adopted the strategy of intracellular life as part of their evolutionary trajectory, it may not be possible to propose an overarching notion of evolution for this microbial set. Consequently, any approach to the subject must be an attempt to discern evolutionary themes in an area where there is no significant fossil record and only a fraction of intracellular pathogens have been studied in detail. Each pathogenic microbe is different and consequently the pathogenic strategy of each intracellular pathogen has unique aspects. This introduces a conundrum because uniqueness argues against generalities in the evolutionary process. However, it is possible to identify generalities by considering the subject from the larger perspective of the mechanisms that are responsible for the virulence and pathogenicity of intracellular pathogens.

The second hurdle in approaching the topic is the designation of a microbe as an intracellular pathogen. There is no good definition for an intracellular pathogen because the term intracellular is vague when considered in the context of microbial life. Intracellular means inside a cell, but when this adjective is applied to pathogenic microbes, one is immediately confronted with many ambiguities. For example, most, if not all, intracellular pathogens must spend some of their life in the extracellular space prior to entry or after cellular exit. An illustrative case of this is the obligate intracellular pathogen *Ehrlichia chaffeensis*, which can be found in extracellular spaces (25). Hence, except for those pathogens that are transferred vertically during host cell replication, the term intracellular refers to one phase of the microbial cycle. Classifying pathogenic microbes as intracellular or extracellular is further complicated because there is often no clear dividing line. By all criteria, viruses are obligate intracellular pathogens, yet viruses are often considered a different class of microbes and are not usually grouped with other intracellular pathogens. In general, microbes are considered intracellular pathogens when their cycle in the host includes residence and/or replication inside host cells. However, this distinction can be blurry. For example, encapsulated bacteria such as *Streptococcus pneumoniae* are generally considered extracellular pathogens, yet these bacteria are often found inside neutrophils. Similarly, *Staphylococcus aureus* is not classically considered

Intracellular pathogen: a microbe capable of causing host damage whereby the lifestyle in the host is associated with intracellular residence, survival, or replication

Pathogen: a microbe capable of causing host damage

Obligate intracellular pathogen: a microbe capable of causing host damage that is completely dependent on a host cell for survival and replication

an intracellular pathogen but this bacterium can survive and replicate inside several types of cells, and intracellular residence is now considered important for persistence and pathogenesis (41). Even more vexing is the example of *Aspergillus fumigatus*, a fungus that is a common cause of life-threatening disease in immunocompromised patients. This organism grows as hyphae in tissue yet is thought to be inhaled as conidia that germinate and replicate in alveolar macrophages (22). *A. fumigatus* does meet some criteria as an intracellular pathogen, yet most authorities do not consider this fungus as such. Furthermore, replication is not required for the designation of intracellular pathogen, as illustrated by *Trichinella spiralis*, the causative agent of trichinosis, which survives but does not replicate in human skeletal muscle cells. The topic is made even more confusing by many reviews on intracellular pathogens that tend to focus on microbes that survive ingestion by phagocytic cells, a quality that is viewed as an ability to subvert professional phagocytes of the innate immune system. Nevertheless, many intracellular pathogens such as *Toxoplasma gondii* and *T. spiralis* can reside in nonphagocytic cells. Consequently, focusing on the host cell type inhabited is not particularly enlightening in resolving the definitional difficulties. Perhaps the greatest problem with the term intracellular is that it connotes a distinction for a set of microbes that may deserve no such distinction. In other words, having an intracellular pathogenic strategy is currently viewed as a special property, yet there is increasing evidence that the capacity for intracellular residence is a common attribute among pathogenic and nonpathogenic microbes. This notion is explored in this review with the proposal that the entire concept of intracellular pathogenesis requires redefinition.

Finally, the word pathogen confers upon microbes a quality that is not their own, as virulence is one outcome of the interaction between a microbe and a host, and requires a susceptible host (10). For the purposes of this review, pathogen is defined as a microbe capable of damaging a host (9). When considering the topic of the evolution of intracellular pathogens, it is worthwhile to remember that no microbe can be a pathogen without a host, and consequently, it is impossible to discuss the subject of evolution from a microbe-centric perspective. Because this review is focused on pathogenic microbes, it does not consider the endosymbiotic bacteria, which are common in many invertebrate and protozoal species (34, 35). Ancient endosymbiotic bacteria are thought to have been critically important in the evolution of eukaryotes and may have been the progenitors of such organelles as mitochondria and plastids (28). The exclusion of endosymbiotic bacteria is done with the acknowledgment that there is often a thin line between symbiotic and pathogenic intracellular microbes, and that these microbes often employ similar mechanisms for intracellular survival. For example, the obligate intracellular bacterium *Wolbachia* spp. is associated with numerous arthropod species where it passed vertically; the outcome of the host-microbe interaction can be symbiotic, neutral, or pathogenic depending on the tissue and the host (32). In fact, it has been argued that the major difference between pathogenic and nonpathogenic (endosymbiotic bacteria) intracellular microbes is their effect on the host, since both must have similar biological requirements for inhabiting intracellular spaces (17).

Considering the diversity of microbes capable of residing in host cells, the ambiguities in the definitions, and the uncertainty in the boundaries of classification, there is a quixotic element to the idea that the topic of evolution of intracellular pathogens can be approached in a short review. In this regard, despite the ambiguities evident in the term intracellular pathogen, this concept has been enormously influential in other fields, such as immunology (11) (see sidebar, Does the Intracellular Lifestyle Warrant a Special Distinctiveness?). On the other hand, an explosion in knowledge of microbial pathogenesis in recent years allows one to begin to discern the outlines of the landscape over which certain microbes evolved to survive and replicate in host cells as part of their pathogenic mechanisms. In particular, progress

DOES THE INTRACELLULAR LIFESTYLE WARRANT A SPECIAL DISTINCTIVENESS?

Distinguishing pathogenic microbes as intracellular or extracellular has been a highly influential division in both microbiology and immunology. For example, immunologists have often viewed humoral and cellular immunity as the immune arms responsible for defense against extracellular and intracellular pathogens, a concept that is now considered too simplistic. Consequently, the designation itself has the potential to affect how we think in entire disciplines. Because concepts often drive research and serve as a basis for understanding, such sweeping generalizations should be made carefully. As discussed in this review there does not seem to be a clear dividing line between extracellular and intracellular pathogens, except possibly for obligate intracellular pathogens. However, some obligate intracellular pathogens, such as *Ehrlichia* spp., have extracellular phases where they are susceptible to antibody-mediated immunity. Hence, classifying organisms as intracellular and extracellular pathogens may be ultimately a futile exercise fraught with error and misconception.

in genomics has yielded such far-reaching concepts as the association of genome reduction with intracellular lifestyle, which provides optimism for the notion that there may be some general rules that apply to the evolution of intracellular life. Furthermore, the recognition that for microbes such as *Legionella pneumophila* (20) and *Cryptococcus neoformans* (48) virulence is likely to be an outcome of selection pressures in the environment brought on by amoeboid predators opens a new conceptual approach to consider how some intracellular pathogens emerged. Consequently, this review tries to identify common themes among the uniqueness of individual host-microbe interactions. The goal is to look at evolution from a distance without getting lost in the intricacies of individual pathogenic microbes. Although generalizations in biology can be treacherous, especially in light of the variability inherent in host-microbe interactions, they also have the potential to provide useful concepts and reveal important principles.

Genome reduction: a phenomenon observed in many microbes that adopt intracellular lifestyles that is associated with gene loss and significantly smaller genomes than free-living phylogenetic relatives

THE INTRACELLULAR LIFESTYLE IS ANCIENT

When approaching the topic of evolution, it is important to ask when the species, characteristic, or phenomenon under study first became apparent in biological history. Establishing a timeline is a critical step for understanding the origin of a phenomenon in the context of evolutionary and geologic time. Unfortunately, there is no fossil record to provide an estimate of when some organisms acquired the capacity to survive inside other microbes. If we accept an endosymbiotic origin for mitochondria and other eukaryotic organelles (28, 29), then we can conclude that the capacity for intracellular residence is ancient and antedated the emergence of eukaryotic organisms as we know them. The emergence of the intracellular lifestyle in ancient microbes appears to have at least three major requirements: (*a*) size differences between microbes such that one can ingest another; (*b*) a mechanism for particle ingestion on the part of the host and/or host invasion on the part of the smaller entity, and (*c*) a capacity for the ingested microbe to survive within the larger host. Such early interactions could have had varied outcomes including survival of both microbes (symbiosis and mutualism), survival of the host (predation), damage to the host (intracellular pathogenesis), or damage to both microbes (incompatibility and antagonism). Presumably, each outcome was subject to selection pressures that directed the emergence of different types of microbe-microbe or host-microbe relationships.

Today, one can discern outcomes that mirror putative ancient outcomes in extant host-microbe relationships. Endosymbiotic bacteria represent mutualistic interactions of a type that may have once been responsible for the origin of the eukaryotic organelles. Bacterial grazing by amoebae represents predatory-type interactions (30). The ingestion by macrophages of pathogenic bacteria with subsequent bacterial survival and damage to the host cell is the type of interaction that falls into the traditional view of intracellular pathogenesis. It has been

suggested that the pre- and postingestional survival strategies of bacterial prey in response to amoeboid predator grazing were the precursors of extra- and intracellular pathogenic strategies, respectively (30). Similarly, postingestional adaptations, such as digestional resistance, intracellular toxin production, and intracellular replication may be linear antecedents of similar phenomena now associated with intracellular pathogens (30). Nevertheless, infection of a host cell with a nonreplication-permissive microbe capable of host damage would represent a deleterious interaction for both entities. An example would be *Toxoplasma gondii* infection of cells in a nondefinitive host that is a biological dead end for the parasite that also damages the host cell. When viewed from the vantage point of evolution, it becomes apparent that what we call intracellular pathogenesis is a part of a continuum of intracellular lifestyle strategies that is both ancient and constantly coevolving.

OBLIGATE AND FACULTATIVE INTRACELLULAR PATHOGENS

Intracellular pathogenic microbes fall into two groups: obligate or facultative. Obligate intracellular pathogens have lost their capacity for living outside of their hosts and these include all viruses, bacteria such as *Rickettsia* and *Chlamydia* spp., and protozoa such as *Plasmodium* spp. In contrast, facultative intracellular pathogens retain the capacity for replication outside their hosts and these include a large number of pathogenic bacteria and fungi. Hence, the designations of obligate and facultative would appear at first glance to represent a clear dividing line for approaching the topic of evolution of intracellular pathogens. However, on closer examination, this distinction is blurred by the phenomenon of genome reduction (see below), suggesting that the difference between obligate and facultative pathogens is simply one of the extent of gene loss that often accompanies host association and dependence. Obligate and facultative intracellular pathogens can also be distinguished by their means of acquisition. Obligate intracellular pathogens are necessarily acquired from other hosts, whereas facultative intracellular pathogens are acquired from other hosts (e.g., *Mycobacterium tuberculosis*) or directly from the environment (e.g., *C. neoformans*). Obligate intracellular pathogens are dependent on their hosts, and consequently these microbes always have intimate and dependent relationships with their hosts. However, these microbes have mastered escaping from host response mechanisms, and, when it occurs, microbial-mediated disease is usually caused by disruption of the host-microbe relationship. In contrast, facultative intracellular pathogens have more varied relationships with their hosts. Some, like *M. tuberculosis*, cause disease only in a minority of infected hosts and this is associated with transmission of infection. Others, like the bacterium *L. pneumophila* and the fungus *C. neoformans*, are acquired from the environment and have no obvious need for mammalian virulence in their replication or survival. Hence, the outcome of the interaction of these microbes with mammalian hosts is usually a function of the immunological status of the host, and both legionellosis and cryptococcosis are often associated with immune impaired hosts.

THE PHENOMENON OF GENOME REDUCTION

Despite the diversity among intracellular pathogenic strategies, evidence from the available sequenced genomes suggests what may be a general rule, namely, that obligate and facultative intracellular pathogens acquired from other hosts often exhibit genome reduction. In other words, the transition from the status of free-living to intracellular life is associated with the loss of large segments of DNA (reviewed in References 15 and 17). These gene deletions often include DNA segments that encode entire metabolic pathways providing nutrients that can then be acquired from the host. For example, *Rickettsia* spp. have lost numerous genes needed for many metabolic pathways including sugar, purine, and amino acid metabolism (42). *Candidatus* Blochmannia, an obligate endosymbiont of ants, has a severely reduced genome

compared with free-living relatives consistent with loss of DNA for host adaptation (53). Another example of genes that are lost in the adoption of the intracellular lifestyle are bacterial toxin-antitoxin loci, which encode for proteins that allow survival in stressful conditions (39).

For some intracellular microbes, genome deletions may have been selected because they make the microbe more fit in its interaction with the host. This phenomenon has been called pathoadaptation, and here the likely mechanism is gene loss that is positively selected because the genes' absence increases microbial fitness in the host (38). An excellent example of this phenomenon is the finding that the gene coding for lysine decarboxylase is lost in *Shigella* spp.; presumably the product of this enzyme inhibits bacterial virulence factors (31). Another example of pathoadaptation is the loss of flagella in many intracellular pathogens, possibly reflecting convergent evolution to shedding a motility mechanism as a trade-off for greater fitness in the host (38). The phenomenon of gene reduction also applies to eukaryotic intracellular pathogens, as revealed from genomic studies of microsporidia, which are highly specialized fungi (23). However, gene gain from eukaryotes can also occur in certain cases, as demonstrated by the finding of numerous eukaryotic-like proteins in *L. pneumophila*, a bacterium that has a long and close evolutionary association with environmental amoebae (12).

Many obligate intracellular pathogenic microbes have also lost mobile elements from their genome, a phenomenon that may translate into a lack of opportunity for gene transfer that in turn reduces the capacity for genetic diversity. An exception to this generalization is *Coxiella burnetii*, which retains mobile elements and has a genome with numerous pseudogenes, suggesting that this organism is a recent convert to obligate intracellular life and may be in the midst of genome reduction (44). In light of these findings with bacterial genomes, it is possible to view viruses as extreme examples of genome reduction, with viral genomes containing only those genes essential for replication and escape of host immune mechanisms.

If the progression from a free-living state to obligate intracellular life is accompanied by irreversible genome loss, then one can envision this evolutionary pathway to be a journey toward niche specialization with no return. In fact, Andersson & Kurland (6) have suggested that the process of genome reduction associated with intracellular pathogenesis may eventually lead to a Muller's ratchet, a phenomenon whereby small asexual genomes accumulate deleterious mutations that are ultimately associated with extinction. This raises the interesting possibility that, for some microbes capable of intracellular survival, the temptation of obligate intracellular life with its bountiful access to host resources and protection may carry with it seeds for their own destruction as distinct biological entities. Even in situations in which the host-microbe relationship evolves into that of mutualistic endosymbionts, such as mitochondria, the obligate intracellular style is associated with a loss of self in the form of genome deletions. Hence, for some pathogenic microbes, the obligate lifestyle may be an evolutionary dead end associated with loss of genome, a narrow ecological niche, dependence on host survival for microbial survival, and the grim possibility of a Muller's ratchet. However, a recent analysis of the *Mycoplasma* genome revealed that it contained a significant proportion of DNA acquired by horizontal gene transfer, suggesting that this outcome may be avoided by certain intracellular pathogens (45). In contradistinction to intracellular pathogens, environmentally ubiquitous microbes, such as *Pseudomonas aeruginosa*, that are capable of occupying diverse ecological niches have large genomes (55).

THE ECOLOGY AT THE SOURCE OF INFECTION

Another distinction that is enormously important when considering the evolution of intracellular pathogens is the environment at the source of infection. In general, microbial

infection is acquired either from other hosts or directly from the environment. In this regard, a host can be considered an ecological niche. Hosts provide microbes with protection, the potential of access to nutrients, and transportation. In contrast to environmental residences where a microbe is subject to numerous conditions and complexities, hosts tend to provide microbes with a more constant and predictable environment, with the caveat that survival in a host usually requires adaptation and specialization, which in turn reduce the number of potential sites that the microbe can inhabit. Hosts that serve as sources of infection can be self or nonself. For example, *M. tuberculosis* is usually acquired from other humans, although the initial strains encountered by human populations may have come from cattle or other vertebrate hosts. An example of infection with intracellular pathogens from nonself hosts would be the acquisition of *Yersinia pestis* from fleas and *L. pneumophila* from environmental amoebae. On the other hand, some intracellular pathogens are acquired directly from environment without having to implicate recent residence in other hosts. Examples of this category include the fungi *Cryptococcus neoformans* and *Histoplasma capsulatum*, which cause human infection after the inhalation of aerosolized spores.

The ecology at the source of infection confers tremendous selection pressures that shape the behavior of intracellular pathogens and the consequences of infection. For intracellular pathogens acquired from other hosts, there must be a premium on pathogenic strategies that are permissive to growth without damaging the host too quickly to insure replication and transmission and, in metazoal hosts, to prevent and/or impair an immune response. For some microbes such as *M. tuberculosis*, damage to the host in the form of cavitary tuberculosis is essential for host-to-host transmission, with the caveat that pulmonary disease occurs in only a minority of infected individuals. For host-acquired microbes, the selection pressures include host immune mechanisms and the optimization of their replicative strategies in their respective hosts. Given that, residence in the intracellular space necessarily reduces the likelihood that such microbes would encounter other microbes to exchange DNA; the intracellular environment is associated with loss of mobile elements from bacterial DNA and possesses the threat of accumulation of deleterious mutations. Some of the facultative intracellular microbes acquired from other hosts, such as *M. tuberculosis*, may be on evolutionary paths to becoming obligate intracellular pathogens, as evidenced by genome reduction relative to other related mycobacteria (21). In this regard, *Mycobacterium leprae* may already have a minimal mycobacterial genome, which precludes growth outside only a few vertebrate hosts (13, 52).

In contrast, intracellular pathogens acquired from the environment are under different types of selection pressures than those acquired from other hosts. For example, microbes acquired directly from the environment would be under biotic and nonbiotic selection pressures in the environment that would not be experienced by microbes that jump from vertebrate to vertebrate host. Soil organisms are exposed to extremes of temperature, light, and humidity, depending on diurnal and weather conditions. Competition for nutrients in soils is fierce given the abundance and diversity of microbial life. Soil-dwelling microbes are under the constant threat of predation from amoebae and small animals. Soil-dwelling microbes need a full array of metabolic machinery to survive in their ecological site and consequently are not under the type of selection pressure that would lead to genome reduction. Hence, one should not anticipate that genome reduction will apply to all intracellular hosts, but rather it should be expected only when the microbe is associated with a host that can potentially supply its metabolic needs. Consistent with this thesis, facultative intracellular pathogens acquired directly from the environment, such as *C. neoformans* and *H. capsulatum*, are autotrophs with minimal nutritional needs that can replicate

across a broad range of environmental temperatures. Hence, it appears that whether a microbe is host- or environmentally acquired provides a clear dividing line for comparing and contrasting the evolution of intracellular pathogens.

The dividing line between host and environmental acquisition is blurred by the observation that enteric human pathogenic bacteria can interact with soil protozoa to emerge in forms that may enhance their survival. For example, coincubation of the human enteric pathogen *Salmonella enterica* with a ciliated protozoan of *Tetrahymena* spp. resulted in the ingestion of the bacteria but they were resistant to digestion and were released in defecation vacuoles containing as many as 50 bacteria per vesicle (8). Bacteria in *Tetrahymena*-derived vesicles were significantly less susceptible to microbicidal chemicals or dehydration (8). This experiment provides an example of how bacterial resistance to intracellular digestion by a predatory protozoan can lead to packaging in vesicles that in turn is likely to affect the persistence and infectivity of this pathogen.

THE INTRACELLULAR ENVIRONMENT

By definition, intracellular pathogens reside inside cells during some part of the pathogenic process. In analyzing the sites of intracellular residence, there are two major types of intracellular locations: vesicular and nonvesicular compartments. Vesicular compartments include phagosomes resulting from host-cell- or microbe-induced ingestion and microbial created compartments such as the parasitophorous vacuole of *Toxoplasma gondii*. Nonvesicular compartments include all intracellular locations where the microbe is not enclosed by a membrane, such as free cytoplasmic residence. The majority of intracellular bacteria occupy vesicular compartments and those that are able to access the cytoplasm have specialized mechanisms that mediate phagosomal escape. Phagosomal compartments are generally viewed as harsh environments where ingested microbes are confronted with acidification, free-radical fluxes, nutrient deprivation, and a battery of antimicrobial proteins. Consequently, intracellular pathogens that inhabit phagosomal compartments interfere with their maturation and/or are resistant to host killing mechanisms. The cytoplasmic environment is generally considered nutrient rich and largely devoid of antimicrobial defenses (37), but two studies that have addressed this issue have provided inconsistent results. Nonpathogenic bacteria expressing *L. monocytogenes* listeriolysin O were found to escape to the cytoplasm after phagocytosis and replicate in there, consistent with the notion that the cytoplasm is a nutrient-rich and relatively unprotected cellular space (reviewed in Reference 37). However, there was no growth when bacteria that do not normally grow in the cytoplasm as part of their life cycle were placed in host cell cytoplasm by microinjection (18). This observation was interpreted to suggest that the ability of certain microbes to grow in the cytoplasm is an acquired trait in evolution (18).

There is no clear correlation between the type of intracellular residence and whether the microbe is an obligate or facultative intracellular pathogen or whether the microbe is acquired from another host or directly from the environment. Because host and/or environmental pressures are likely to have played a critical part in the emergence of the pathogenic strategy, the inability to correlate intracellular location with either category suggests that this distinction is not a dominant factor in the emergence of an intracellular pathogenic strategy. Instead, intracellular location is likely to be a distal outcome of adaptation to a host rather than a dominant engine driving evolution.

THE FOUR PHASES OF INTRACELLULAR PATHOGENESIS

Taking a bird's eye view of intracellular pathogenesis, it is possible to divide this process into four phases: (*a*) entry, (*b*) survival,

(*c*) replication, and (*d*) exit from the host cell. Entry, survival, and exit would appear to be essential for all intracellular pathogens, whereas most pathogens, but not all, also replicate in their host cells. Entry involves the steps necessary for the transition from the extracellular to the intracellular space. Each of these phases is primarily microbe-active or host-active depending on the specific host-microbe interaction. Entry can be achieved by the microbe by active or passive mechanisms. Examples of microbe-active entry are the invasion of host cells by *T. gondii* or the inducement of ingestion by cytoskeletal rearrangement in *Salmonella* spp. Microbe-passive entry mechanisms include opsonin-mediated phagocytosis, which delivers the microbe to a phagosome where it is not killed, as exemplified by the interaction of *C. neoformans* with macrophages. Microbes such as *H. capsulatum* manifest both microbe-active and microbe-passive mechanisms, whereby the fungal cell binds to the complement receptor through a direct fungal adhesion-receptor interaction that then leads to ingestion.

In contrast to entry, which can be a microbe- or host-active process, the type of survival strategy is determined largely by the microbe. Some microbes survive by subverting intracellular antimicrobial mechanisms such as phagosome-lysosome fusion, modulating phagosomal pH, damaging phagosomal membranes, and/or quenching microbicidal oxidative bursts. Others escape from the vesicular compartment, such as a phagosome to the nonvesicular compartment of the cytoplasm. Other microbes, like *T. gondii*, survive by creating their own compartments that are impervious to host microbicidal mechanisms. For all obligate intracellular pathogens and most facultative intracellular pathogens, survival in a permissive cell leads to replication with an increase in the intracellular microbial burden. Again, numerous exit strategies are dictated largely by the specific microbe in question. Some bacteria such as *Listeria monocytogenes* spread from cell to cell by actin tails. Others exit by inducing host cell lysis with release of microbial progeny. Yet others, like *C. neoformans*, exit the host cell by inducing phagosomal extrusion (5). Because intracellular residence could trap a microbe in its host, it is intriguing to consider that those microbes with specialized exit strategies represent that subset which is fully adapted to the intracellular lifestyle. Again, like the situation with the location of intracellular residence, no overriding theme emerges in the comparison of the various pathogenic strategies. Hence, apart from the overarching themes of entry, survival, replication, and cellular exit, the details associated with these phases of intracellular life for specific pathogens appear to be adaptations suitable for specific host-microbe interactions.

Despite the menagerie of variations used to achieve entry, survival, replication, and exit by different intracellular pathogens, there are only a few general solutions to these problems. Entry requires attachment to cellular receptors, and several facultative intracellular pathogens, including *M. leprae* (43), *H. capsulatum* (26), and *C. neoformans* (50), exploit the complement receptor. Others, including several gram-negative bacteria, induce their own uptake by manipulating host cytoskeletal functions (3). Once inside the cell, many intracellular pathogens ensure their own survival by interfering with the process of phagosome maturation. This strategy is used by such different intracellular pathogens as *M. tuberculosis* (facultative intracellular, mycobacteria, self host-acquired), *L. pneumophila* (facultative intracellular, bacteria, nonself host-acquired), *Chlamydia* spp. (obligate intracellular, bacteria, self host-acquired), *T. gondii* (facultative intracellular, protozoa, nonself host-acquired), and *H. capsulatum* (facultative intracellular, fungus, environmentally acquired). Given that this group includes diverse bacteria, fungi, and protozoa, a likely explanation for the commonalities in the process is convergent evolution.

ORIGIN OF VIRULENCE IN INTRACELLULAR PATHOGENS

The interaction of an intracellular microbe with a host can have positive, neutral, and

deleterious outcomes for each of the participants. Positive interactions include mutualism and are illustrated by the existence of endosymbiotic bacteria in invertebrate hosts. Neutral interactions are harder to define without more knowledge of the systems involved, because neutrality implies absence of damage for either party, which is akin to proving a negative. Deleterious interactions include the death of the microbe and damage to the host. Because this review is about intracellular pathogens, the discussion is focused on those interactions that are deleterious to the host.

Virulence is one outcome of the host-microbe interaction whereby the result is host damage, and this damage can come from the microbe, the host immune response, or both (9). Because host-acquired intracellular pathogens are host dependent, there is no obvious requirement for the host-microbe interaction to be deleterious to the host unless host damage is required for microbial replication or transmission. In general, the virulence of host-acquired microbes is a result of an unbalanced interaction whereby microbe- and/or host-mediated damage affects homeostasis. Two well-studied facultative intracellular pathogens acquired from other hosts include *M. tuberculosis* and *L. pneumophila*, which occupy the ecological sites of humans and amoebae, respectively. These organisms manifest similar intracellular survival strategies, and, for both, lung disease has strong components of host-mediated damage. For *M. tuberculosis* pulmonary cavitary formation with cough and the formation of infective aerosols is a critically important mechanism for host-to-host spread. For *L. pneumophila*, human infection is a dead-end event that is unlikely to be a significant selection force for this protozoal associated microbe.

For environment-acquired intracellular pathogens, the encounter with a potential host can have different implications. Using *C. neoformans* and *H. capsulatum* as examples of environmentally acquired facultative intracellular pathogens, one can discern significant differences from host-acquired microbes. Neither of these fungi requires other hosts for completion of its life cycle. For environmentally acquired microbes their interactions with animal hosts are a potential dead end that results in the death of the microbe. Because these interactions are likely to be rare and to involve only a minute fraction of the individuals in the populations, they cannot be expected to provide a major selection pressure for the evolution of intracellular pathogenic strategies. Instead, it is more likely that intracellular pathogenic strategies of environmentally acquired microbes are the result of adaptations to their ecological niche. In this regard, protozoal grazers such as amoebae have emerged as an important biotic force that could select for bacterial characteristics that translate into fitness in vertebrate hosts (34). For example, *Escherichia coli* strains harboring the Shiga toxin genes are more resistant to predation by the protozoan *Tetrahymena pyriformis*, raising the intriguing possibility that this important virulence factor was initially selected for environmental survival and only accidentally functions to harm animal hosts (49). Similarly, the interactions of several pathogenic fungi are similar to those with macrophages, and passage of an avirulent strain of *H. capsulatum* in amoebae restored its virulence (27, 47, 48). For *C. neoformans*, the interactions with amoeboid cells of *Dictyostelium discoideum* also enhanced virulence for mice (46).

HOW COMMON IS THE CAPACITY FOR INTRACELLULAR LIFE?

The phenomenon of intracellular pathogenesis is often viewed as a specialized host-microbe interaction despite considerable evidence that most, if not all, microbes have some capacity for intracellular survival. Host-associated microbes, whether endosymbionts, commensals, or pathogens, all require some capacity for intracellular life. Many human

Table 1 Human pathogenic microbes that can survive ingestion by amoebae[a]

Type	Organism	Reference
Bacteria	*Chlamydia pneumonia*	(14)
	Legionella pneumophila	(34)
	Listeria monocytogenes	(56)
	Escherichia coli	(4)
	Mycobacterium avium	(36)
	Coxiella burnetii	(24)
	Francisella tularensis	(1)
	Vibrio cholerae	(2)
	Helicobacter pylori	(54)
	Pseudomonas aeruginosa	(19)
Fungi	*Cryptococcus neoformans*	(48)
	Histoplasma capsulatum	(47)
	Sporothrix schenckii	(47)
	Blastomyces dermatidis	(47)

[a]Not a complete list. For a more extensive reference list see Reference 19.

intracellular pathogens are capable of survival in amoebae (**Table 1**). Hence, it should not be surprising that investigators often discover that common pathogens not considered intracellular pathogens can survive and often replicate in host cells. Examples of such organisms classified as extracellular that have been recently found to survive and replicate inside cells include *Staphylococcus aureus* (41), *Streptococcus pyogenes* (33), *Enterococcus* spp. (51) and *Helicobacter pylori* (40). In fact, analysis of gene expression patterns of *S. aureus* upon entry into human epithelial cells suggests a response that is adapted to the intracellular environment (16). Environmental microbes such as bacteria and fungi are subject to grazing by protista such as amoebae. Once ingested, their survival is a function of their ability to escape from the host cell and/or inhibit the host microbicidal mechanisms that are presumably designed for nutrient acquisition (30).

Because all soil microbes are in an ecological niche inhabited by innumerable species of amoeboid predators, survival in such an environment would almost certainly require the selection of effective mechanisms for intracellular survival. If this is the case, then the capacity for intracellular life must be a common and necessary attribute for survival in both environmental and host ecological niches. Hence, the ability of microbes to survive inside other cells might be the rule rather than the exception, with intracellular pathogens representing the subset of microbes that are capable of both intracellular survival and mediating damage to the host, either inadvertently or as a necessary condition for replication and survival. Such a view would redefine the outcome of evolution of intracellular pathogens and shift it from a specialized survival strategy to a common microbial survival mechanism that is sometimes associated with deleterious effects on the host.

CONCLUSION

In the introduction, we considered some of the problems inherent in the terminology of the phrase evolution of intracellular pathogens. However, in approaching this subject it is apparent that there is also a problem of perspective, whereby intracellular pathogenic strategies are seen as specialized microbial adaptations rather than common interactions that sometimes are deleterious to the host. Such viewpoints are critically important to how one might view the evolution of intracellular pathogens. If the capacity for intracellular lifestyle is a common microbial attribute, then similarities in the host subversion mechanisms observed for intracellular pathogens are likely a consequence of divergent evolution whereby the outcome of pathogenicity can arise by chance or as part of microbial specialization to a particular host. However, if the capacity for intracellular lifestyle is relatively rare among the microbiota, then the similarities among numerous unique intracellular survival strategies described for intracellular pathogens represent convergent evolution to solve the problem of intracellular survival. At this time, we do not have sufficient information to choose between these possibilities, and both may apply to different sets of intracellular pathogens. For example, similarities

in the intracellular pathogenic strategy of phylogenetically distant microbes such as fungi and bacteria may represent convergent evolution, whereas the variations in intracellular survival mechanisms among gram-negative bacteria may represent divergent evolution from ancient ancestor strategies to survive phagocytic predators. To investigate these possibilities, one would need additional information on the innate capacity of pathogenic and nonpathogenic microbes to survive in both vertebrate and nonvertebrate hosts.

More definitive conclusions about the evolution of intracellular pathogens will require a better delineation of the diversity of microbial life on earth combined with an assessment of potential intracellular living opportunities and threats at various ecological sites. For example, current views on the association of amoebae with the emergence of virulence in such organisms as *L. pneumophila* and *C. neoformans* have been inferred from the interactions of these microbes with only a few amoeboid species. Because the biota contains vast numbers of amoeboid species, it would seem logical to ascertain the generality of observations made with such common laboratory species as *Acanthamoeba castellani* with wild amoebae. Given the immense number of host-microbe interactions, such a project appears to involve a staggering amount of work. However, it is possible that the outlines of the problem will emerge after studying only a few more interactions in the same manner that the completion of a few microbial genomes provided fundamental insights into the relationship of facultative and obligate intracellular pathogens and illustrated the phenomenon of genome reduction.

SUMMARY POINTS

1. Each intracellular pathogen likely adopts a unique intracellular survival strategy. The uniqueness of each microbial strategy follows from the uniqueness of each microbial species and its niche.
2. Despite the uniqueness of each host-microbe interaction, there are relatively few solutions to the problem of intracellular entry, survival, and escape. For phylogenetically distant microbial species, similarities in intracellular pathogenic strategies are probably most easily explained by convergent evolution.
3. For a microbial species, the benefits of intracellular life are balanced by the loss of potential free-living habitats and by genome reduction. Organisms such as *Mycobacterium leprae* have lost their capacity for living independently of their hosts, and increased host specialization severely limits the number of host species available for infection and survival. Loss of identity is exemplified by mitochondria and may be one outcome of the endosymbiotic relationship. Hence, the attraction of intracellular life may ultimately doom such microbes through dependence on vulnerable hosts, Muller's ratchet phenomena, or loss of identity.
4. The ecological site from which a microbe is acquired during infection is an important consideration when analyzing the selection forces responsible for the evolution of intracellular pathogens. Specifically, it is important to consider whether the microbe is under active predation by larger microbes capable of ingesting it, and to identify those phagocytic predators. Amoeboid predators can be found within animal hosts, where they could conceivably provide selection pressures on host-associated flora.

5. Whereas intracellular pathogenesis is often viewed as a specialized lifestyle, there is emerging evidence that most pathogenic microbes are capable of intracellular survival, at least during some stages of the infection and disease cycle. For example, organisms such as *S. aureus*, *S. pyogenes*, *H. pylori*, *B. anthracis* and *Aspergillus fumigatus* are found inside host cells and most can replicate in that environment. Even ciliated organisms that are almost always found in extracellular spaces like *Trypanosoma* spp. have sophisticated mechanisms for invading vertebrate cells (7). Hence, the capacity for intracellular life may be the rule rather than the exception.

DISCLOSURE STATEMENT

The author is not aware of any biases that might be perceived as affecting the objectivity of this review.

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