Advances in Experimental Medicine and Biology 710

Eleftherios Mylonakis
Frederick M. Ausubel
Michael Gilmore
Arturo Casadevall *Editors*

Recent Advances on Model Hosts

Aegean Conferences

Springer

ADVANCES IN EXPERIMENTAL MEDICINE AND BIOLOGY

For further volumes:
http://www.springer.com/series/5584

Eleftherios Mylonakis • Frederick M. Ausubel
Michael Gilmore • Arturo Casadevall
Editors

Recent Advances on Model Hosts

Springer

Editors
Eleftherios Mylonakis
Harvard Medical School
Massachusetts General Hospital
Boston, MA, USA
emylonakis@partners.org

Frederick M. Ausubel
Harvard Medical School
Massachusetts General Hospital
Boston, MA, USA
ausubel@molbio.mgh.harvard.edu

Michael Gilmore
Harvard Medical School
Schepens Eye Research Institute
Boston, MA, USA
mgilmore@vision.eri.harvard.edu

Arturo Casadevall
Department of Microbiology
and Immunology
Albert Einstein College of Medicine
Yeshiva University of Medicine
Bronx, NY, USA
arturo.casadevall@einstein.yu.edu

ISSN 0065-2598
ISBN 978-1-4419-5637-8 e-ISBN 978-1-4419-5638-5
DOI 10.1007/978-1-4419-5638-5
Springer New York Dordrecht Heidelberg London

Library of Congress Control Number: 2011939199

Contents

Chapter 1
Amoeba Provide Insight into the Origin of Virulence in Pathogenic Fungi

Arturo Casadevall

Abstract Why are some fungi pathogenic while the majority poses no threat to humans or other hosts? Of the more than 1.5 million fungal species only about 150–300 are pathogenic for humans, and of these, only 10–15 are relatively common pathogens. In contrast, fungi are major pathogens for plants and insects. These facts pose several fundamental questions including the mechanisms responsible for the origin of virulence among the few pathogenic species and the high resistance of mammals to fungal diseases. This essay explores the origin of virulences among environmental fungi with no obvious requirement for animal association and proposes that selection pressures by amoeboid predators led to the emergence of traits that can also promote survival in mammalian hosts. In this regard, analysis of the interactions between the human pathogenic funges *Cryptococcus neoformans* and amoeba have shown a remarkable similarity with the interaction of this fungus with macrophages. Hence the virulence of environmental pathogenic fungi is proposed to originate from a combination of selection by amoeboid predators and perhaps other soil organism with thermal tolerance sufficient to allow survival in mammalian hosts.

The Pathogenic Fungi

The human pathogenic fungi comprise a highly diverse group of organism that can be broadly classified into two broad groups: dermatophytes and systemic mycoses. The dermatophytes are relatively common pathogens and include such agents as Tinea pedis, a cause of athlete's feet. Dermatophytes cause troublesome conditions but are rarely life-threatening. In contrast, systemic mycoses are rare in immunologically intact human populations. In comparison to bacterial and viral diseases that are known since antiquity, systemic appear to be relative latecomers to the parade of human pathogens. Diseases such as cryptococcosis, blastomycosis, histoplasmosis and coccidiomycosis were only described in the late nineteenth or early twentieth centuries. In fact, most pathogenic fungal species were identified in the twentieth century and the overwhelming majority is exceedingly rare, thus related to case reports. Although sporadic systemic fungal diseases almost certainly

A. Casadevall (✉)
Departments of Microbiology and Immunology and Medicine (Division of Infectious Diseases), Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461, USA
e-mail: arturo.casadevall@einstein.yu.edu

E. Mylonakis et al. (eds.), *Recent Advances on Model Hosts*,
Advances in Experimental Medicine and Biology 710, DOI 10.1007/978-1-4419-5638-5_1,

must have affected certain individuals in human populations since the emergence of humanity, these were not recognized as such, probably because of the relatively paucity of cases and the fact that their non-contagiousness meant the absence of epidemics with recognizable common symptoms that would have led to their identification as cause of disease.

Endothermy and Susceptibility to Fungal Diseases

The paucity of fungal diseases in mammals relative to other classes of pathogenic microbes is striking given that fungi are major pathogens for plants, insects and amphibians. In fact, fungi are the major pathogens of plants and a chytrid fungus has caused the extinction of numerous amphibian species in recent years. Given the susceptibility of amphibians the relative resistance of mammals to fungi cannot be attributed to be a general characteristic of vertebrates. When considering the mammals relative to other vertebrates and animals they have several striking characteristics such as being endothermic, homeothermic and possession an immune system capable of both innate and adaptive immunity. The same characteristics also apply to birds, which are also relative resistant to fungal infections except for thermophilic fungal genera such as *Aspergillus* spp.

Evidence for the synergistic contribution of temperature and sophisticated immunity to host resistance against fungi is provided by the work of Dr. John Perfect using rabbits as a model for cryptococcosis (Perfect et al. 1980). Rabbits have the relatively high body temperature for mammals with core temperatures of 41°C, a temperature that is near the temperature tolerance limit for many cryptococcal strains. Hence, it is very difficult to induce systemic cryptococcosis in rabbits and this presumably is a consequence of the fact that fungi near their thermal limit of viability are in such stressed conditions that they are highly vulnerable to host defense. However, localized infection can be caused in rabbits by injecting yeast cells into testis, an organ that is several degrees cooler than the core, implying that the immune system alone cannot clear the infection when the fungus is in a less thermally stressed condition. However, when rabbits are immunosuppressed with corticosteroids it is possible to induce meningoencephalitis by direct injection of cryptococci into the cerebrospinal fluid, such that even thermally stressed yeast cells can persist in tissue in the setting of impaired immunity.

Additional evidence for the importance of the contribution of endothermy to mammalian host defense against fungi comes from the ongoing decline of bats in North America as a consequence of a new fungal disease caused by Geomyces destructans (Blehert et al. 2009). Beginning in 2006 bat die offs were reported in the Northeast of the United States accompanied by fungal growth in the bat nostrils in a disease named ‘white nose syndrome’. *Geomyces destructans* is a cold loving fungus that grows optimally at 12°C. During the summers bats are awake and maintain mammalian range temperatures but they hibernate during winters and their core temperature drops to the 6–11°C range, a permissive temperature for *Geomyces destructants* (Chaturvedi et al. 2010). Core temperatures have been shown to be important in both susceptibility and resistance to fungal diseases even among ectothermic vertebrates such as reptiles and amphibians. Lizards are unable to survive experimental fungal infection unless they can induce behavioral fevers. Similarly, frogs can be cured of chytrid infection when placed at 37°C.

Analysis of the thermal tolerance of several thousand fungal strains in a culture collection revealed that most thrived at ambient temperatures while undergoing a rapid decline in their growth viability at temperatures above 30°C (Robert and Casadevall 2009). A plot of the proportion of viable fungal strains versus temperature revealed a linear relationship such that for each degree in temperature increase between 30°C and 42°C there was a corresponding decline of 6% in terms strain viability (Robert and Casadevall 2009). Consequently, mammalian temperatures were sufficient to inhibit the majority of fungal strains. However, the mammalian lifestyle is characterized by a high basal metabolic rate that demands a high food energy intake. There is no good explanation

for the high temperatures associated with mammalian endothermy and homeothermy. However, solving for the temperature that optimally restricts fungal growth and metabolic costs yielded a temperature of 37°C, providing the tantalizing suggestion that the costly mammalian lifestyle may have been selected for the protection it provides against fungal diseases (Bergman and Casadevall 2010). If the temperature gradient between mammalian temperatures and the environment provides protection against environmental pathogens there is concern that global warming will reduce it and bring forth new fungal diseases (Garcia-Solache and Casadevall 2010).

Origin of Fungal Infection

When considering the virulence of pathogenic fungi it is instructive to categorize them by the place from which fungal infection is contracted. There are two major sources of fungal infection: other hosts and the environment. Humans are born sterile but are then rapidly infected with a large number of microbes that eventually becomes the commensal or host-associated microbiota. In this regard, Candida albicans is often acquired from the maternal flora and dermatophytes are probably acquired from other infected hosts. In contrast, most organisms responsible for systemic mycoses are probably acquired directly from environmental reservoirs. For example, a study of the age of seroconversion for cryptococcal infection of children from New York City revealed that the majority acquired serological evidence of infection in early life (Goldman et al. 2001), presumably by infection from urban sources of C. *neoformans* (Currie et al. 1994). Similarly, such organisms as *Blastomyces dermatitidis*, *Cocciodiodes immitis*, and *Histoplasma capsulatum* are likely to be acquired directly from environmental sources.

In general, human disease from fungi acquired from other hosts usually results from an alteration of the host–microbe relationship such that tissue damage affects homeostasis. For example, mucosal and/or systemic candidiasis is often a consequence of immune suppression, alterations in host bacterial flora and comprised integument. In recent years evidence has accumulated suggesting that some cases of chronic candidiasis are associated with genetic traits, such as mutations in the dectin signaling pathways. Such mutations are believed to impair host responses and thus alter the normal balance tipping the outcome towards disease. Similarly, dermatophyte-related disease such as athlete's foot occurs when the pedal environment is disturbed by shoe usage thus creating conditions for fungal proliferation and skin damage. In contrast, human disease from fungi acquired from the environment is usually self limited with severe cases reflecting infection in hosts with impaired immunity or large innocula as are sometimes encountered in special situations such as histoplasmosis following cave explorations and coccidiomycosis following paleontological excavations.

Fungi acquired from other hosts such as *Candida albicans* are already host adapted and they do not cause disease in the majority of infected hosts. In contrast, when fungi acquired from the environment infect a mammalian host they must adapt and survive in an environment that is very different than their natural habitat. Hence, the origin of virulence for fungi acquired from other hosts or directly from the environment must have fundamental differences with regards to fungal adaptation to the host and microbial traits necessary for survival in the host. In the case of *C. albicans* there is conclusive evidence that the same strains that exist in a commensal state in one individual can be associated with disease in the same individual with there is an alteration of the host–microbe relationship. Consequently, the same organism is pathogenic or non-pathogenic depending on the host state. However, for those organisms acquired directly from the environment inhabiting a mammal is not part of their life cycle and carries considerable danger of lethality from elevated temperatures and powerful immune defenses. Hence, the few environmental fungi capable of causing human disease must be endowed with traits that allow survival in mammalian hosts, and these traits must be independent of their ability to survive in such hosts given than neither animal virulence or carriage can be considered essential to their survival in the soil.

The Soil Environment

The human pathogenic fungi acquired directly from the environment inhabit soils, but these soils can differ significantly. For example, *C. neoformans* and *H. capsulatum* are often found in soils contaminated with bird and bat guano, respectively, while *C. immitis* is found in desert soils of the North American southwest. Soils are remarkably complex ecologic niches occupied by a complex biota with which such fungi must interact. A fascinating aspect of these soil-dwelling fungi is that they can recovered from soils in a fully virulent form. For example, early techniques for the isolation of *C. neoformans* and *H. capsulatum* from soils included injecting a soil slurry into rodents, which cleared other soil microbes but developed infection with those specific organisms. Similarly, placement of rodents in soils known to be infected with *C. immitis* resulted in their acquiring coccidiodiomycosis. These observations are significant because they indicate that soil residence is associated with the maintenance of pathogenic potential for mammalian hosts. Consequently, there is a high likelihood that selection pressures in the environment are responsible for the emergence and maintenance of traits that confer upon some soil microbes the capacity for survival in animal hosts. In considering the types of interactions that could have selected for attributes of virulence we have focused on amoeboid predators for these inhabit soils and share certain characteristics with cells of the host innate immune system.

Amoeba and Fungi Coexist in Many Ecologic Sites

Amoeba and fungi are found throughout the terrestrial environment and their co-existence in many ecologic niches provides them with the opportunity to interact. Although amoebae are generally associated with watery and moist environments they have the capacity to survive in dry conditions as a result of encystment. Consequently, amoeba can be found even in arid soils (Robinson et al. 2002), and this is important because it implies that even desert-inhabiting species like *C. immitis* can come into contact with them. Some species of amoeba are thermotolerant such that they can inhabit hot springs (Sheehan et al. 2003). Amoeba are frequent on the surfaces and internal tissues of edible mushrooms (Napolitano 1982), attesting to a close connection between the kingdoms. Amoebae are also found in the human mouth and nasal mucosa (Rivera et al. 1984).

When considering fungal–amoeba interactions is it important to note that there is tremendous diversity among amoebae species and that the biological diversity for this group of organisms is poorly understood. Estimates of the number of amoebae species in the biosphere prior to the development of deep sequencing techniques ranged from 40,000 to 100,000, with the understanding that such numbers were likely to be underestimates (Couteaux and Darbyshire 1988). In general, amoeba feast on bacteria and their prevalence in an ecologic site is often directly proportional to the numbers of bacteria found (Ettinger et al. 2003). However, there some types of amoebae that specialize in eating fungi and these are known as mycophageous amoebae (Couteaux and Darbyshire 1988).

The majority of laboratory studies of fungal–amoeba interactions have focused on only a few species of amoebae such as *Acanthamoebae castellanii* and *polyphag*a. The extent to which these species provide information that is representative of the types of fungal–amoeba interactions found in the environment is not known. Of these two amoebae *A. castellani* provides the major system because it has been adapted to grow in an axenic medium, an adaptation that greatly simplifies experimental design by eliminating the need for feeder bacteria but also provides for a much more artificial type of situation. Hence, when considering results obtained in laboratory conditions it is important to remember that such systems are quite distant from natural systems and that there is little or no information about the generalizability of observations to other amoebae species.

When provided with a choice of food prey amoebae can be choosy and different amoebae have different culinary interests. For example when the soil amoebae *Hartmannella glebae* is offered a choice of menus it prefers gram positive bacteria over gram negative bacteria and ignores algae, yeasts and molds (Upadhyay 1968). On the other hand, *A. castellanii* was originally isolated discovered as a contaminant in cultures of a *Cryptococcus* sp., where it was probably feasting (Castellani 1931), a finding supported by subsequent investigators who established that it preyed on yeast (Nero et al. 1964). *A. castellani* recognizes fungal cells through a mannose-binding receptor and phagocytosis places ingested yeast in the lysosomal pathway, which produces nutrients and activates phosphoinositide metabolism (Allen and Dawidowicz 1990a, b. The presence of surface receptors presumably allows *A. castellani* to discriminate between yeast particles and latex beads, such that it preferentially ingests the former (Bowers and Olszewski 1983). There is indirect evidence that yeast cells can produce products that interfere with phagocytosis, possibly as a defense mechanism against ameboid predators. For example, co-incubation of *Entoamoeba histolytica* with *Saccharomyces boulardii* and erythrocytes reduces erythrocyte ingestion, suggesting that yeast cells produce soluble factors that reduce amoeba phagocytic capacity (Rigothier et al. 1994).

Amoebae as Model Hosts for the Study of Fungal Virulence

The fact that amoebae resemble host phagocytic cells such as macrophages, which are essential cells in containing fungal infection, together with knowledge that they interact with fungi in ecologic sites and in the laboratory suggested their potential usefulness for studying fungal virulence. Early studies on the interaction of amoebae with human pathogenic fungi were carried out in the 1950s when Castellani demonstrated that *A. castellani* could devour and kill a strain of *Cryptococcus neoformans* (Castellani 1955). Subsequently, Bulmer and colleagues followed up the interesting observation that attempts to culture *C. neoformans* from mouse feces were often complicated by contamination of recovery plates with amoeba (Neilson et al. 1978). They identified the contaminating amoeba as *A. polyphaga* and proceeded to demonstrate that this strain devoured yeast cryptococcal cells but avoided rare pseudohyphal forms and proposed that a morphological transition from yeast to pseudohyphal provided an ‘escape hatch’ for *C. neoformans* to survive amoeba predation (Neilson et al. 1978; Bunting et al. 1979). When pseudohyphal forms were tested for virulence in a mouse model of infection they were found to be significantly less virulent than yeast forms, establishing that amoeba predation could select for variants with altered virulence. Apart from establishing the precedent of altered virulence following predation, the relevance of pseudohyphal strains in infection is unclear since such forms are very rare with *C. neoformans* and do not seem to play a major role in pathogenesis. The observation that some amoeba preyed on *C. neoformans* led Bulmer and colleagues to propose that these protists were major biological control agents in the environment, a finding supported by the observation that cryptococcal numbers in soils were inversely proportional to the presence of amoeba (Neilson et al. 1978; Bunting et al. 1979; Ruiz et al. 1982).

After the studies of Bulmer work with amoeba ceased until the late 1990s when these hosts again became interesting objects for investigation as a result of progress in understanding *C. neoformans*–macrophage interactions. At that time it was becoming apparent, and increasingly accepted, that *C. neoformans* was a facultative intracellular pathogen in mammalian hosts (Feldmesser et al. 2000, 2001). Furthermore, it was clear that *C. neoformans* survived in intracellular spaces with a unique pathogenic strategy that included no interference with initial phagosome maturation followed by later phagosomal membrane leakiness and the release of a large number of polysaccharide-containing vesicles into the host cell cytoplasm. The recognition that *C. neoformans* was an intracellular pathogen with a sophisticated and unique strategy introduced a conundrum since it was not immediately clear why a soil organism with no requirement for mammalian hosts for survival of completion of

its life cycle would possess such attributes. This observation combined with earlier findings that *C. neoformans* and amoeba interacted led to the hypothesis that such pathogenic strategies are the result of environmental selection for characteristics that accidentally led to traits that permitted survival in host phagocytic cells. The hypothesis was tested by investigating *C. neoformans* interactions with *A. castellani* and it was found that features of macrophage infection such as intracellular vesicular release were faithfully reproduced in amoebae (Steenbergen et al. 2001). Of note, *C. neoformans* attributes needed for mammalian virulence, such as the capsule, melanin synthesis and phospholipase were also found to be important for virulence in amoeba (Steenbergen et al. 2001). Those observations led to a formal proposal that virulence in *C. neoformans* and other environmental organisms with no requirements for animal hosts was a consequence of selection of traits that serendipitously also provided the means to survive animal immune defense mechanisms (Casadevall et al. 2003; Steenbergen and Casadevall 2003). In subsequent essays the proposal was expanded and modified to emphasize the point that such interactions may arise stochastically in the environment and result in the steady emergence of microbes with virulence potential for other hosts (Casadevall and Pirofski 2007; Casadevall 2007).

Subsequent studies have shown that not all virulence factors for mammals are necessary for virulence in amoebae. For example, the alpha mating factor locus is important has been shown to contribute to virulence in mice but no difference was observed in the interaction of congenic a and a strains in amobae (Nielsen et al. 2005). Similarly, the Ca^{2+}–ATPase (*ECA1*) type calcium pump of *C. neoformans* is involved in stress tolerance and is critical for thermolerance. *C. neoformans* strains deficient in ECA1 are avirulent for mice because of reduced thermotolerance but hypervirulent for A. castellani (Fan et al. 2007). The finding that some but not all virulence factors are required for animal and protist virulence can be expected from the enormous differences inherent in the comparison between these types of hosts. Certainly, amoeba have no immune systems and virulence factors that promote fitness in animal hosts by modulating immune function would not be expected to be relevant for amoeba pathogenesis. Similarly, fungal structures that affected amoeba receptors for yeast cell phagocytosis would not be relevant for mammalian virulence unless they had a similar impact on macrophages through the targeting of conserved pathways.

In an effort to broaden the scope of this concept our group also studied the interactions with amoeba and several other pathogenic fungi: *Candida albicans* (Steenbergen et al. 2001), *Blastomyces dermatitidis* (Steenbergen et al. 2004), *Sporothrix schenkii* (Steenbergen et al. 2004), and *H. capsulatum* (Steenbergen et al. 2004). *C. albicans* cells were killed by *A. castellani* while cells of the three dimorphic pathogenic fungi each survived the interaction with amoeba and killed their hosts. Cells of *S. schenkii* and *H. capsulatum* were readily ingested by amoeba but survived intracellularly while *B. dermatitidis* was seldom ingested. Of great interest was the serendipitous observation that coincubation of amoebae and cells of the three dimorphic fungi led to an increase in hyphal forms even at 37°C (Steenbergen et al. 2004). This observation was important because the yeast-hyphal transition is associated with virulence in several dimorphic fungi and this transition occurs as the temperature is changed from ambient (25°C; hyphal form) to mammalian (37°C; yeast form). Given that the yeast morphology is the type most likely associated with virulence it was always assumed that this transition was somehow related to the capacity for mammalian virulence but the mechanisms for evolutionary emergence where unknown, since again, none of these fungi has a requirement for an animal host for survival. Hence, the finding that the hyphal form could be induced at the nonpermissive temperature of 37°C by amoebae implied that this trait was also a consequence of environmental selection, possibly another form of the ‘escape hatch’ view first proposed by Bulmer when he noted that pseudohyphal forms of *C. neoformans* resisted predation (Neilson et al. 1978).

In contrast to *C. neoformans*, the interaction of *C. gattii* with *A. castellani* appears to be qualitatively different (Malliaris et al. 2004). Whereas, *C. neoformans* is readily ingested and appears to kill the amoeba as a consequence of intracellular pathogenesis, *C. gattii* is seldom found internalized and kills amoeba through an extracellular mechanism. The resistance of *C. gattii* to phagocytosis appeared

related to differences in the capsular polysaccharide structure. Subsequent studies established that the capsule protected against ingestion by amoeba providing another correlate between the function of this structure in mammalian pathogenesis and interactions with amoebae (Chrisman et al. 2010; Zaragoza et al. 2008). Another important parallel between *C. neoformans* interactions with amoebae and macrophages was related to the phenomenon of exiting from phagocytic cells. *C. neoformans* has been shown to be capable of non-lytic exocytosis from murine and human macrophages in a remarkable phenomenon where the host cells survive the event (Ma et al. 2006; Alvarez and Casadevall 2006; Alvarez et al. 2009). This phenomenon involves a complex cellular choreography whereby phagosomes containing *C. neoformans* are expulsed from the macrophages, either before or after phagosome to phagosome fusion, by a mechanism that must involve phagosomal-cell membrane fusion in a manner that preserves the integrity of the host cells. Non-lytic exocytosis has now been described in amobae (Chrisman et al. 2010). Although details of the non-lytic exocytosis phenomenon in amoeba and macrophages differ, such as their discordant response to cytochalasin D, the finding of same general phenomenon in such evolutionarily distant cells, one of which is commonly found in cryptococcal ecologic niches, suggests that this mechanism was initially selected for escape from environmental predators.

Other Single Celled Hosts for Fungi

In addition to amoeba two other single celled hosts have been evaluated for their interactions with *C. neoformans*: *Paramecium* spp. (Frager et al. 2010) and *Dictyostelium discoideum* (Steenbergen et al. 2003). Like some species of amoebae, *Paramecium* spp. were efficient grazers of *C. neoformans*, ingesting and digesting fungal cells. Hence, apart from a temporary cessation of movement after Paramecia encountered the fungal cells, no damage was apparent from their interaction with *C. neoformans*. Like *A. castellani*, *D. discoideum* ameboid cells were susceptible to *C. neoformans* although the much smaller cells of this social amoeba were significantly less efficient at phagocytosing yeast cells. Co-cultivation of an avirulent *C. neoformans* cells with *D. discoideum* cells resulted in an increase in virulence for mice, implying that exposure to *C. neoformans* to social amoebae cells resulted in changes that translated into increased fitness in the mammalian host. Although the mechanism responsible for the increase in virulence is not understood the relatively short incubation interval suggests that this effect did not involve selection for more virulent genetic variants but was perhaps mediated by epigenetic changes.

The studies with *D. discoidum* revealed that the concept of microbial opportunism could be extended to unicellular level. *C. neoformans* acapsular mutants that were not pathogenic for wild type *D. discoideum* were pathogenic for slime mold strains defective myosin VII suggesting that notion that avirulent microbes for normal hosts can be virulent for impaired hosts also occurs at the unicellular level (Steenbergen et al. 2003). The experience with *Paramecium* spp. and *D. discoiduem* suggests that the outcome of the protist model host interaction with *C. neoformans* is likely to reflect the specific characteristics of the interacting pair and it is likely that each different fungal–protist interaction is unique. This concept is important because there are innumerable protist species in the environment and caution is warranted in generalizing results from specific interactions.

Thoughts Towards a Synthesis on the Origin of Virulence

The observations that: (1) many aspects of *C. neoformans* interactions with amoebae closely resemble interactions with macrophages (Steenbergen et al. 2001; Zaragoza et al. 2008); (2) virulence factors of *C. neoformans* for mammals are also virulence factors for amoebae (Steenbergen et al. 2001);