Chapter I

Introduction

Penicillium marneffei is the only dimorphic species of the genus *Penicillium* that causes penicilliosis marneffei in HIV-infected individuals living in the Far East, including Thailand, Myanmar (Burma), Cambodia, southern China, Indonesia, Laos, Malaysia and Vietnam. The number of cases of penicilliosis has increased dramatically over the past several years in Thailand. Between 1991 and 1997, penicilliosis was diagnosed in 1,173 HIV-infected patients at Chiang Mai University hospital alone (Supparatpinyo *et al.*, 1992). Other countries in the endemic area reporting cases of penicilliosis in HIV-infected patients include Hong Kong, Taiwan and Malaysia. Cases have also been reported in HIV-infected patients in Australia, France, Germany, Italy, the Netherlands, Sweden, Switzerland and the United Kingdom (Duong, 1996). All of these penicilliosis patients had previously visited the endemic area.

1. *Penicillium marneffei* -general introduction

P. marneffei is unique in its genus in being dimorphism by growing in living tissue or in culture at 37°C as yeast-like organisms (although the latter are more properly described as fission arthroconidia) or in culture at environmental temperatures as a mycelial phase. The ability of *P. marneffei* to grow at 37°C must play a major role in its infectivity (Hamilton, 2003). At 37° C *in vitro*, *P. marneffei* is grown as arthroconidia divided by fission to produce yeast cells. The morphology of yeast cells grown in vitro differs from that found *in vivo* (Cánovas & Andrianopoulos, 2007). It depends on the nutrition conditions of culture media which influence the morphology of *P. marneffei* yeast cells (Tongchusak *et al.*, 2004). We investigated the effect of different morphological yeast cells against antifungal susceptibility.

2. Melanin–its definition

Melanins are dark brown or black pigments which are formed by the oxidative polymerization of phenolic or indolic precursors. They are widely distributed in all living kingdoms and are negatively charged, hydrophobic pigments, which are recalcitrant to degradation. Typically they are insoluble in aqueous and organic solvents (Jacobson, 2000). Melanins are generally subdivided into 3 typical types: eumelanins, which are formed from quinines and free radicals by a complex

1

polymerization process, phaeomelanins, which are formed from tyrosine and cysteine precursors, and allomelanins, which are produced from nitrogen free precursors (Hamilton and Gómez, 2002). The identification of a pigment as a melanin relies on various biophysical techniques such as electron spin resonance (ESR) spectroscopy, which establishes that the particular compound under study is a stable free radical (Enochs *et al.*, 1993).

2.1 Immunological behavior of melanins

Melanins are immunologically active molecules, which are capable of inducing strong T-cell independent antibody responses (Nosanchuk *et al.*, 1998); they also contain anti-inflammatory properties (Mohagheghpour *et al.*, 2000). *C. neoformans* melanin has been shown to activate the alternative complement cascade (Rosas *et al.*, 2002) and has ability to inhibit the recognition of the organism by host defenses (Huffnagle *et al.*, 1995). In contrast, grape melanin has anti-inflammatory and immunosuppressive properties–injection of grape melanin into rats inhibits the development of primary and secondary adjuvant induced disease, and results in a significant decrease in the detectable levels of various cytokines in these animals (Avramidis *et al.*, 1998). Production of the pro-inflammatory cytokines IL-1β, IL-6, IL-10 and TNF–α by lipopolysaccharides (LPS) stimulated human peripheral blood monocytes has also shown to be inhibited by synthetic melanin (Mohagheghpour *et al.*, 2000).

2.3 Resistance to antimicrobial compounds

Several recent studies have been undertaken to determine whether melanin produced by fungal pathogens alters their susceptibility to anti-fungal drugs. Melanized *C. neoformans* cells have been shown to be less susceptible to amphotericin B (AmB) when compared with non-melanized cells (Wang and Casadevall, 1994). Such observations suggest that melanin may protect the organism against the effects of AmB by binding the drug and preventing its penetration; alternatively it may reduce cell wall permeability to AmB. Subsequently the effects of melanin synthesis by *C. neoformans* and *H. capsulatum* on their susceptibilities to the antifungal drugs; AmB, caspofungin, fluconazole, itraconazole and flucytosine have been investigated using the standard minimal inhibitory concentration (MIC) M27A protocol (van Duin *et al.*, 2002). However, more detailed killing assays demonstrated that melanin can indeed protect *C. neoformans* and *H. capsulatum* against the activity

of AmB and caspofungin by decreasing the susceptibilities of both fungi to these drugs. In addition, this suggested that these drugs were in fact being absorbed by melanin (van Duin *et al.*, 2002). In contrast, exposure of melanin to other antifungals, such as fluconazole, itraconazole or flucytosine had no detectable effect on the C:N ratio, which indicated that there was no binding of melanin to these drugs. More recently, Ikeda and colleagues (2003) have confirmed the role of *C. neoformans* melanin in resistance to AmB. These authors found that pigmented *C. neoformans* cells were much more resistant to killing by AmB when compared with non-pigmented cells. It thus seems likely that infections with melanized *C. neoformans* and *H. capsulatum* cells may be more difficult to treat given that they are less susceptible to killing by both AmB and caspofungin (van Duin *et al.*, 2002). Interestingly, although caspofungin exhibits measurable *in vitro* activity it has been shown to be ineffective in treatment using *in vivo* models of *C. neoformans* (Abruzzo *et al.*, 1997) or *H. capsulatum* (Kohler *et al.*, 2000).

Our previous study, *P. marneffei* had been found to produce melanin or melanin-like compounds in vitro and during infection (Youngchim *et al.*, 2005). In this respect, melanin presumably contributes to *P. marneffei* virulence by promoting survival within host tissue. Given the potential role of melanin in virulence of *P. marneffei,* we investigated the effect of melanin on the susceptibility to antifungals.

3.   Maintenance of virulence for human pathogenic fungus

The idea that pathogenic microbes are endowed with certain components that confer upon them the capacity for virulence is the central theme of the virulence factor concept. Although the definition of what constitutes a virulence factor is varied and debatable (Casadevall & Pirofski 1999), and this idea has been undermined by the finding that commensal organisms cause disease (Casadevall & Pirofski 2001), the concept maintains a powerful understanding in the imagination of investigators and continues to drive much of the intellectual and experimental energy in the field of microbial pathogenesis. The virulence factor concept has unquestionably led to the identification of important microbial attributes of virulence that have greatly furthered our understanding of microbial pathogenesis. Nevertheless, the virulence factor concept has significant limitations for a global understanding of microbial virulence.

Most of human-pathogenic fungi that are attained from the environment dwell in ecological niches defined by soils, trees, and decaying vegetation. Soils are extreme environments, and soil-dwelling microbes must alter to rapidly changing, harsh conditions. Soil microbes occupy an environment where there must be brutal competition for nutrients. In addition to these nutritional and physical stresses, soil-dwelling microbes must cope with predators in the form of amoebae and other protista, which feed on bacteria and fungi. Consequently, soil-dwelling microbes must develop ways to escape phagocytosis and/or survive ingestion through mechanisms for intracellular survival.

The incidence of invasive fungal disease has significantly increased over the past few decades corresponding to the rising number of immunocompromised patients. The major risk factors for severe fungal disease include administration of broad-spectrum antibiotics, corticosteroids and cytotoxic agents, invasive medical procedures, and HIV infection. The rise in the incidence of fungal infection has led researchers to identify virulence determinants and to examine why environmental fungi cause disease.

The amoebae are an extremely diverse group of eukaryotic microorganisms that constitute a major class of phagocytic organisms in soils. *Acanthamoeba* is a free-living, ubiquitous ameba that occurs in trophozoite and cyst stages during its life cycle. In addition, *Acanthamoeba castellanii* is a soil amoeba that feeds on bacteria and fungi that was originally isolated from cultures of *Cryptococcus* neoformans, and has been used to study bacteria–amoeba interactions. Both plant and animal pathogenic fungi that reside in soils and vegetation inhabit extreme environments where they must compete with other microbes, endure extremes of humidity and survive predation by amoeboid organisms and small animals such as nematodes. Hence, both share comparable risks and selection pressures. For several human pathogenic fungi it has been demonstrated that determinants of virulence needed for mammalian pathogenicity are also important for surviving predation by amoeba, slime molds, and nematodes (Steenbergen, *et al.*, 2001; 2003; 2004).

Most common pathogenic fungi are considerate saprophytic because they are free living and do not require an animal host for propagation. Likewise, several dimorphic fungi are important human pathogens, but the origin and maintenance of virulence in these organisms is mysterious, since an interaction with a mammalian

host is not a required for fungal survival. For instance, *Cryptococcus neoformans* was shown to interact with macrophages, slime molds (Steenbergen, *et al.*, 2003) and amoebae (Steenbergen, *et al.*, 2001) in a similar manner, suggesting that fungal pathogenic strategies may arise from environmental interactions with phagocytic microorganisms.Since thermally dimorphic fungi are found primarily in the soil, we hypothesized that *A. castellanii*, an environmental phagocytic predators could place selective pressures on *P. marneffei* soil fungi. Here, we investigated the interaction of *P. marneffei* with *A. castellanii*. According to global warming has been assumed to bring about new fungal diseases in the coming century, to understand the mechanisms by which virulence emerges in environmental microbes is necessary (Garcia-Solache, & Casadevall, 2010).