Arthroconidia production in *Trichophyton rubrum* and a new *ex vivo* model of onychomycosis

S. Amir Yazdanparast1 † and Richard C. Barton2

1Department of Microbiology, University of Leeds, Leeds LS2 9JT, UK
2Mycology Reference Centre, Department of Microbiology, Leeds General Infirmary, Leeds LS1 3EX, UK

The dermatophyte fungus *Trichophyton rubrum* often produces arthroconidia in *vivo*, and these cells are thought to be involved in pathogenesis, and, in shed skin scales, to act as a source of infection. The purpose of this study was (i) to examine the environmental and iatrogenic factors which affect arthroconidiation in *T. rubrum in vitro*, (ii) to look at arthroconidia formation in a large number of clinical isolates of *T. rubrum* and (iii) to develop a new model for the study of arthroconidia formation in nail tissue. Arthroconidia production was studied in *T. rubrum* grown on a number of media and under conditions of varying pH, temperature and CO2 concentration. The effect of the presence of antifungals and steroids on arthroconidia formation was also examined. Nail powder from the healthy toenails of volunteers was used as a substrate for arthroconidial production. On Sabouraud dextrose agar in the presence of 10 % CO2 plus air, arthroconidial formation occurred optimally at 37 °C and pH 7-5, and was maximal at 10 days. Most isolates of *T. rubrum* showed a similar level of arthroconidial production, and only two out of 50 strains were unable to produce arthroconidia. Subinhibitory levels of some antifungals and betamethasone resulted in the stimulation of arthroconidia formation. Arthroconidial production in ground nail material also occurred under the same optimal conditions, but took longer to reach maximal levels (14 days). These *in vitro* and *ex vivo* results provide a useful basis for the understanding of arthroconidium formation *in vivo* in infected tissues such as nails.

**INTRODUCTION**

The high incidence of dermatophyte infections makes them an important public health problem (Aly, 1994), and studies estimate that between 10 and 15 % of the population worldwide is affected (Matsumoto, 1996). In spite of rapid improvements in treatment during the past decade, many aspects of dermatophyte infections remain poorly understood. Dermatophyte infection of the nail, known as tinea unguium or onychomycosis, is perhaps the most refractory to treatment, and requires several months of systemic therapy, with terbinafine being the most effective agent (Evans, 1999), even though a success rate of greater than 80–85 % cannot be expected. Possible reasons for the 20 % failure rate include the development of antifungal resistance, poor pharmacokinetics due to the characteristics of the nails (i.e. excessive thickness) and the presence of resistant structures such as subungual dermatophytomas (Evans & Roberts, 1998), and arthroconidia, which are frequently seen in infected nails, and whose role in disease has long been debated (Chin & Knight, 1957). Arthroconidium formation, characteristic of dermatophyte infection of skin, hair or nails, results from the fragmentation of a hypha following multiple septum formation. Growth in the stratum corneum of skin, nails and hair occurs in the form of hyphae, which may then form arthroconidia. It has been suggested that arthroconidia are more commonly found in wet skin lesions and nails (Miyazi et al., 1971). The potential of arthroconidia to contribute to resistance to treatment is suggested by the fact that having no immediate exogenous nutritional requirements, they are resistant to adverse environmental conditions (Hashimoto & Blumenthal, 1978). Arthroconidia shed from lesions are likely to be the form involved in the spread of infection. The development of arthroconidia in *Trichophyton mentagrophytes* has been observed *in vitro* by a number of authors (Bibel et al., 1979; Emyanitoff & Hashimoto, 1979; Weigl & Hejtmanek, 1979; Wright et al., 1984) and in *Trichophyton rubrum* (Miyaji & Fujiwara, 1971). Here we describe a detailed examination of the environmental and iatrogenic factors which affect arthroconidiation *in vitro* in *T. rubrum*, and describe a new *ex vivo* model for the study of arthroconidium formation in nails.

**METHODS**

**Organism.** A total of 50 strains of *T. rubrum*, isolated from the skin...
and nails of patients, were used. Cultures were maintained at 27 °C by monthly subculture on Sabouraud dextrose agar (SDA), and permanent cultures were stored at −70 °C in 1-6 % DMSO.

**Media and culture conditions.** For arthroconidium formation, a standard pH of 5-6 was employed for SDA (Oxoid); other pHs were obtained by the addition of sodium hydroxide. When medium without glucose was required, peptone agar (Difco) at 1, 3, 5 and 10 % was used. Brain heart infusion (BHI, Oxoid), Trichophyton no. 1 (Difco), and SDA plus 1, 3, 5 and 10 % NaCl, were also used. Betamethasone 17-valerate, amphotericin B, itraconazole, griseofulvin and terbinafine were added to SDA to investigate their effects on arthroconidium formation. Each antifungal was dissolved in DMSO, and DMSO at the same concentration was used as a control. Cultures were incubated for up to 10 days at various temperatures, either under aerobic conditions or in air plus various CO2 concentrations using a LEEC CO2 incubator in which the CO2 concentration was set and controlled automatically by the incubator.

**Microscopic examination.** Adhesive tape was cut in to 1 cm squares, and was placed with sterile forceps on the surface of a colony, lifted off, and mounted colony side up, and a drop of 70 % ethanol and a drop of lactophenol cotton blue were placed on top. The number of arthroconidia was determined after examining four randomly selected fields of view under ×400 magnification. Arthroconidia were defined as any hyphal compartment < 4 μm long or any spherical cell representing a disarticulated arthroconidium that was not obviously an aleuroconidium (microconidium).

**Ground nails for ex vivo experiment.** Clippings from several healthy toenails previously found free from fungi when examined in 10 % KOH were cleaned with 70 % ethanol, dried in a sterile Petri dish at 37 °C, and ground with liquid nitrogen to a powder with a pestle and mortar. Plates of *T. rubrum* on SDA grown aerobically were harvested at 2 weeks by gently scraping the surface of the agar with a glass rod. The cells were suspended in distilled water, the pH was adjusted to 7-5 with NaOH, and the concentration of microconidia was standardized at 1 × 107 cells ml−1. Microconidial suspension of *T. rubrum* (20 μl) was spread over the surface of 0.2 g nail powder on a sterile microscope slide, and the inoculated slide placed on a glass rod within a glass Petri dish. Sterile water was added to the Petri dish to maintain a humid atmosphere, and then incubation was carried out aerobically or in 10 % CO2 at 37 °C for 14 days. The powder was then mounted, and examined microscopically in 10 % KOH, with or without lactophenol cotton blue staining.

**RESULTS AND DISCUSSION**

**Factors affecting arthroconidium formation in *T. rubrum***

Arthroconidium formation occurred in all five isolates of *T. rubrum* used in preliminary studies, when 10 % of the incubation atmosphere was replaced by CO2 on SDA media at 37 °C after 10 days. These conditions were used as standard in the comparison of the effect of different factors on arthroconidium formation. When cultured in normal air, the frequency of arthroconidium formation was zero. The frequency of arthroconidium formation was at 10 days (Fig. 1), and the optimal percentage of CO2 for arthroconidium formation in *T. rubrum* N250 was 10 % (Fig. 2). Arthroconidium production in *T. mentagrophytes* in vitro at 31 °C is also induced by cultivation in an atmosphere with higher CO2 tension (Bibel et al., 1979). Curiously, Gupta et al. (2003) have found that *Trichophyton rautbeschkii*, which some authors consider to be conspecific with *T. rubrum*, produces arthroconidia under normal aerobic conditions, and that this is not stimulated by 10 % CO2. Whether the stimulated arthroconidiogenesis is due to the presence of CO2 itself or reduced oxygen tension has been the subject of some controversy (Barrera, 1986). Some investigators (Emyanitoff & Hashimoto, 1979) have shown that exogenous CO2 is not the absolute requirement, and that replacement of air by either N2 or CO2 stimulates arthrosporulation in *T. mentagrophytes* to the same extent. However, King et al. (1976) have shown that while helium and methane, like CO2, tend to inhibit the growth of *T. mentagrophytes*, they do not induce arthroconidial formation. CO2 diffuses physiologically from normal skin (Frame et al., 1972), and its level rises in damaged or occluded skin (Malten & Thiele, 1973). Thus a rise in CO2 tension together with a reduction in oxgen tension during infection may cause the transformation of dermatophyte hyphae to arthroconidium chains (Allen & King, 1978).
Table 1. Effect of five different media on arthroconidial production in five strains of T. rubrum

The mean and 95% confidence limits are shown (n=3).

<table>
<thead>
<tr>
<th>Strain</th>
<th>BHI agar</th>
<th>Trichophyton agar no. 1</th>
<th>Medium</th>
<th>SDA</th>
<th>SDA + 1% NaCl</th>
<th>1% peptone agar</th>
</tr>
</thead>
<tbody>
<tr>
<td>N250</td>
<td>95.0±17.4</td>
<td>20.0±7.4</td>
<td>N250</td>
<td>283.0±17.4</td>
<td>185.0±11.4</td>
<td>110.0±10.8</td>
</tr>
<tr>
<td>N251</td>
<td>75.0±19.7</td>
<td>45.0±10.8</td>
<td>N251</td>
<td>269.7±5.2</td>
<td>160.0±41.8</td>
<td>115.0±11.4</td>
</tr>
<tr>
<td>15477</td>
<td>80.0±13.8</td>
<td>59.0±12.4</td>
<td>15477</td>
<td>180.0±4.3</td>
<td>130.0±10.8</td>
<td>85.0±23.7</td>
</tr>
<tr>
<td>14686</td>
<td>77.0±24.4</td>
<td>34.0±13.1</td>
<td>14686</td>
<td>160.0±11.4</td>
<td>155.0±11.4</td>
<td>150.0±227</td>
</tr>
<tr>
<td>13626</td>
<td>60.0±5.0</td>
<td>25.0±25.9</td>
<td>13626</td>
<td>268.7±10.0</td>
<td>140.0±11.4</td>
<td>103.0±16.3</td>
</tr>
</tbody>
</table>

In the presence of 10% CO₂, SDA was the optimal medium for arthroconidium formation for most strains. No growth was seen in media containing >3% NaCl or >3% peptone, and the addition of 1% NaCl did not increase arthroconidium formation (Table 1). Levels of arthroconidium formation were reduced even further with other media. Miyazi & Fujiwara (1971) have reported that T. rubrum produces a greater number of spherical cells (arthroconidia) on BHI at 37°C than on other media, and that the presence of 0.85% NaCl or ornithine is important for arthrosporulation.

When cultures were incubated at various temperatures with 10% CO₂, a temperature of 37°C was found to be maximal for arthroconidium formation (Fig. 3). At 42°C, no growth occurred. Temperatures between 32 and 39°C also strongly favour the arthroconidiation of T. mentagrophytes on SDA, and no or less arthroconidium formation is seen in T. mentagrophytes at 30°C (Emyanitoff & Hashimoto, 1979); in addition, arthroconidium formation in T. rubrum was seen at 37°C but not at 25°C by Miyazi & Nishimura (1971). The temperature required for optimal arthroconidium formation in vitro, 37°C, is higher than those recorded for the surface of the human body, typically 33±1°C, and near the upper limit for growth for this species. Although, generally, slightly acidic conditions favoured arthrosporulation, the optimal pH for arthroconidium formation was found to be 7.5 (Fig. 4). The optimal pH of the medium for arthroconidium formation in T. rubrum was found to be 7.4 by Miyazi et al. (1971).

Effect of antifungal agents and betamethasone on arthroconidia formation

Subinhibitory levels of several antifungal agents were tested for their effects on arthroconidium formation on SDA at 37°C. At subinhibitory concentrations, some of these antifungal agents, including griseofulvin and clotrimazole, stimulated the level of arthroporation, but itraconazole, and particularly terbinafine, suppressed arthroconidium production (Table 2). Similar effects have been seen in T. mentagrophytes, in which griseofulvin (0.5 μg ml⁻¹), clotrimazole (0.1 μg ml⁻¹), and particularly amphotericin B (5 μg ml⁻¹), stimulate a low level of arthroporation (Emyanitoff & Hashimoto, 1979). Compared to other antifungals, itraconazole, and particularly terbinafine, have significantly increased rates of cure and shorter treatment times (Roberts, 1999), and interestingly, in our study, only itraconazole, and particularly terbinafine, suppressed arthroconidium production in T. rubrum N250. These results may be clinically significant: if sufficiently fungicidal concentrations are not attained in some areas of the lesion, some antifungals may enhance the conversion of hyphae to arthroconidia. Hashimoto &
Blumthenthal (1978) have reported that dormant arthroco-
nidia of *T. mentagrophytes* are resistant to common anti-
mycotics such as griseofulvin, clotrimazole, miconazole and
nystatin. This resistance may be due to the nature of the
arthroconidial wall. The steroid betamethasone 17-valerate
at 1 mg ml⁻¹ caused appreciable stimulation of arthroco-
nidial wall. The steroid betamethasone 17-valerate
at 1 mg ml⁻¹ caused appreciable stimulation of arthroco-

<table>
<thead>
<tr>
<th>Antifungal</th>
<th>Concentration (µg g⁻¹)</th>
<th>Mean no. arthroconidia per field of view*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphotericin B</td>
<td>1·0</td>
<td>395±5·4</td>
</tr>
<tr>
<td></td>
<td>5·0</td>
<td>340±9·3</td>
</tr>
<tr>
<td>Griseofulvin</td>
<td>0·25</td>
<td>364±11·3</td>
</tr>
<tr>
<td></td>
<td>0·5</td>
<td>295±14·2</td>
</tr>
<tr>
<td>Clotrimazole</td>
<td>0·1</td>
<td>373±6·1</td>
</tr>
<tr>
<td></td>
<td>0·5</td>
<td>314±8·8</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>0·25</td>
<td>210±10·1</td>
</tr>
<tr>
<td></td>
<td>0·5</td>
<td>175±17·7</td>
</tr>
<tr>
<td>Terbinafine</td>
<td>0·001</td>
<td>120±8·1</td>
</tr>
<tr>
<td></td>
<td>0·05</td>
<td>95±7·3</td>
</tr>
<tr>
<td>DMSO (10%)</td>
<td>NA</td>
<td>275±19·3</td>
</tr>
<tr>
<td>Sabouraud broth control</td>
<td>NA</td>
<td>280±5·4</td>
</tr>
</tbody>
</table>

*The mean and 95% confidence limits are shown (n=3).

**Clinical isolates of *T. rubrum***

Of a total of 50 clinical isolates, obtained before, during and
after treatment with itraconazole and terbinafine, only two
strains were unable to produce arthroconidia under the
previously defined optimal conditions. Of these isolates, 45
were from nine patients (two to six per patient), and
therefore not necessarily independent isolates. However, the
two isolates that failed to produce arthroconidia came from
patients from whom arthroconidium-producing isolates
had also been obtained. This might represent the suppres-

**Arthroconidial formation in ex vivo nails**

Arthroconidial production in ground nails from the toenails of
volunteers occurred under the same optimal conditions,
and again did not occur unless the atmosphere was replaced
with 10% CO₂, although it took longer (14 days) to reach
maximal levels. This suggests that during invasion of the
nail by hyphal growth, changes to the environment of the
fungus caused by the reaction of the host tissue to the fungus
induce the formation of arthroconidia. For example,
hyperkeratosis, often seen in onychomycosis, may lead to
a decrease in local O₂ concentration and/or increase in
CO₂ concentration, as seen in damaged skin (Malten &
Thiele, 1973). It has recently been demonstrated in a similar
nail model (though without arthroconidial formation) that
the minimum fungicidal concentration of terbinafine
against *T. rubrum* within nails is increased up to 100-fold
compared with standard media (Osborne et al., 2004). This
suggests, in general, that the properties of this dermatophyte
in nail models are likely to differ significantly from those of
fungi grown on laboratory media and to be more
representative of the situation in vivo.

It has yet to be demonstrated that arthroconidia of *T.
rubrum* formed in vitro, and therefore presumably in vivo,
are more resistant to antifungal activity than hyphae.
Terbinafine, as one of the main therapeutic agents for
onychomycosis, has been demonstrated to be fungicidal,
though this has only been shown using actively growing
hyphae (Hazen, 1998). The thick walls of arthroconidia may
resist antifungal treatment, only to germinate following the
cessation of treatment. Indeed, an approach designed to
stimulate the germination of resistant propagules in
onychomycosis patients who failed antifungal therapy by
applying slides of Sabouraud agar to diseased nails for 24 h
prior to topical antifungal treatment has been shown to have
a high cure rate (Pierard et al., 2000).

In conclusion, this study has shown how certain conditions,
including physiological changes within the nail and anti-
fungal agents, may trigger the formation of arthroconidia
from hyphal cells, and that a key factor is the presence of
10% CO₂. Further work is needed to examine the widely
assumed importance of the role of arthroconidia in patho-
genesis and the resistance of dermatophytes to effective
antifungal treatment.

**REFERENCES**

Allen, A. M. & King, R. D. (1978). Occlusion, carbon dioxide and

Aly, R. (1994). Ecology and epidemiology of dermatophyte infec-

Barrera, C. R. (1986). Formation and germination of fungal arthro-


