Hydrocortisone-enhanced growth of *Aspergillus* spp.: implications for pathogenesis

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***Aspergillus fumigatus* and *Aspergillus flavus* are the most common cause of invasive mould infections worldwide and carry a high mortality. Corticosteroid therapy and Cushing's disease are associated with an increase in invasive aspergillosis. Corticosteroids impair immune function in mammals and, specifically, the conidicidal activity of human macrophages, which was thought to be sufficient explanation for this increased risk. However, we have found a 30–40% increase in growth rate of *A. fumigatus* and *A. flavus* exposed to pharmacological doses of hydrocortisone (a human glucocorticoid), suggesting an alternative or additional mechanism for the association. No significant effect was observed with other human steroids such as testosterone, oestradiol or progesterone, though a smaller (21%) but significant growth rate increase was obtained with the fungal sterol ergosterol. The presence of a ligand/receptor system is therefore possible in pathogenic *Aspergillus* spp. Although corticosterone-binding proteins have been identified in some yeast species, a demonstrable physiological effect has been lacking. Interruption of the putative ligand/receptor interaction could have a major effect on the growth and pathogenicity of *A. fumigatus*, providing opportunities for the development of alternative therapeutic strategies to those currently available.**

Keywords: *Aspergillus* spp., hydrocortisone, aspergillosis, ergosterol, growth

INTRODUCTION

Aspergillus spp. are remarkable pathogens, affecting a wide range of hosts including plants, insects, birds and marine and land-based mammals, including man. Increasing numbers of immunocompromised patients and an increased success in treating bacterial infections have led to the emergence of invasive aspergillosis (most commonly caused by *Aspergillus fumigatus*) as one of the most common life-threatening opportunistic infections. The proportional incidence of disseminated aspergillosis at autopsy has risen from 17% in 1978–1982 to 60% in 1987–1992 of all systemic mycoses (Groll *et al.*, 1993). Characteristically, neutropenic and bone marrow transplant patients, solid organ transplant recipients (Denning & Stevens, 1990) (especially liver transplant patients) and patients with AIDS (Denning *et al.*, 1991) or chronic granulomatous disease are afflicted. Mortality rates vary from 30–95%.

In particular, endogenous hypercortisolaemia or pharmacological doses of corticosteroid have been identified as a risk factor for disseminated aspergillosis (Graham & Tucker, 1984; Palmer *et al.*, 1991). This predisposition to fungal infection was thought to be primarily due to the inhibitory effect of corticosteroid on the monocyte-mediated damage to fungal hyphae (Diamond, 1983) and the conidicidal activity of tissue macrophages (Schaffner, 1985). A direct effect of human cortisol on the growth or metabolism of *A. fumigatus* has so far not been demonstrated.

This study was conducted to explore the possibility of a direct interaction between human cortisol and *A. fumigatus* by measuring the effect of pharmacological doses of hydrocortisone on the *in vitro* growth of two clinical isolates of *A. fumigatus*.

METHODS

Isolates. Five isolates of *Aspergillus* were studied. Two were clinical isolates of *A. fumigatus*, AF-6 and AF-10. The former was the cause of fatal disseminated invasive aspergillosis in a renal transplant recipient; autopsy showed disseminated disease

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in the brain, lung and heart valve. AF-10 was the cause of invasive pulmonary aspergillosis in a woman with multiple sclerosis treated with adrenocorticotrophic hormone (ACTH), which raises endogenous cortisol concentrations. She responded to withdrawal of ACTH and amphotericin B therapy. AF-10 is accessioned as ATTC 90240. One isolate each of *A. flavus*, *A. oryzae* and *A. niger* were also tested. The *A. flavus* isolate was a laboratory contaminant (AFL14/92). *A. oryzae* (IFO 1477) and *A. niger* (AB4.1; from Dr D. Archer, AFRC, Norwich) were standard laboratory isolates.

Growth measurement. We measured the effect of hydrocortisone sodium succinate (Solucortef; Upjohn) on the growth of *Aspergillus*. First, we measured the specific growth rate of fungal mycelia as described previously by Robson *et al.* (1991). Briefly, inocula were made up by diluting the spore suspension (final spore concentration, 10^3 c.f.u. ml⁻¹) in 10^{-6} M hydrocortisone solution or PBS (0.145 M NaCl, 0.15 M sodium phosphate; control) and then spreading onto agar-solidified Vogel's medium (containing 5 mM glucose) prepared with or without hydrocortisone (overlaid with sterile Cellophane to ensure that the mycelia grew in a single plane). Plates were incubated at 32 °C or 37 °C in a thermostatically controlled electrically heated box. Growth in exponential-phase was monitored by measuring the total hyphal length of germlings (each arising from a viable spore) at hourly intervals over a 5 h period using a Measure Mouse graphics system (Analytical Measuring Systems, Cambridge) and an Amstrad PC 1520 connected to a Nikon microscope. A Panasonic WV-CD20 video camera relayed the microscope image to a computer monitor on which it could be traced using a computer mouse. At each time-point, five replicate germlings per plate were measured. Specific growth rate was calculated from the slope of the natural logarithm of total hyphal length versus time as described by Trinci (1974). The difference between the mean specific growth rates in the presence and absence of hydrocortisone was analysed using analysis of covariance (ANCOVA).

The second method measures growth indirectly by the rate of incorporation of *N*-acetyl-D-[1-¹⁴C]glucosamine (Amersham International). Chitin, which makes up 20% of the fungal cell wall, is largely composed of polymers of acetylglucosamine. The inoculum (10^7 c.f.u. ml⁻¹; 0.1 ml) was diluted in 2 ml Vogel's medium in the presence or absence of various concentrations of hydrocortisone and incubated at 37 °C on a rotary shaker after the addition of 0.1 µCi (3.7 kBq) of the labelled substrate. In exponential phase, at hourly intervals, four tubes, each containing a different concentration of hydrocortisone (10^{-8} , 10^{-7} , 10^{-6} , 10^{-5} M), were retrieved from the incubator along with the control. Trichloroacetic acid (2 ml) was added to each to remove any free substrate that was not incorporated in the cell wall. Tubes were centrifuged at 2000 r.p.m. for 5 min and supernatants discarded. Pellets were each washed three times with PBS, mixed with 2.2 ml 1 M sodium hydroxide and then sonicated for 1–2 min. The homogeneous solution obtained (2 ml) was placed into 2 ml Ultima Gold (Packard Instrument) in a scintillant vial and radioactive emission measured for 10 min in a 1211 Minibeta liquid scintillation counter. Counts per minute (c.p.m.) were converted into disintegrations per minute (d.p.m.) using the quench correction curve previously derived. The rate of cell-wall uptake of radioactive substrate was derived from the slope of the natural logarithm of sample radioactivity versus time. The difference in slopes (Gardner & Altman, 1989) was analysed using Fig-P (Biosoft).

The effects of ergosterol (a fungal sterol) and three other human steroids, namely 17β-oestradiol (E₂; Sigma), progesterone

(Sigma) and testosterone (Sigma) on *A. fumigatus* (AF-10) were also investigated using a simple screening test adapted from the radiometric assay. For each sterol (at 10^{-6} M concentration), a set of four replicate tubes were prepared as described and grown at 37 °C along with four replicate controls (live conidia without added steroid). These were retrieved at mid-exponential phase, processed and the total amount of assimilated radioactivity (r^{total}) measured. Growth increase (%) was defined by the equation:

$$\frac{[(\text{mean } r^{\text{total}} \text{ of the four replicates}) / (\text{mean } r^{\text{total}} \text{ of the four controls}) - 1] \times 100 \%}{}$$

RESULTS AND DISCUSSION

Mean specific growth rate (at 37 °C) was found to increase, in the presence of 10^{-6} M hydrocortisone, by 40 % (from 0.62 h⁻¹ to 0.87 h⁻¹; $P = 0.0001$ by ANCOVA) (Fig. 1). The effect of hydrocortisone is independent of temperature as similar increases in growth were obtained in two subsequent experiments repeated at 32 °C [36 %; from 0.478 h⁻¹ to 0.652 h⁻¹ ($P = 0.0008$) and 43 %; from 0.426 h⁻¹ to 0.607 h⁻¹ ($P = 0.0002$)]. Similarly, the rate of cell-wall uptake of labelled acetylglucosamine was found to increase by 140 % with 10^{-6} M hydrocortisone [difference in slopes = 0.111 (0.193–0.082) h⁻¹, 95 % CI = 0.008–0.214 h⁻¹] (Fig. 2a). In the repeat experiment, a 70 % increase [difference in slopes = 0.081 (0.197–0.116) h⁻¹, 95 % CI = 0.012–0.15 h⁻¹] was observed. This effect was shown to be dose-dependent (Fig. 2b), with 10^{-6} M being the concentration that corresponded to the maximum growth increase. Pharmacokinetically, this concentration is roughly equivalent to the peak serum level achieved in humans following an intravenous administration of 20 mg hydrocortisone (Derendorf *et al.*, 1991). No effect on time to germination was observed (data not shown).

Similar experiments with other species of *Aspergillus* showed that the effect of hydrocortisone is probably species-specific in that it had no effect on the growth rate of *A. oryzae* or *A. niger* whereas the specific growth rate of a laboratory strain of *A. flavus* was increased by 30 % from 0.45 h⁻¹ to 0.593 h⁻¹ (Fig. 3). This difference was not statistically significant, $P = 0.3$ by ANCOVA, but un-

Fig. 1. Increase in total hyphal length (µm) with time (h) in germlings of isolate AF-6 grown at 37 °C with (○) or without (●) 10^{-6} M hydrocortisone. At each time point the mean $\pm$ SEM of five replicate germlings was plotted.

Fig. 2. Effect of the addition of hydrocortisone on the rate of incorporation of radiolabelled *N*-acetylglucosamine into the cell wall in isolate AF-10. (a) 10⁻⁶ M hydrocortisone (○) versus no steroid control (●). (b) Dose responsive increase in growth rate with hydrocortisone (10⁻⁸–10⁻⁵ M).

Fig. 3. Increase in total hyphal length (μm) with time (h) in germlings of a laboratory strain of *A. flavus* grown at 32 °C with (●) or without (○) 10⁻⁶ M hydrocortisone. At each time point the mean ± SEM of three or four replicates was plotted.

Fig. 4. Effect of the addition of 1 μg ergosterol ml⁻¹ (●) on the specific growth rate of AF-10 grown at 30 °C. ○, No steroid control.

Table 1. Effects of different sterols on the growth of AF-10 as measured by a simplified radiometric assay

Sterol (10 ⁻⁶ M)	Growth increase relative to control*	<i>P</i> value
Hydrocortisone	44 %	0.03
Ergosterol†	30 %	0.183
17β-oestradiol	8 %	0.277
Progesterone	3 %	0.937
Testosterone	15 %	0.211

* Mean r^{total} (c.p.m.) of controls = 15927 and 9209, respectively, in two experiments.

† Actual concentration 2.5×10^{-6} M.

fortunately it was only possible to do one experiment with three or four replicates per time point.

Table 1 compares the effects of different sterols on the growth of *A. fumigatus* (AF-10) detected by the simplified

radiometric assay. The figures presented represent the mean of two experiments. Among the five sterols tested, only hydrocortisone produced a significant growth increase (44%). The results were analysed by two-way analysis of variance (ANOVA), which showed that the effect of hydrocortisone was the same in each experiment ($F_{1,8} = 0.1$, $P = 0.76$) and there was a significant increase in growth with hydrocortisone in comparison to the control ($F_{1,9} = 6.5$, $P = 0.03$). The percentage growth increase (30%, $P = 0.183$) induced by the addition of 10⁻⁶ M (1000 ng ml⁻¹) ergosterol was second to that of hydrocortisone. These results were similar to those obtained using the computer Measure Mouse system, which showed a 21 % increase (from 0.369 to 0.448 h⁻¹; $P = 0.0001$ by ANCOVA) in specific growth rate with the addition of 10⁻⁶ M ergosterol (Fig. 4). Small growth-rate increases were seen at ergosterol concentrations of 2.5×10^{-7} M (0.422 h⁻¹; $P \geq 0.05$) and 2.5×10^{-5} M (0.434 h⁻¹; $P > 0.05$).

In this study we have unequivocally demonstrated, for the first time, a direct interaction between human cortisol and *A. fumigatus* resulting in a significant increase in growth. Several other interactions between human hormones and

fungi have been documented, some of which have proved to be important in the pathogenesis of certain fungal diseases. For instance, progesterone and 17β -oestradiol (E_2) both promote the growth and endospore release of *Coccidioides immitis* (Powell *et al.*, 1983), therefore accounting for the increased incidence of coccidioidomycosis in pregnancy. Infections with *Paracoccidioides brasiliensis*, on the other hand, occur predominantly in males (among those who acquire the disease after puberty), possibly as a result of the inhibitory effect 17β -oestradiol has on its mycelium- to yeast-form transformation (Restrepo *et al.*, 1984), an essential preliminary step in the establishment of infection. In addition, specific binding proteins for human hormones have been found in various fungi, e.g. corticosterone-binding protein (CBP) in *Candida albicans* and six other *Candida* spp. (Loose *et al.*, 1983a); specific binders for E_2 in *Paracoccidioides brasiliensis* (Loose *et al.*, 1983b), *C. albicans*, *Candida glabrata* (Powell *et al.*, 1984) and *Saccharomyces cerevisiae* (Feldman *et al.*, 1982); luteinizing hormone/human chorionic gonadotropin binding sites in *C. albicans* and *Candida tropicalis* (Bramley *et al.*, 1990). Some of these binding proteins, upon hormonal binding, appear to mediate the morphological changes characteristic of the pathological process seen in the corresponding fungal disease, while others, such as the CBP in *C. albicans*, have no demonstrable effect upon fungal growth, phase conversion or glucose oxidation (Loose *et al.*, 1983a). Unlike yeasts, we found no significant effect of E_2 , progesterone or testosterone on the growth of AF-10. The effect of cortisol on *Aspergillus* may therefore be unique in that it is not mimicked by other human steroids. Interestingly, ergosterol also increases the growth of AF-10 in a dose-dependent manner. Maximum increase in specific growth rate was obtained at $1 \mu\text{g ml}^{-1}$ ($2.5 \times 10^{-6} \text{ M}$), the same concentration that was associated with an increase in protein kinase activity in *S. cerevisiae* (Dahl *et al.*, 1987). The intracellular signalling pathways of ergosterol (at least in *Saccharomyces*) and some of the mammalian hormones/cytokines are similar in that both result in an increase in phosphoinositide hydrolysis [i.e. conversion of phosphatidylinositol bisphosphate (PIP_2) to inositol triphosphate (IP_3) and diacylglycerol], which in turn leads to protein kinase activation and cell proliferation (Dahl & Dahl, 1985). The mechanism(s) by which hydrocortisone affects fungal growth is, however, unknown, but may involve binding to a glucocorticoid receptor similar to that found in *Candida* and mammals. The mammalian glucocorticoid receptor includes, for maintaining its high-affinity steroid binding conformation, the highly conserved heat-shock-protein 90 complex (hsp90) (Meschini *et al.*, 1990). There is considerable immunological cross-reactivity between the hsp90 antigens of *C. albicans* and mammals and some of the immunodominant antigens of *A. fumigatus*, including the 88 kDa antigen (Riehl *et al.*, 1985; Burnie & Matthews, 1991). These data lend credence to the suggestion that *A. fumigatus* possesses a glucocorticosteroid receptor which may be linked to hsp90.

Our discovery that corticosteroid promotes the growth of

A. fumigatus may have important implications for the pathogenesis of aspergillosis, as the specific growth rate of 0.9 h^{-1} of *A. fumigatus* grown in the presence of hydrocortisone at 37°C (corresponding to a doubling time of 48 min) makes it one of the fastest growing fungi described. Currently we are working to identify and characterize the putative corticosteroid-binding receptor in *A. fumigatus*, which may enable us to develop alternative therapeutic agents to those currently available.

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REFERENCES

- Bramley, T. A., Menzies, G. S., Williams, R. J., Adams, D. J. & Kinsman, O. S. (1990). Specific, high-affinity binding sites for human luteinizing hormone (hLH) and human chorionic gonadotrophin (hCG) in *Candida* species. *Biochem Biophys Res Commun* **167**, 1050–1056.
- Burnie, J. P. & Matthews, R. C. (1991). Heat shock protein 88 and *Aspergillus* infection. *J Clin Microbiol* **29**, 2099–2106.
- Dahl, C., Biemann, H. P. & Dahl, J. (1987). A protein kinase antigenically related to pp60 v-src possibly involved in yeast cell cycle control: positive in vivo regulation by sterol. *Proc Natl Acad Sci USA* **84**, 4012–4016.
- Dahl, J. S. & Dahl, C. E. (1985). Stimulation of cell proliferation and polyphosphoinositide metabolism in *Saccharomyces cerevisiae* GL7 by ergosterol. *Biochem Biophys Res Commun* **133**, 844–850.
- Denning, D. W. & Stevens, D. A. (1990). Antifungal and surgical treatment of invasive aspergillosis: review of 2121 published cases. *Rev Infect Dis* **12**, 1147–1201.
- Denning, D. W., Follansbee, S., Scolaro, M., Norris, S., Edelstein, D. & Stevens, D. A. (1991). Pulmonary aspergillosis in AIDS. *N Engl J Med* **324**, 654–662.
- Derendorf, H., Mollmann, H., Barth, J., Mollmann, C., Tunn, S. & Krieg, M. (1991). Pharmacokinetics and oral bioavailability of hydrocortisone. *J Clin Pharmacol* **31**, 473–476.
- Diamond, R. D. (1983). Inhibition of monocyte-mediated damage to fungal hyphae by steroid hormones. *J Infect Dis* **147**, 160.
- Feldman, D., Do, Y., Burshell, A., Stathis, P. & Loose, D. S. (1982). An estrogen-binding protein and endogenous ligand in *Saccharomyces cerevisiae*: possible hormone receptor system. *Science* **218**, 297–298.
- Gardner, M. J. & Altman, D. G. (1989). *Statistics with Confidence – Confidence Intervals and Statistical Guidelines*. London: BMJ.
- Graham, B. S. & Tucker, W. S. (1984). Opportunistic infections in endogenous Cushing's syndrome. *Ann Intern Med* **101**, 334–338.
- Groll, A., Shah, P. M., Menzel, C., Just, G., Schneider, M. & Hubner, K. (1993). Invasive mycosis in post-mortem findings. *Trends in Invasive Fungal Infections II* (Manchester, UK). Abstract no. 1.
- Loose, D. S., Stevens, D. A., Schurman, D. J. & Feldman, D. (1983a). Distribution of a corticosteroid-binding protein in *Candida* and other fungal genera. *J Gen Microbiol* **129**, 2379–2385.
- Loose, D. S., Stover, E. P., Restrepo, A., Stevens, D. A. & Feldman, D. (1983b). Estradiol binds to a receptor-like cytosol binding protein and initiates a biological response in *Paracoccidioides brasiliensis*. *Proc Natl Acad Sci USA* **80**, 7659–7663.

- Meshinchi, S., Sanchez, E. R., Martell, K. J. & Pratt, W. B. (1990).** Elimination and reconstitution of the requirement for hormone in promoting temperature-dependent transformation of cytosolic glucocorticoid receptors to the DNA-binding state. *J Biol Chem* **265**, 4863–4870.
- Palmer, L. B., Greenberg, H. E. & Schiff, M. J. (1991).** Corticosteroid treatment as a risk factor for invasive aspergillosis in patients with lung disease. *Thorax* **46**, 15–20.
- Powell, B. L., Drutz, D. J., Huppert, M. & Sun, S. H. (1983).** Relationship of progesterone- and estrogen-binding proteins in *Coccidioides immitis* to coccidioidal dissemination in pregnancy. *Infect Immun* **40**, 478–485.
- Powell, B. L., Frey, C. L. & Drutz, D. J. (1984).** Identification of a 17-beta-estradiol-binding protein in *Candida albicans* and *Candida (Torulopsis) glabrata*. *Exp Mycol* **8**, 304–313.
- Restrepo, A., Salazar, M. E., Cano, L. E., Stover, P., Feldman, D. & Stevens, D. A. (1984).** Estrogens inhibit mycelium-to-yeast transformation in the fungus *Paracoccidioides brasiliensis*: implications for resistance of females to paracoccidioidomycosis. *Infect Immun* **46**, 346–353.
- Riehl, R. M., Sullivan, W. P., Vroman, B. T., Bauer, V. J., Pearson, G. R. & Toft, D. O. (1985).** Immunological evidence that the nonhormone binding component of avian steroid receptors exists in a wide range of tissues and species. *Biochemistry* **24**, 6586–6591.
- Robson, G. D., Wiebe, M. G. & Trinci, A. P. J. (1991).** Exogenous cAMP and cGMP modulate branching in *Fusarium graminearum*. *J Gen Microbiol* **137**, 963–969.
- Schaffner, A. (1985).** Therapeutic concentrations of glucocorticoids suppress the antimicrobial activity of human macrophages without impairing their responsiveness to gamma interferon. *J Clin Invest* **76**, 1755–1764.
- Trinci, A. P. J. (1974).** A study of the kinetics of hyphal extension and branch initiation of fungal mycelia. *J Gen Microbiol* **81**, 225–236.

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