Methylprednisolone Enhances the Growth of *Exserohilum rostratum* In Vitro, Attenuates Spontaneous Apoptosis, and Increases Mortality Rates in Immunocompetent *Drosophila* Flies

Dimitrios Farmakiotis,¹ Fazal Shirazi,¹ Yanan Zhao,²,³ Peguy J. Saad,¹ Nathaniel D. Albert,¹ Emmanuel Roilides,⁴ Thomas J. Walsh,³ David S. Perlin,² and Dimitrios P. Kontoyiannis

¹The University of Texas M.D Anderson Cancer Center, Houston; ²Public Health Research Institute Center, New Jersey Medical School, Rutgers—The State University of New Jersey, Newark; ³New York–Presbyterian/Weill Cornell Medical Center, New York City; and ⁴Aristotle University of Thessaloniki, Greece

High concentrations of methylprednisolone (0.32 mg/mL) accelerated growth and attenuated spontaneous apoptosis of *Exserohilum rostratum* in vitro. Injection of *E. rostratum* conidia preexposed to 0.32 mg/mL of methylprednisolone for 7 days in immunocompetent flies led to increased mortality and a higher fungal burden. Exposure to methylprednisolone could enhance the virulence of *E. rostratum*.

**Keywords.** *Exserohilum rostratum*; virulence; corticosteroids; animal models; apoptosis.

Contamination of preservative-free methylprednisolone acetate (MP)—containing vials from a single compounding pharmacy resulted in a multistate outbreak of central nervous system, bone, and joint fungal infections, affecting >700 patients [1–3]. Although a variety of molds were isolated from contaminated vials, the predominant organism was the dematiaceous mold *Exserohilum rostratum*. Previously, *E. rostratum* had only been sporadically reported to cause human disease, most commonly in patients with significant immune dysfunction [4, 5]. Data regarding the pathophysiologic mechanisms and, more specifically, the effect of corticosteroids on the growth and lethality of this unusual pathogen are scarce [4].

In previous public health emergencies, data from in vitro experiments and animal models have been useful in providing the scientific basis for effective management of life-threatening infections [6]. As fungi have been postulated to express steroid receptors that could affect their growth and/or virulence in the presence of corticosteroids [4, 7, 8], we investigated the effect of MP on in vitro growth, spontaneous apoptosis, and in vivo lethality of *E. rostratum*. To that end, we inoculated *E. rostratum* conidia, with or without 7-day exposure to MP, into *Drosophila melanogaster* flies, a minihost model that has been previously shown to simulate the pathogenesis of a variety of invasive mold infections [9, 10].

**METHODS**

A clinical isolate of *E. rostratum* from the cerebrospinal fluid of infected patients (provided by T. J. W., Weill Cornell Medical Center, New York) was grown on potato agar plates for 5 days at 37°C to obtain conidia. Conidia were collected and washed twice in sterile phosphate-buffered saline (PBS) and resuspended in Roswell Park Memorial Institute (RPMI) medium, with 2% glucose, at a concentration of 10⁵ *E. rostratum* conidia/mL in the presence of different MP concentrations (0, 0.16, and 0.32 mg/mL). Subsequently, 300 µL were dispensed in 96-well plates and incubated in a plate reader at 37°C for 96 hours; RPMI medium alone was used as the standard control. *E. rostratum* growth rates were estimated by analyzing absorbance at a wavelength of 600 nm. Spontaneous apoptosis of *E. rostratum* conidia that were or were not exposed to MP was assessed with DiBAC staining (to determine cell death), dihydrorhodamine 123 (DHR-123) staining (to determine reactive oxygen species [ROS] accumulation), TUNEL staining (to determine DNA fragmentation), and CaspACE FITC-VAD-FMK staining (to determine metacaspase activity), as previously described (Supplementary Materials) [11].

Oregon¹ wild-type and Toll-pathway-deficient *D. melanogaster* female flies were used, and standard procedures for their manipulation, feeding, and housing were implemented [9, 10]. We inoculated 2–5-day-old flies (25–30 per group) with *E. rostratum* conidia, with or without preexposure to high-dose MP (0.32 mg/mL) in liquid PBS for 7 days at room temperature, by injecting their lateral thoracic wall with a 10-µm needle that had been dipped in a solution containing 10⁶ conidia/mL (Supplementary Figure 1).
Survival rates were recorded daily for 7 days after infection. Less than 5% of flies died within 3 hours of injection, and they were excluded from survival analysis. In separate experiments, 5 flies per group were killed by freezing on day 3 after infection, fixed in 10% formaldehyde, and embedded in paraffin. Tissue sections were stained with hematoxylin-eosin and Gomori methenamine silver stains and were examined for visible fungal burden under a light microscope. In another experiment, quantitative real-time polymerase chain reaction (PCR) analysis was performed on whole flies in groups of 50 at the Public Health Research Institute (Newark, NJ), using a previously described protocol for assessment of E. rostratum fungal burden (Supplementary Materials) [12].

Both in vitro and in vivo experiments were performed at least 3 times, each on a different day. All experiments were performed between 11 AM and 12 PM to minimize circadian rhythm variability. Statistical analysis was performed using the In Stat software program (GraphPad Software, San Diego, CA). E. rostratum growth rates were compared by means of 2-way analysis of variance. PCR-based conidial equivalent counts were compared with the Mann–Whitney test. Kaplan–Meier survival curves for flies were compared with the log-rank test. For all comparisons, 2-tailed P values of <.05 were considered statistically significant.

RESULTS

Compared with controls (which were not exposed to MP), the growth of E. rostratum was significantly enhanced in the presence of a high (0.32 mg/mL) but not low (0.16 mg/mL) concentration of MP (P = .0046; Figure 1A). MP attenuated spontaneous apoptosis of conidia, as evidenced by the significantly lower proportion of apoptotic dead cells (determined by DiBAC staining; Figure 2A and 2E), ROS accumulation (determined by DHR-123 staining; Figure 2B and 2E), DNA fragmentation (determined by TUNEL staining; Figure 2C and 2E), and decreased metacaspase activity (determined by CaspACE.

Figure 1. Methylprednisolone (MP) enhances the in vitro growth of Exserohilum rostratum and increases the mortality rate in an immunocompetent fly model of disseminated infection. A, Growth curves of E. rostratum in the absence of corticosteroids or in the presence of 2 different doses of MP. B, Survival curves of wild-type (WT) Drosophila flies injected in their lateral thoracic wall with 10⁶ E. rostratum conidia/mL (P < .001 by the log-rank test). C, Representative hematoxylin-eosin (HE) and Gomori methenamine silver (GMS) stains of WT fly abdominal sections, showing a significantly higher fungal burden (arrows) in a fly infected with conidia of E. rostratum preexposed to 0.32 mg/mL of MP in phosphate-buffered saline (PBS) for 7 days (bottom, MP), compared with a fly infected with conidia of E. rostratum incubated for 7 days in PBS alone (top, control). D, Results of quantitative polymerase chain reaction analysis of whole flies injected with E. rostratum preexposed to 0.32 mg/mL of MP for 7 days in PBS (E. rostratum + MP) or PBS alone (control). Data are conidium equivalents/500 µL of fly homogenate, and horizontal lines denote median values (P = .008, by the Mann–Whitney test).
FITC-VAD-FMK staining; Figure 2D and 2E), in conidia preexposed to MP for 7 days, compared with conidia preexposed only to PBS for 7 days.

In addition, we observed a significantly higher mortality rate in flies infected with E. rostratum conidia preexposed to 0.32 mg/mL of MP in PBS for 7 days than in flies injected with conidia incubated in PBS alone (P < .001; Figure 2B). There was no mortality in flies infected with <10⁶ spores/mL, independent of preexposure to MP (data not shown). Histopathology sections also showed significantly more tissue invasion by E. rostratum hyphae in flies injected with E. rostratum conidia preexposed to MP (0.32 mg/mL) for 7 days, compared with those injected with PBS alone (Figure 1C). Whole-fly quantitative PCR confirmed a significantly higher fungal burden in flies infected with conidia preexposed to MP for 7 days than in those preexposed to PBS alone (P = .008; Figure 1D). In comparison to wild-type (immunocompetent) flies, injection of E. rostratum in Toll-deficient flies was highly lethal, and we did not observe a significant difference in mortality between isolates preexposed to MP, compared with those exposed to PBS alone (Supplementary Figure 2).

**DISCUSSION**

The recent epidemic of iatrogenic fungal meningitis caused by E. rostratum is by far the largest infectious outbreak related to the administration of compounded steroids to date [1–5]. A similar, more limited cluster occurred in 2002 as a result of contamination of MP-containing vials with another pigmented mold, Exophiala dermatitidis [13]. Those 2 public health tragedies show that preservative-free steroids could serve as growth media for molds. This agrees with the results of the present study, in which the in vitro growth of E. rostratum was accelerated in the presence of corticosteroids (Figure 1A).

Our findings are in agreement with those reported by Ng et al [7], who were the first to document a significant increase in the growth rate of Aspergillus fumigatus or Aspergillus flavus exposed to hydrocortisone. There might be organism-specific differences regarding the effects of corticosteroids on fungal biology, as Ng et al did not observe the same effect of hydrocortisone on other Aspergillus species (ie, A. oryzae or A. niger) [7]. This phenomenon might account for the predominance of a single fungus species in both steroid-related iatrogenic...
outbreaks [1, 2, 4, 12], although environmental sources of contamination would be expected to harbor an abundance of different molds [4].

Of >14 000 patients exposed to MP from the contaminated lots in the 2012–2013 outbreak, 751 cases of fungal infections were reported, although subtle manifestations, such as minimal symptoms with abnormal imaging results, are being increasingly recognized in additional individuals [14]. The reasons for this relatively low infection/exposure rate are unclear, but clinical data are strongly suggestive of a significant inoculum and time-exposure effect: repeated injections and older vials were independent predictors of developing disease [2]. Moreover, a recent clinicopathological report identified higher fungal burdens not only in the cerebrospinal fluid, but also at the injection sites in patients with fungal meningoencephalitis, compared with those with less severe infections [3].

Our findings are also consistent with the effects of both high fungal inoculum and the organism’s preexposure to corticosteroids. Specifically, we observed corticosteroid-related increased proliferation (Figure 1A) and decreased spontaneous apoptosis (Figure 2) of E. rostratum conidia, which likely accounted for increased lethality and fungal burdens in vivo (Figure 1B–D). As the biological effects observed in E. rostratum at the concentrations used in this study were considerably below that in the contaminated vials (8 mg/mL), the effect of dilution in injected tissue would still permit ongoing modification of this pathogen. While the exact mechanism of growth enhancement and attenuation of E. rostratum spore apoptosis by high concentrations of MP remains unknown, those properties would be compatible with binding to corticosterone-binding protein, similar to that of Candida species, with repression of growth-regulatory genes [4].

In Toll-deficient Drosophila flies, injection of E. rostratum incubated in PBS alone was highly lethal (Supplementary Figure 2), indicating that the Toll pathway is an important host defense mechanism against E. rostratum, similar to other molds [9, 10]. However, there was no significant difference in survival of Toll-deficient flies between E. rostratum isolates that were and those that were not preexposed to MP. This is likely due to the increased baseline mortality in Toll-flies infected with E. rostratum. Nevertheless, it is also possible that the effect of steroids on the lethality of E. rostratum could be attenuated in the setting of a deficient Toll pathway. Therefore, a potential mechanism through which steroids make E. rostratum more lethal in immunocompetent flies could be alteration of the fungal surface and/or increased secretion of virulence factors, triggering a hyperinflammatory activation of the Toll pathway, which could have a U-shaped effect on fly survival.

Our study has limitations that should be taken into consideration. First, although the injected inoculum of spores was similar in the MP-treated group and controls, on the basis of our in vitro results (Figure 1A), we cannot exclude the possibility that, shortly after infection, more spores in the MP arm were germinating, resulting in increased hyphal invasion of fly tissue. Second, our model did not address the concomitant effect of corticosteroids on host defense mechanisms. In the recent outbreak, some degree of systemic immunosuppression due to comorbidities and advanced age (identified as an independent risk factor [2]) or corticosteroid-induced mitigation of local phagocytic activity in closed spaces likely contributed to the development of clinical symptoms [1–4]. In animal models, intrathecal MP leads to detectable plasma levels of MP for up to 2 weeks [15], and the potential immunomodulatory effects of epipual glucocorticoids have not been well investigated. However, during the recent outbreak, the incubation periods of 4–6 weeks were longer than the expected duration of corticosteroid-related immunosuppression were frequently reported, indicating that factors other than the effect of the injection of corticosteroids on host defenses must have played an important role [1, 2, 4, 5].

In conclusion, high concentrations of MP accelerated the in vitro growth of E. rostratum and attenuated spontaneous apoptosis of its conidia. Moreover, corticosteroid preexposure led to increased hyphal tissue invasion and death in an immunocompetent fly model of disseminated Exserohilum infection. Further studies using minihost and vertebrate animal models with several independent Exserohilum isolates may help elucidate virulence factors for black molds, host immunity against invasive mycoses, and the potential for pharmacologic interventions and drug development.

**Supplementary Data**

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyrighted. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

*Financial support.* This work was supported by the Frances King Black Endowed Professorship for Cancer Research (to D. P. K.), the Henry Schueler Foundation (to T. J. W.), the Sharpe Family Foundation (to T. J. W.), and the Save Our Sick Kids Foundation (to T. J. W.).

*Potential conflicts of interest.* D. P. K. has received research support and honoraria from Pfizer, Astellas Pharma US, Gilead, and Merck. D. S. P. has received research support from Astellas Pharma US, Merck, bioMérieux, Myconostica, and Pfizer. T. J. W. has received research grants for experimental and clinical antimicrobial pharmacotherapeutics from Astellas Pharma US, Novartis, Merck, ContraFect, and Pfizer and has served as a consultant for Astellas Pharma US, ContraFect, Daiichi Pharmaceuticals, iGo, Novartis, Pfizer, MethylGene, Sigma Tau Pharmaceuticals, and Trius. E. R. has received research grant support from Pfizer, Gilead, Merck; has served as a consultant to Gilead, Astellas, Cephalon, and Pfizer; and has been on the speakers’ bureaus of Merck, Pfizer, Gilead, and Astellas. All other authors report no potential conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.
References