Mucormycosis during Deferoxamine Therapy Is a Siderophore-mediated Infection

In Vitro and In Vivo Animal Studies

Johan R. Boelaert,* Marielle de Locht,‡ Jan Van Cutsem,§ Véronique Kerrels,‖ Brigitte Cantinieaux,‖ Ann Verdonck,¶ Herman W. Van Landuyt,¶ and Yves-Jacques Schneider‡

**Unit of Renal and Infectious Diseases, Algemeen Ziekenhuis St-Jan, B-8000 Brugge, Belgium; ‡Laboratory of Cell Biochemistry, Université Catholique de Louvain, B-1348 Louvain-la-Neuve, Belgium; §Department of Bacteriology and Mycology, Janssen Research Foundation, B-2340 Beerse, Belgium; ‖Department of Hematology, University Hospital St Pierre, B-1000 Brussels, Belgium; and ¶Department of Microbiology, Algemeen Ziekenhuis St-Jan, B-8000 Brugge, Belgium*

Abstract

This study investigates the pathophysiology of mucormycosis caused by *Rhizopus,* which has been reported in 46 dialysis patients, while treated with deferoxamine (DFO). This drug aggravates mucormycosis, which we experimentally induced in guinea pigs and which lead to a shortened animal survival ($P \leq 0.01$). The drug's effect on *Rhizopus* is not mediated through the polymorphonuclear cells. Fe.DFO, the iron chelate of DFO, abolishes the fungistatic effect of serum on *Rhizopus* and increases the in vitro growth of the fungus ($P \leq 0.0001$). This effect is present at Fe.DFO concentrations ≥ 0.01 μM, at which fungal uptake of radioiron from ^{55}Fe.DFO is observed. A 1,000-fold higher concentration of iron citrate is required to achieve a similar rate of radioiron uptake and of in vitro growth stimulation as observed with Fe.DFO. These in vitro effects of Fe.DFO (1 μM) in serum on radioiron uptake and on growth stimulation are more striking for *Rhizopus* than for *Aspergillus fumigatus* and are practically absent for *Candida albicans.* For these three fungal species, the rates of radioiron uptake from ^{55}Fe.DFO and of growth stimulation in the presence of Fe.DFO in serum are directly related ($r = 0.886$). These results underscore the major role of Fe.DFO in the pathogenesis of DFO-related mucormycosis. Pharmacokinetic changes in uremia lead to a prolonged accumulation of Fe.DFO after DFO administration, which helps explain the increased sensitivity of dialysis patients to DFO-related mucormycosis. (*J. Clin. Invest.* 1993. 91:1979–1986.) Key words: rhizopus • zygomycosis • iron • desferrioxamine • chelator

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Address reprint requests to Dr. Johan R. Boelaert, Unit of Renal and Infectious Diseases, Algemeen Ziekenhuis St. Jan, Ruddershove 10, B-8000 Brugge, Belgium.

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Introduction

Mucormycosis is an infection caused by fungi of the class *Zygomycetes,* order *Mucorales.* Prior to 1986, the development of mucormycosis in dialysis patients had been reported only once (1). Since then, a substantial number of such cases have been recognized in dialysis patients treated with deferoxamine B (DFO)[1] for either aluminum or iron overload. An international registry of mucormycosis in dialysis patients was established in order to gain more clinical information on the possible association with this drug therapy (2). Up to March 1991, 59 dialysis patients who had developed mucormycosis were reported to this registry. 46 of them (78%) were receiving DFO at the time the infection became clinically apparent, which suggests a possible role of DFO therapy in the pathogenesis of this infection. Furthermore, it was reported that the administration of DFO aggravates experimentally induced mucormycosis in the guinea pig (3). We have hypothesized that mucormycosis in DFO-treated patients is mediated by the siderophore activity of the feroxamine complex (Fe.DFO) toward the causative fungi (3).

DFO is known to exert disparate effects on microorganisms (4). For some microorganisms, DFO suppresses in vitro growth and protects against experimental infection; for another group of microorganisms, DFO enhances in vitro growth and aggravates experimental infection. *Klebsiella spp., Salmonella typhimurium, Vibrio vulnificus* and some serotypes of *Yersinia enterocolitica* belong to the latter group, in which microorganisms are supposed to be able to take up iron from Fe.DFO.

Feroxamines are siderophores which have, up to now, not been isolated from fungi but are the main siderophores produced by *Streptomyces* and *Nocardia* species, being Gram-positive soil bacteria, and by some Gram-negative enterobacteria, such as *Enterobacter* (5–10). Uptake of radioiron from ^{55}Fe.DFO has been found not only in *Streptomyces pilosus,* the producer strain (11), but also in some bacterial and fungal species that lack the capacity to synthesize DFO but are able to utilize this exogenous siderophore.

The mechanism of the Fe.DFO mediated iron uptake varies from species to species. In *Streptomyces pilosus,* the intact Fe.DFO complex has been shown to be taken up (11). In *Saccharomyces cerevisiae* and in *Geotrichum candidum,* two mechanisms coexist: on the one hand, there is entry of the iron-chelator complex, and on the other hand, there is reduction of iron

1. *Abbreviations used in this paper:* BDM, basal defined medium; DFO, deferoxamine B.

from the Fe.DFO complex prior to iron being transported across the cellular membrane (12, 13). Fungi belonging to the genus *Rhizopus,* the most important species causing mucormycosis in humans, are also able to take up iron from Fe.DFO (14).

The aim of the present study was to test the hypothesis that iron uptake from the Fe.DFO complex by *Rhizopus* leads to stimulation of its in vitro growth as well as to enhancement of its in vivo pathogenicity.

Methods

Strains. Four strains of *Rhizopus* (class *Zygomycetes,* order *Mucorales,* family *Mucoraceae*), were studied, including 2 of *Rhizopus microsporus var. rhizopodiformis* (*R. microsporus*) and 2 of *Rhizopus arrhizus* (formerly called *Rhizopus oryzae*) (15). For both species, one strain was a clinical isolate, while the other one was of environmental origin. *R. microsporus* B 51321 (ATCC 66276; American Type Culture Collection, Rockville, MD) was isolated from a dialysis patient who died from mucormycosis while on DFO treatment (16); *Rhizopus arrhizus* B 51322 (ATCC 66275) was isolated from a leukemic patient who died from mucormycosis in the absence of DFO treatment. Both clinical isolates were provided by Prof. Ch. De Vroey (Laboratory of Mycology, Institute for Tropical Medicine, Antwerp, Belgium). *R. microsporus* B 58739 (IHEM 4770) and *Rhizopus arrhizus* B 58735 (IHEM 4769) were environmental strains, isolated from the air and from a menth leaf, respectively. These two environmental strains were provided by Dr N. Nolard (Laboratory of Mycology, Institute of Hygiene and Epidemiology [IHEM], Brussels, Belgium).

For comparison, 3 non-*Zygomycete* strains were used: *Candida albicans* (B 2630, ATCC 44858), *Aspergillus fumigatus* (B 19119, IHEM 3772) and *Cryptococcus neoformans* (B 42419, ATCC 32665). Characteristics of these strains have been reported elsewhere (17–20).

Culture conditions. Strains belonging to the genus *Rhizopus* were maintained on Sabouraud dextrose agar (Difco Laboratories, Inc., Detroit, MI). Sporangiospores (spores) were prepared by growing the fungi on the same medium for 10 d at 37°C. The mycelium was scraped in sterile deionized water (Milli Q; Millipore Corp., Bedford, MA); the sporulated mycelial suspension was vigorously agitated for 10 min in the presence of glass beads (mean diameter of ~2 mm) and then filtered through gauze. Eluate containing spores was washed twice with water and concentrated by centrifugation to obtain a suspension free of hyphal fragments which contained about 10^7 spores/ml, as estimated with a Bürker hemocytometer; spores were stored at 4°C in the presence of penicillin and streptomycin at 30 and 70 μg/ml, respectively (21). Culture conditions of the non-*Zygomycete* strains were as previously reported (17, 19).

Chelators. DFO was used as the commercially available Desferal[R] (Ciba Geigy, Basel, Switzerland). Fe.DFO was prepared as previously described (22), by mixing stoichiometrical amounts of DFO and $^{55}FeCl_3$ (Amersham International, Buckinghamshire, England). Aluminoxamine (Al.DFO) was provided by Prof. R. R. Crichton (Université Catholique de Louvain, Louvain-La-Neuve, Belgium). Rhizoferrin, a siderophore isolated from *R. microsporus* B 58739 (14), was provided by Prof. G. Winkelmann (Institut für Mikrobiologie und Biotechnologie, Tübingen, Germany). ^{55}Fe. rhizoferrin and ^{55}Fe. citrate complexes were prepared by mixing $^{55}FeCl_3$ and rhizoferrin or citrate with a stoichiometry 1:1 and 1:2, respectively. All ferric chelator complexes were formed before dilution in BDM.

In vivo experiments. Male albino guinea pigs (Pirbright strain), weight 500±50 g, were injected in the lateral vein of the penis with the following filamentous fungi or yeasts: *Rhizopus microsporus, Rhizopus arrhizus, Candida albicans, Aspergillus fumigatus* or *Cryptococcus neoformans.* The number of CFU injected per gram body weight in 1 ml saline is shown in Table I. For experimental aspergillosis, four different doses were studied. 12 infected guinea pigs were used for each experiment: 6 controls and 6 others treated with DFO (100 mg/kg in 0.5 ml of saline, injected intramuscularly during four successive days, starting 24 h before the infection). With *Cryptococcus neoformans* infection, groups of nine animals were used, and DFO was administered during nine successive days.

After infection with *Rhizopus* or *Aspergillus fumigatus,* animals were observed twice daily. Survival was recorded up to day 28 after infection. After *Candida albicans* infection, the number of CFU per

Table I. Experimental Systemic Mycoses in the Guinea Pig (Groups of 6). Effects of DFO

Fungus/Yeast	No. of CFU injected/g body wt	Controls	DFO-treated	P*
		(mean±SEM)	*(100 mg/kg for 4 d)*	
Survival up to 28 d (in days)				
R. microsporus	293	4.2±0.41	3.0±0	§
R. arrhizus	3,550	8.8±0.41	4.8±0.41	§
A. fumigatus	25,000	5.5±0.55	3.8±0.41	§
	12,500	5.7±0.52	4.0±0.63	§
	6,250	14.0±0.51	5.2±0.75	§
	3,125	21.2±10.63	6.0±1.67	‡
CFU/g organ				
C. albicans	8,000			
		skin: 139,850±71,434	107,519±90,072	NS
		kidney: 96,054±72,065	44,656±57,515	NS
Survival up to 42 d (in days, median value)				
C. neoformans‖	200	> 42	36	‡
Score of skin granulomas (at day 28)		3.3±0.71	4.0±0	‡

* Mann-Whitney U test (2-tailed probability) ‡ $P \leq 0.05$, § $P \leq 0.01$. ‖ Groups of nine guinea pigs; DFO administration for nine days in the DFO treatment group.

gram of organ weight was determined for the skin and the kidney, once the animals were killed on day 16. Colony counting on Sabouraud agar plates supplemented with antibacterial antibiotics was performed with serial 10-fold dilutions. The plates were incubated at 37°C for 1 wk.

After infection with *Cryptococcus neoformans*, animals were observed twice daily. Survival was recorded up to day 42 after infection. The numbers and extent of cutaneous cryptococcal granulomatous lesions were scored semiquantitatively on day 28. Details on methods used for experimental fungal infections have been reported extensively (17–20).

Effect of polymorphonuclear granulocytes. Human polymorphonuclear granulocytes (PMNs) were harvested and purified as previously reported (23). In order to study the effect of PMNs on spore germination, spores were washed in HBSS and incubated in 10% normal human pooled serum during 10 min at 37°C, washed in HBSS and diluted to 1,000/μl. Spores were mixed with PMNs (10^7 PMN/ml) and 10% pooled serum to a final volume of 1 ml and incubated for 0 (baseline), 2.5, and 5.5 h, respectively. At these intervals, 20 μl were plated on Sabouraud broth agar. After one night at 37°C, the germinated spores were counted by microscopy and, the number of spores germinated were compared with the number germinated at baseline.

The inhibitory effect of PMNs on the growth of *R. microsporus* hyphae was studied by mixing hyphae and PMN suspensions at several dilutions. *Rhizopus* spores were diluted to 1,000/μl in Sabouraud broth and incubated for 8 h at 37°C. Under these conditions, ≥ 90% of the spores had germinated to a length of ≥ 30 μm without appreciable clumping of the organisms (24). Hyphae (10 μl) in Sabouraud broth were mixed with 0.3 ml of PMN suspensions, yielding PMN/hyphae ratios of 600/1, 300/1, 150/1, 75/1, 37.5/1, and 18.75/1 in 24-well dishes. 1 ml of alpha-MEM No. 041-02561 containing 21 amino acids and 11 vitamins (Gibco, Gent, Belgium) + 10% fetal calf serum was added to each dish and the mixtures were incubated at 37°C in 5% CO_2. After 20 h, the PMN/hyphae ratio at which no hypal growth could be observed (inhibitory ratio) was determined macroscopically.

The effects of PMNs on spore germination and hyphal growth were studied with: (*a*) PMNs isolated from six healthy donors, preincubated in vitro with DFO, Fe.DFO, or ferric chloride at 0 (control), 0.5, 1, 10, and 100 μM for 20 h at 37°C; and (*b*) PMNs isolated from six hemodialysis patients. Three of them were taking daily oral aluminum hydroxide as phosphate binder and were not clearly iron overloaded (serum ferritin < 1,000 μg/liter; the other three patients had not been taking aluminum salts but had transfusional iron overload (serum ferritin > 2,000 μg/liter). Blood was withdrawn prior to and 44 h after a single intravenous administration of DFO at 30 mg/kg at the end of hemodialysis session ("DFO test"). The serum concentration of the iron chelate of DFO, Fe.DFO, was measured as reported elsewhere (25).

In vitro growth. Fungal and yeast growth was studied by a turbidimetric determination performed after cultivation in BDM (basal defined medium). This synthetic medium is designed for mammalian cell cultivation, is buffered by Hepes and sodium bicarbonate, and contains 1.36 μM of ferric iron (26). Spores (*Rhizopus*), conidia (*Aspergillus*) and yeasts (*Candida*), all at 10^6, were incubated in 2.1 ml of BDM in the presence or absence of different concentrations of pooled serum from healthy human volunteers (iron content, 16.7 μM; transferrin content, 2.4 mg/ml, i.e., 0.3 μM; iron saturation of transferrin, 24%) and/or chelators. The in vitro growth was assayed at different pH values in the presence of human serum, apotransferrin or fully saturated transferrin (Sigma Immunochemicals, St Louis, MO). After 24 h at 37°C, the culture medium was centrifuged twice for 15 min at 2,000 *g*. Pellets were harvested in PBS and sonicated (50 W, 1 min); absorbance was read at 400 nm and background absorbance due to the medium was subtracted (27). Other culture media (alpha-MEM and Wickerham's medium) were also used. Instead of normal human serum, serum from six hemodialysis patients was also studied before, as well as 44 h after, a single in vivo administration of 30 mg/kg DFO.

In vitro iron uptake. For uptake experiments, 10^6 fungal particles (see above) in 2.1 ml BDM, supplemented or not with 40% of human serum, were incubated for different intervals at 37°C in the presence of chelators bound to ^{55}Fe. At the end of incubation, fungal elements were filtered under vacuum through cellulose acetate membranes (Millipore Corp., pore diameter of 0.22 μm), after 3 washings with 5 ml of PBS, filters and fungal elements were assayed for radioactivity, after dispersion with 5 ml of Aqualuma cocktail (Lumac-Lsc, Olen, Belgium) in a Tri Carb 460CD scintillation counter (Packard Instruments, Meriden, CT).

Table II. Inhibition of the Germination of R. microsporus Spores by PMNs (5.5 h of Incubation)

Origin of PMNs	% Inhibition	P*
	(mean±SEM)	*(versus control)*
Normal subjects (*n* = 6)		
Not preincubated	64±8.6	
Preincubated for 20 h (control)	62±7.8	
Preincubated for 20 h with		
DFO (1 μM)	58±6.1	NS
Fe.DFO (1 μM)	54±8.6	NS
Fe^{3+} (1 μM)	60±3.6	NS
Hemodialysis patients (*n* = 6)		
Pre-DFO (control)	71±3.2	
Post-DFO	67±1.6	NS

* Friedman 2-way analysis of variance by ranks.

Morphological analysis. Spores of *R. microsporus* were incubated for 24 h at 37°C in BDM supplemented or not with 40% human serum in the presence or absence of Fe.DFO (1 μM). The fungal suspension was centrifuged twice for 15 min at 2,000 *g*, and the pellets were harvested in a lugol solution (Merck, Rahway, NJ) and put on slides. Slides were washed, dehydrated, and cleared with toluol, and the material was covered with a mounting medium.

Statistics. Mean values are given together with SEM. Statistical significance between study groups was determined by either the unpaired *t* test, the Friedman two-way analysis of variance by ranks, the two-tailed Mann-Whitney U test, or the one factor- or two factors-ANOVA tests (28).

Results

Effect of DFO on experimental mycosis. Table I shows that DFO, administered to normal immunocompetent guinea pigs, aggravates the course of experimental mycosis due to some but not all studied fungi or yeasts. The administration of DFO at the time of induction of the experimental infection leads to a significant shortening in survival, when the infection is caused by *Rhizopus microsporus, Rhizopus arrhizus, Aspergillus fumigatus* and *Cryptococcus neoformans.* DFO therapy, however, has no significant effect upon the course of systemic mycosis caused by *Candida albicans.*

Effect of PMNs. Table II shows that normal PMNs inhibit the germination of *R. microsporus* spores by 64±8.6% after 5.5 h of incubation. This inhibition is not affected by preincubation of the PMNs for 20 h nor by their preincubation for 20 h in the presence of either DFO, Fe.DFO or ferric chloride (at concentrations ranging from 0.5 to 100 μM). Similar results are observed with *Rhizopus arrhizus* (not shown). In vivo administration of DFO to hemodialysis patients also does not signifi-

cantly modify the PMN effect on *R. microsporus* spore germination.

Similarly, the minimum PMN/*R. microsporus* hyphae ratio, able to inhibit the growth of hyphae of *R. microsporus,* is influenced neither by preincubation of normal PMNs with DFO, Fe.DFO, or ferric chloride, nor by DFO administration to hemodialysis patients (not shown). These data indicate that the development or aggravation of mucormycosis during DFO therapy does not result from a DFO-induced decrease in the defense capacity of PMNs toward *Rhizopus.*

Synergistic effect of feroxamine and serum on Rhizopus growth. The growth of *R. microsporus* has been measured quantitatively by a turbidimetric assay. When cultivated in BDM, a nutritive medium designed for the cultivation of mammalian cells, and in the absence of human serum, neither Fe.DFO, Al.DFO nor the *R. microsporus* own siderophore Fe.rhizoferrin (all at 1 μM) significantly influence the growth of *R. microsporus;* only ferric chloride or citrate slightly but significantly increases the growth of *R. microsporus* ($P \leq 0.0005$ and 0.0001, respectively, Fig. 1). Similar results are obtained when the spores are cultivated for 24 h in Wickerham's medium, a classical culture medium for fungi, in the presence of Fe.DFO at concentrations ranging from 0.01 to 10 μM (not shown).

Normal human serum is fungistatic toward *R. microsporus,* leading to a decreased fungal growth ($P \leq 0.0001$). As shown in Fig. 2, fungistasis in BDM is already reached at a serum concentration ≤ 10%. A similar serum fungistatic effect is observed when *R. microsporus* is cultivated in α-MEM or Wickerham's medium. Fungistasis is observed at a serum concentration ≤ 10% with α-MEM, whereas a serum concentration ≥ 30% is required with Wickerham's medium.

Prior dialysis of the serum for 48 h against PBS does not affect serum fungistasis (not shown). Human apotransferrin (Fig. 3), but not fully saturated transferrin (not illustrated), causes a fungistatic effect similar to that of serum; this effect is observed when the incubation is carried out at pH 7.4 or 6. However, lowering the pH to 5, at which transferrin loses its capacity to bind iron, abolishes the fungistatic effect of both serum and apotransferrin and increases fungal growth (Fig. 3; $P \leq 0.0001$). This suggests that serum fungistasis could be mediated, at least partially, through the iron-binding capacity of apo- or incompletely saturated transferrin present in serum.

The fungistatic effect of serum added to BDM is lost upon addition of 1 μM Fe.DFO (Fig. 2; $P \leq 0.0001$). Furthermore,

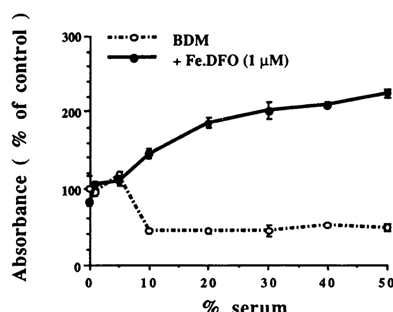

Figure 2. Effect of human serum and Fe.DFO on *Rhizopus* growth. *R. microsporus* spores were incubated in BDM supplemented with different concentrations of human serum with or without 1 μM of Fe.DFO for 24 h at 37°C. Growth was evaluated by turbidimetry at 400 nm. Mean results±SEM of 8 independent experiments are given.

this addition results in a significant increase in the growth of *R. microsporus* (Figs. 1 & 2); this effect is observed from a serum concentration of 10% and is related to the serum concentration, reaching a level of 230% at 50% serum (Fig. 2). In contrast to Fe.DFO, Fe.rhizoferrin at 1 μM does not promote growth in BDM with 40% serum (Fig. 1). Al.DFO and iron salts, tested at 1 μM with human serum, restore the growth of *R. microsporus* to the level reached in the absence of serum but have no synergistic effect as observed with Fe.DFO (Fig. 1). In the presence of apotransferrin at pH 7.4 or 6, the addition of Fe.DFO restores the growth of *R. microsporus* to the control level; nevertheless, the effect of Fe.DFO is considerably more pronounced in the presence of serum than of apotransferrin (Fig. 3).

The fungistatic effect of serum toward *Rhizopus* was also studied after one single administration of DFO to 6 hemodialysis patients. Hyphal growth in α-MEM is inhibited by serum at 5% ($n = 3$) or at 10% ($n = 3$) prior to DFO administration. However, serum, even at a concentration of 20%, loses its fungistatic activity 44 h after DFO administration ($P = 0.02$ post- versus pre-DFO). The Fe.DFO serum concentration 44 h after DFO administration was 3.96±1.25 μM.

Phase contrast microscopy indicates that, after 24 h cultivation of *R. microsporus* in BDM, the presence of human serum and/or Fe.DFO (1 μM) affects the fungal development. Indeed, the proportion of spores transformed into hyphae is considerably decreased in the presence of serum (Figs. 4 *A* and *B*).

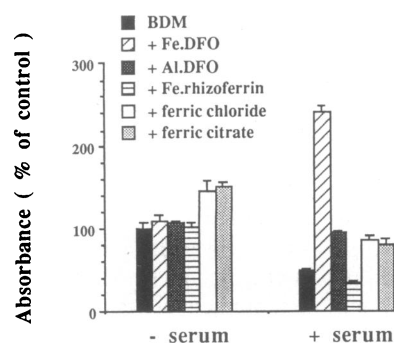

Figure 1. Effects of iron chelators and salts on *Rhizopus* growth. *R. microsporus* spores were cultivated for 24 h at 37°C in BDM, supplemented or not with 40% human serum and in the presence of 1 μM Fe.DFO, Al.DFO, Fe.rhizoferrin or iron salts. Growth was evaluated by turbidimetry at 400 nm. Results were normalized, with the growth in BDM but without serum considered to be 100%. Mean results±SEM of eight independent experiments are given.

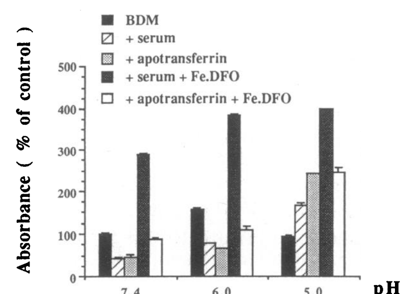

Figure 3. Effects of pH and apotransferrin on *Rhizopus* growth. *R. microsporus* spores were cultivated for 24 h at 37°C in BDM at different pH in the presence of either serum or apotransferrin (1 mg/ml) with or without supplementation with Fe.DFO at 1 μM. Growth was evaluated by turbidimetry at 400 nm. Mean results±SEM of four independent experiments are given.

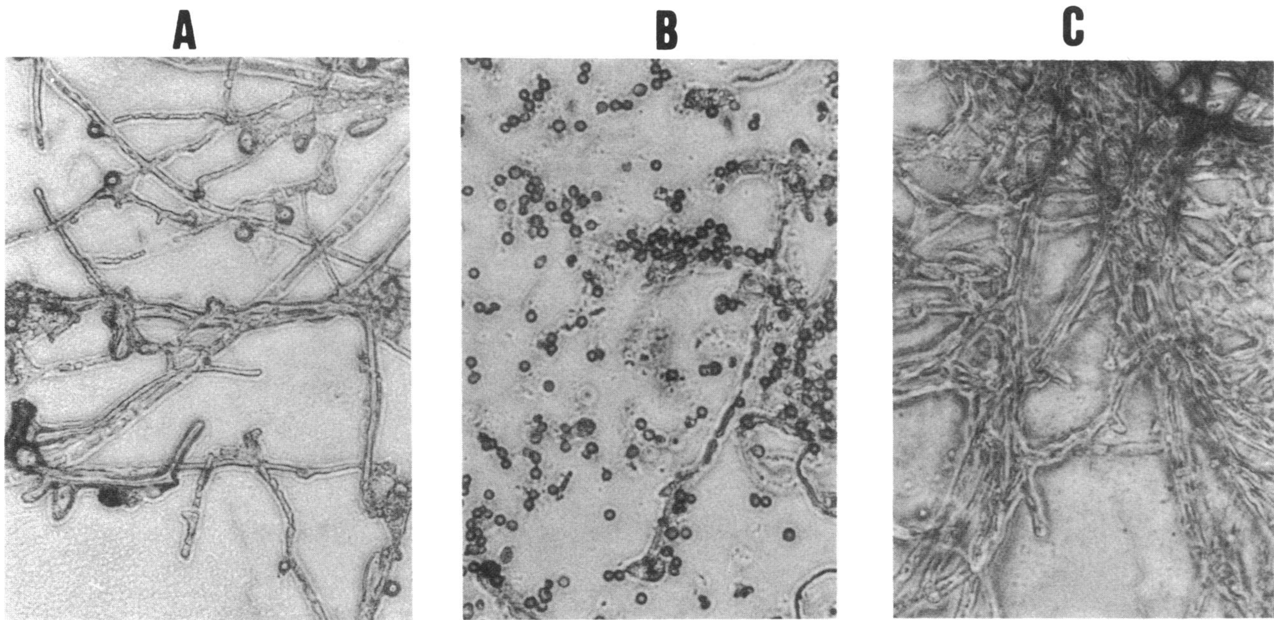

Figure 4. Morphological effect of serum and Fe.DFO on *Rhizopus* growth. *R. microsporus* spores were cultivated for 24 h at 37°C in BDM alone (*A*), in BDM with 40% human serum (*B*) or in BDM with 40% serum + 1 μM Fe.DFO (*C*). Lugol stain, ×500.

However, when Fe.DFO is added to this medium, the morphological transformation is restored, and the growth rate is increased (Fig. 4 *C*). In the absence of serum, Fe.DFO has no effect (not shown).

Iron uptake by Rhizopus. In order to establish a possible correlation between *Rhizopus* growth and iron uptake, *R. microsporus* spores were incubated in BDM supplemented with 40% human serum, in the presence of different concentrations of either ^{55}Fe.DFO, ^{55}Fe citrate, or ^{55}Fe.rhizoferrin; after 24 h incubation, radioiron uptake and *Rhizopus* growth were determined in parallel. In the case of Fe.DFO, a growth-promoting effect is observed at a concentration as low as 0.01 μM and is maximal at 0.5 μM (Fig. 5 *A*); iron accumulation is proportional to the Fe.DFO concentration from 0.01 to 15 μM (Fig. 5 *B*). In the presence of Fe.rhizoferrin or ferric citrate, by contrast, the growth remains inhibited by serum up to a concentration of ca. 5 μM for both iron complexes; no significant accumulation of radioiron is detected. At concentrations > 5 μM, iron is accumulated and the growth of *R. microsporus* is stimulated. Similar rates of iron uptake (18 and 51 pmols ^{55}Fe/10^6 fungal particles, respectively) occur at very different concentrations of Fe.DFO (0.01 μM) and Fe.citrate (10 μM).

Specificity of feroxamine effect on Rhizopus. Human serum at 40% in BDM reduces the growth of all four strains of *Rhizopus* studied, the effect being more pronounced for the clinical than for the environmental isolates; serum also slightly reduces the growth of *Aspergillus fumigatus;* however, it does not significantly affect the growth of *Candida albicans* (Fig. 6 *A*).

The addition of 1 μM Fe.DFO to BDM containing 40% serum has no effect on *C. albicans,* leads to a moderate increase in growth of *A. fumigatus,* but more markedly increases the growth of *R. arrhizus* and *R. microsporus,* for both the clinical and the environmental strain (Fig. 6 *A*).

Fig. 6 *B* illustrates the iron uptake by the organisms after 24 h incubation in BDM supplemented with 40% serum and containing 1 μM ^{55}Fe.DFO. Iron uptake by *R. microsporus* and *R. arrhizus* (both environmental and clinical strains) is much higher than that by *A. fumigatus* and *C. albicans* ($P \le 0.0001$). Both *R. microsporus* strains take up significantly more iron than either strain of *R. arrhizus* ($P \le 0.0001$).

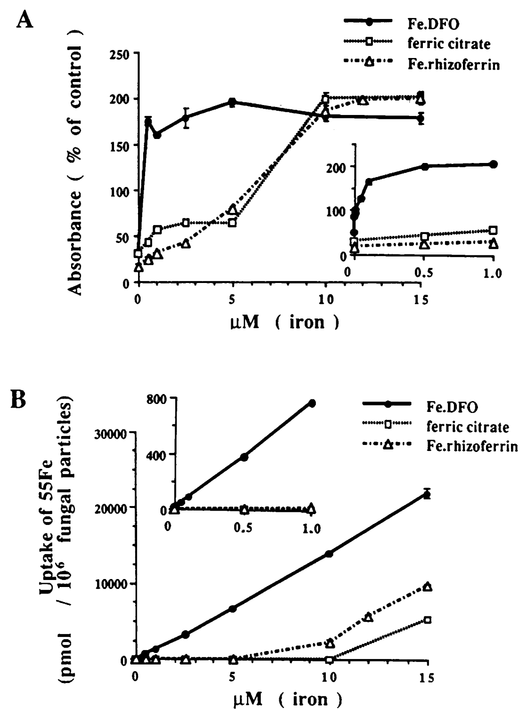

Figure 5. Iron uptake and *Rhizopus* growth. *R. microsporus* spores were cultivated for 24 h at 37°C in BDM, supplemented with 40% human serum, in the presence of different concentrations of ^{55}Fe.DFO, ^{55}Fe.rhizoferrin or ^{55}Fe citrate. The effect of these iron chelators on *Rhizopus* growth was evaluated by turbidimetry at 400 nm (*A*) and the radioiron uptake by *Rhizopus* was measured (*B*). Inset figures show results obtained at low chelator concentration (≤ 1 μM Fe). Mean results±SEM of eight independent experiments are given.

Fig. 7 shows that, for the different fungal species tested, there is a significant positive correlation ($r = 0.886$) between the radioiron uptake from 1 μM ^{55}Fe.DFO and the growth stimulation in the presence of Fe.DFO in a serum-containing medium.

Discussion

The present investigation was prompted by several reports of fulminant and commonly fatal mucormycosis developing in patients on maintenance dialysis. The seminal observation is the association between this infection and treatment with DFO, as found in 46/59, i.e., 78% of the patients (2). It therefore seemed important to investigate the mechanism of this iatrogenic pathology.

When mucormycosis, caused by two different species of *Rhizopus*, was experimentally induced in the guinea pig, DFO aggravated the infection, with significant shortening of survival (3). A similar aggravation of experimental mucormycosis by DFO was found in the mouse by an independent group of investigators (29). In uninfected animals, DFO at the same dosage did not influence survival (3).

As DFO can produce several effects on the immune system, a possible effect of DFO upon PMNs, which are assumed to play an important role in the host defense against *Rhizopus* (30), was examined. Neither in vitro preincubation with DFO or its chelates, nor a single administration of DFO to hemodialysis patients, modified the rate of inhibition of PMNs on either

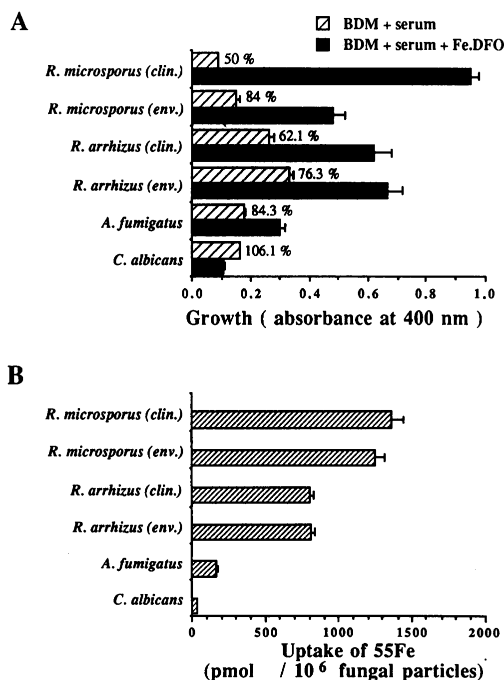

Figure 6. Effect of serum and Fe.DFO on growth of *R. microsporus* (clinical and environmental), *R. arrhizus* (clinical and environmental), *A. fumigatus* and *C. albicans*. Spores, conidia, and yeasts were incubated for 24 h at 37°C in BDM supplemented or not with 40% human serum and with Fe.DFO (1 μM). Growth was evaluated by turbidimetry at 400 nm. The effect of serum on growth is given by the % value of growth with vs. without serum (*A*). At the same time, the iron uptake from ^{55}Fe.DFO was measured (*B*). Mean results±SEM of eight independent experiments are given.

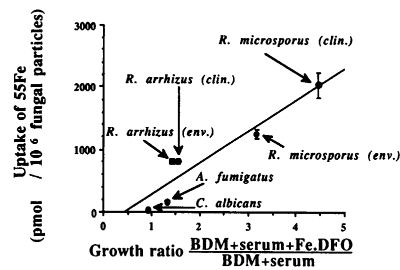

Figure 7. Correlation between the effects of Fe.DFO upon iron uptake and upon growth for different fungal species. Spores, conidia, and yeasts were incubated for 24 h at 37°C in BDM containing 40% human serum supplemented or not with 1 μM Fe.DFO or ^{55}Fe.DFO. The simultaneous effects of Fe.DFO on fungal growth and on radioiron uptake were measured.

the germination of *Rhizopus* spores or hyphal growth. Therefore, a direct effect of DFO or one of its chelates upon *Rhizopus* was postulated.

Our study confirms the observation, known for three decades (31, 32), that human serum inhibits the growth of *Rhizopus*. Serum also retards the morphological transformation of *Rhizopus* spores into hyphae. The precise mechanism(s) of serum fungistasis toward *Rhizopus* is (are) presently unknown. According to Artis et al., serum fungistasis requires a direct contact between transferrin and *Rhizopus* (33). Apotransferrin exerts a similar degree of fungistasis as whole serum. Dialysis of the serum does not alter the fungistatic effect, but the latter is lost at pH 5, at which transferrin's capacity to bind iron is decreased (34). These data support the concept that serum fungistasis toward *R. microsporus* is mediated, at least partially, by the capacity of (apo)transferrin to bind serum iron and to make it inaccessible to the fungus. However, at pH 5, serum decreases the growth of *R. microsporus* more than apotransferrin does. This suggests that serum contains unidentified factors other than apotransferrin, which can inhibit the growth of *Rhizopus*.

In the absence of serum, Fe.DFO does not significantly stimulate the growth of *R. microsporus*, an observation published in two earlier reports (35, 36). Likewise, Fe.DFO does not affect the rate of morphological transformation of spores into hyphae. However, when Fe.DFO is added to several serum-containing media, not only does the serum-induced growth inhibition disappear, but also a highly significant growth stimulation of *Rhizopus* spores is observed; the rate of their morphological transformation into hyphae is also restored. A concentration of Fe.DFO as low as 0.01 μM increases the in vitro growth when compared with control growth in the absence of serum. The synergy between serum and Fe.DFO is specific, as Al.DFO, ferric chloride, and ferric citrate each restore the fungal growth only to the control level. More surprisingly, 1 μM Fe.rhizoferrin, the siderophore produced by *R. microsporus*, has no effect. The in vivo correlate of this synergy between serum and Fe.DFO is the observed loss of fungistatic activity of the serum from hemodialysis patients after a single administration of DFO, with leads to the in vivo generation of Fe.DFO.

The precise mechanism by which a nanomolar concentration of Fe.DFO, but not of ferric citrate nor of Fe.rhizoferrin, can disrupt the serum fungistasis is presently unclear. It could relate to the ability of serum apotransferrin to take up iron from the two latter complexes, making iron unavailable to *R. microsporus*, and to its inability to take up iron from Fe.DFO (De Locht, M., manuscript in preparation). Whatever the

mechanism, our results show that the growth stimulation of *R. microsporus* by iron complexes is linked to fungal iron uptake. When comparing Fe.DFO with ferric citrate, both allow a similar degree of growth promotion in serum at a similar rate of iron accumulation by *R. microsporus.* However, the concentration of both iron complexes needed to reach these effects is very different: 0.01 μM for Fe.DFO, which allows an uptake of 18 pmols ^{55}Fe/10^6 fungal particles, vs. 10 μM for ferric citrate, which allows an accumulation of 51 pmols ^{55}Fe/10^6 fungal particles. These results strongly suggest that iron is a growth factor for *Rhizopus.* Some promotion of *Rhizopus* growth due to iron salts in BDM without serum could been explained by the presence of an exogenous iron source. The lack of growth promotion with Fe.rhizoferrin in serum is possibly related to a lower stability of the iron complex in the presence of serum. The fact that the degree of iron uptake from ^{55}Fe.DFO correlates directly with Fe.DFO-associated growth promotion in totally different fungal species adds further strength to the concept that Fe.DFO is a growth factor for some fungi.

Patients treated with DFO and reported to develop mucormycosis are mainly dialysis patients, whereas DFO-treated patients with normal renal function only exceptionally develop this infection (2, 37). This is in keeping with the central role played by Fe.DFO in the pathogenesis of this infection when developing during DFO therapy. Indeed, pharmacokinetic changes in uremia lead to a sixfold prolongation of the elimination halftime and a 4.9-fold increase in the area under the concentration-time curve for Fe.DFO in dialysis patients, when compared with subjects with normal renal function, after one single DFO administration (38). 44 h after one DFO administration at 30 mg/kg, hemodialysis patients have serum levels of Fe.DFO > 1 μM; when these patients are iron overloaded, the Fe.DFO serum levels at 44 h are > 10 μM (38). Such serum levels suggest that, in the case of once weekly DFO dosage, dialysis patients may continuously have Fe.DFO serum levels > 0.01 μM, levels which are sufficient to reverse serum fungistasis to *Rhizopus* and to promote the growth of the fungus. On the other hand, patients with normal iron stores have an unbound iron-binding capacity of transferrin, which keeps the level of other iron salts (such as ferric citrate) well below a concentration of 5 μM, a level at which the growth of *Rhizopus* is not stimulated.

In dialysis patients with aluminum overload, a part of the administered DFO is converted to Al.DFO, while another part becomes Fe.DFO. Similarly, the addition of 1 μM Al.DFO to the culture medium could lead to the generation of some Fe.DFO and to an equilibrium between the two complexes. It is therefore not possible to ascertain whether the slight increase in *Rhizopus* growth observed with Al.DFO in serum is really due to Al.DFO or to the formation of some Fe.DFO. Because of the unavailability of a stable aluminum isotope, it was possible neither to answer this question nor to measure aluminum uptake by *Rhizopus* directly. In vivo pretreatment of mice with aluminum salts has no effect upon the susceptibility to *Rhizopus* infection (39), whereas pretreatment with iron salts aggravates the infection (3, 29). This supports the idea that the slight effect of Al.DFO in vitro could be due to formation of some Fe.DFO. These data would underscore the critical role played by Fe.DFO and not by Al.DFO in the pathogenesis of this infection.

A few DFO-treated dialysis patients have been reported to develop bacteremia caused by *Yersinia enterocolitica* of some serotypes (40). These strains, which do not synthesize a siderophore, are able to take up radioiron from ^{59}Fe.DFO, which stimulates their in vitro growth and their pathogenicity in experimental animals (41, 42). In the present study, the in vivo effect of DFO upon the course of infection caused by several fungal species not belonging to the *Zygomycete* class has been examined. DFO is found to enhance the pathogenicity of *Aspergillus fumigatus* and of *Cryptococcus neoformans* but has no effect upon that of *Candida albicans.* Similarly, we and others found that the in vitro growth is increased by Fe.DFO in the first two (43) but not in the latter species (44). Radioiron uptake from ^{55}Fe.DFO, measured for *A. fumigatus* and *C. albicans,* was much lower (8.3-fold and 40.1-fold respectively) than for the clinical strain of *R. microsporus.* This is in agreement with a previous report (45). The good correlation between the iron uptake from Fe.DFO and the degree of growth promotion by Fe.DFO among the studied fungal species does not prove but is compatible with a cause-and-effect relationship, iron uptake being responsible for the growth stimulation. Furthermore, these results could explain why mucormycosis is reported to have occurred in at least 46 cases of dialysis patients treated with DFO, (2), whereas disseminated aspergillosis developed in only one case (46) and systemic candidiasis has never been reported in this clinical setting.

In conclusion, this study underscores the siderophore function of Fe.DFO in the pathogenesis of DFO-induced mucormycosis, as it develops in man, and of DFO-aggravated mucormycosis, as observed in animals. In the presence of serum, Fe.DFO allows a significant uptake of iron by *Rhizopus* and it promotes its in vitro growth. This growth stimulation is already present at a Fe.DFO concentration as low as 0.01 μM. Renal dysfunction allows Fe.DFO to accumulate, so that this concentration is exceeded for several days after DFO administration, explaining the particular sensitivity of this patient population to this infectious complication of DFO therapy. Given the present interest in chelation therapy, it is obvious that studies on new chelators (DFO derivatives or others) will have to take into account the possibility that specific microorganisms may utilize chelator-bound iron. Our results have shown that in vitro testing of iron uptake and of growth in appropriate media may be highly predictive of the in vivo consequences of siderophore-mediated iron uptake.

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