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Fungistatic Mechanism of Human Transferrin for *Rhizopus oryzae* and *Trichophyton mentagrophytes*: Alternative to Simple Iron Deprivation

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Human serum, human transferrin (TF), and the iron chelator 1,10-phenanthroline (OP) produce iron-reversible fungistatic activity which has been attributed to simple iron deprivation. In this study, the influence of the size of the inoculum on the inhibitory activity of serum, TF, and OP prepared with the same iron-binding capacity (2.5 μg/ml) for *Rhizopus oryzae* and *Trichophyton mentagrophytes* was examined. Inhibition was monitored in liquid microcultures maintained at 37°C and pH 7.4 to 7.5 by measuring the change in absorbance density. Increasing the number of spores in the inoculum disrupted the fungistatic activity of serum and TF, but not that of OP. The dilution at which OP lost fungistatic activity was not affected by the number of spores in the inoculum and was the same for both fungi. The dilution at which TF and serum lost fungistatic activity was dependent upon both the quantity of the inoculum and the species of fungus. The number of viable spores, rather than the total number of spores in the inoculum, was determined to be important in overcoming the inhibition of fungal growth by serum and TF. The fungistatic activity of serum and TF could be diminished by the preexposure of the serum to viable but nongrowing spores. Direct and indirect fluorescence studies indicated that both *T. mentagrophytes* and *R. oryzae* absorbed TF. Glucose uptake by *R. oryzae* was inhibited by a 4-h exposure to 5.0 to 0.15 mg of apotransferrin per ml. These results suggest that the fungistatic activity of TF for *R. oryzae* and *T. mentagrophytes* may not be attributable to simple iron deprivation and raise the possibility of a requirement for a direct interaction.

Human serum in vitro has fungistatic properties which have been attributed to the iron-binding glycoprotein transferrin (TF) (7, 10, 12, 21, 22). Two lines of direct evidence have implicated TF in this activity. First, purified TF added to medium has been demonstrated to inhibit fungal growth (12, 20, 21). Second, the removal of TF from serum by affinity chromatography abolished the fungistatic activity of serum (12). The mechanism by which TF inhibits fungal growth is thought to be related to the capacity of the protein to tightly bind and thus reduce the presence of available iron to a level below that required to support fungal growth (22). The previously observed disruption of antifungal activity by the addition of iron-containing compounds to either serum- or TF-containing media is consistent with this explanation of the mechanism (7, 10, 12, 20, 21).

Several reasons lead us to suspect that the mechanism by which TF in serum inhibits fungal growth may be more complex than the simple deprivation of iron. First, recent reports suggest that the amount of non-TF-bound iron in serum and plasma may range from 10^{-12} to 10^{-10} M (17, 18). These values are significantly greater than the 10^{-18} M value reported previously (6) and are much nearer to the estimated $\leq 10^{-9}$ M iron requirement value for fungal growth recently reported (T. C. Granada, H. E. Jones, and W. M. Artis, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, Abstr. no. F62, p. 336). Second, elevating the serum iron of guinea pigs 50 to 140 times above normal levels by the subcutaneous injection of the soluble iron compounds ferric citrate and ferric ammonium citrate did not correlate with the disruption of serum fungistatic activity for *Trichophyton mentagrophytes* and *Rhizopus oryzae* (9). Third, the removal of TF from TF-supplemented medium by careful ultrafiltration restored the capacity of the medium to support fungal growth, despite a significant lowering of the iron content, suggesting that TF may require intimate contact with

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fungi to exert an inhibitory effect (R. L. Duncan, Jr., F. Rastinejad, and W. M. Artis, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, abstr. no. F63, p. 337).

In this paper, we present our investigation of the mechanism by which TF exerts an inhibitory effect on the growth of the dermatophyte *T. mentagrophytes* ATCC 18748, for which TF has been clearly established as the serum inhibitory factor, and on that of *R. oryzae*, a causative agent of mucormycosis, for which there is also good evidence that TF is the inhibitory component in serum (5, 12). The results are consistent with the possibility that TF may suppress the growth of these fungi by a mechanism(s) other than iron deprivation. The possibility of a requirement for a direct interaction is considered.

MATERIALS AND METHODS

Fungi. *T. mentagrophytes* var. *granulare* (ATCC 18748) and *R. oryzae* (clinical isolate; Mycology Division, Centers for Disease Control, Atlanta, Ga.) were maintained on Sabouraud agar. The spores were prepared by growing the fungi on potato dextrose agar for 2 weeks and scraping the sporulated mycelium from the agar into sterile deionized water. The sporulated mycelial suspension was agitated in the presence of glass beads and filtered through a column containing glass wool. The spores contained in the eluate were washed and concentrated by centrifugation in sterile deionized water, adjusted to a concentration of 10^7/ml, and stored at 4°C. Nonviable spores were obtained by heating spores (10^7/ml in deionized water) at 80°C for 2 h with occasional mixing. After the heating, the spores were washed two times with sterile deionized water and stored at 4°C. Viability was assessed by the capacity to initiate growth in nutrient broth and on Sabouraud agar after 72 h of incubation at 35°C and by the limiting dilution method in nutrient broth. Spore viability was reduced to ≤5%. Some lots showed no viability.

Human sera. Nonheparinized venous blood was collected in 50-ml conical centrifuge tubes (Falcon Plastics; Oxnard, Calif.) and allowed to clot at room temperature. The clots were removed after 2 h, and the remaining suspension was centrifuged at 400 × *g* for 10 min. Sera fractions were decanted, sterilized by passage through a 0.22-μm syringe filter unit (Millipore Corp., Bedford, Mass.), and stored in 6-ml aliquots at −80°C. The serum iron (SI), unbound iron-binding capacity (UIBC), and total iron-binding capacity were determined as described below.

TF and albumin. The purified human TF (Sigma Chemical Co.; St. Louis, Mo.) used in this investigation was approximately 98% iron free, contained no preservatives, and formed one precipitate band against rabbit antiwhole human serum. The TF was stored at 4°C in its lyophilized form. The final concentration of TF was adjusted to yield the same UIBC (2.5 μg/ml) as the human sera used. TF solutions were sterilized by passage through 0.22-μm syringe filter units.

Purified human albumin (Sigma) was essentially globulin free, formed one precipitate band against rabbit anti-whole human serum, and showed no cross-reactivity with TF. The albumin was stored in its lyophilized form at 4°C until used.

For conjugation with fluorescein isothiocyanate isomer I (Sigma), TF and albumin were adjusted to 20 mg/ml in 0.25 M carbonate-bicarbonate buffer (pH 9.0). This was mixed overnight at 4°C with 0.05 mg of fluorescein isothiocyanate per mg of protein. The conjugated protein was separated from the free fluorochrome by passing the mixture down a Sephadex G-25 column equilibrated with 0.15 M phosphate-buffered saline (PBS; pH 7.4).

Antisera. Goat antihuman TF and goat antihuman albumin (Cappel Laboratories, Westchester, Pa.) were stored at −20°C until used. Both the anti-TF and anti-albumin formed one precipitate band in immunodiffusion against human serum and showed no indication of cross-reactivity. Fluorescein-conjugated immunoglobulin G (IgG) fractions of goat antihuman TF and antihuman albumin (10 mg/ml of IgG; molar F/P ratio, 2.5) were obtained from Calbiochem-Behring (La Jolla, Calif.).

Chemicals. A stock solution (0.885 mg/ml) of the chelator 1,10-phenanthroline (OP) (Matheson, Coleman, and Bell, Norwood, Ohio) was prepared by dissolving 9.34 mg in 0.5 ml of 70% ethanol and diluting this in 10.05 ml of nutrient broth. The chelator was further diluted in medium (described below) so that the final concentration was 8.85 μg/ml. The iron-binding capacity of OP was calculated on a mole per mole basis (1 molecule of ferric iron per molecule of OP). The addition of iron to the chelator resulted in a vivid pink-orange color which reached maximum intensity near the theoretical iron saturation point. This reaction was used to confirm the iron-binding activity of the chelator.

Iron nitrilotriacetic acid (Fe-NTA) was used to disrupt the fungistatic activity of human serum and TF-supplemented medium by exact saturation of the UIBC. A stock solution of Fe-NTA was prepared by reacting 2.67 g of disodium NTA (Sigma) with 1.45 g of ferric chloride in 100 ml of deionized water. After 1 h, the pH of the reaction mixture was adjusted to 7.5 by the slow addition of 1.0 N NaOH. Insoluble iron hydroxide complexes were removed by centrifugation at 20,000 rpm, using a J-21 Beckman high-speed centrifuge equipped with a JA-21 head. The final iron content was determined by a ferrozine chlorometric assay and was found to be 1.75 mg/ml.

SI and UIBC. SI levels were determined spectrophotometrically by a modification of the procedure described by Shade et al. (19). Serum (0.125 ml) was pipetted into disposable semi-microcuvettes (VWR Scientific Apparatus; Atlanta, Ga.). Iron bound to TF was made available for measurement by the addition of 0.5 ml of acetate buffer (pH 4.5) prepared by dissolving 6.0 g of sodium acetate in 100 ml of deionized water containing 4.0 ml of glacial acetic acid. The contents of the cuvette were mixed, and an initial absorbance was determined with a Beckman model 35 spectrophotometer set to read at 562 nm. After 5 min, 0.025 ml of the chelator ferrozine [3-(2-pyridyl)-5,6 bis-(4-phenyl-sulfonic acid)-2,4-triazine] (Sigma) was added to the cuvette. The final absorbance was determined 5 min later. The change (final absorbance minus initial) was compared with values obtained with stan-

dard iron solutions to determine the iron present in the serum.

The amount of additional iron that can be bound by TF in serum (UIBC) was determined in a manner similar to that described for the SI measurement, except that a 1.0 M Tris buffer (pH 8.3) containing 5.0 μg of iron per ml in the form of ferric citrate was used. Final absorbance readings were made after 45 min. The decrease in the amount of free iron present after 45 min was proportional to the amount of iron taken up by TF (initial free iron minus final free iron = UIBC).

Medium. The medium was prepared from 8 g of nutrient broth (Difco Laboratories, Detroit, Mich.), 2 g of sodium bicarbonate, 10 ml of fetal calf serum (Flow Laboratories, Inc., McLean, Va.), brought to 1,000 ml with deionized water. The medium was sterilized by filtration through a 0.22-μm membrane filter (Millipore). The medium did not exhibit any iron-binding activity. Medium supplemented with TF was prepared by adding apotransferrin until a UIBC of 2.5 μg/ml was reached. This usually required 2.5 to 3.0 mg of TF protein per ml. Medium supplemented with the chelator OP (8.85 μg/ml) was prepared by adding 1.0 ml of the OP stock solution to 99.0 ml of the medium. Noninhibitory serum and TF-supplemented medium were prepared by adding ≤10-μl portions of Fe-NTA with mixing to 100 ml of whole human serum or apotransferrin-supplemented medium. The mixture was allowed to equilibrate for 20 min, and the UIBC was determined. When no UIBC was detectable, the mixtures were allowed to equilibrate for 24 h at 4°C after which time a final adjustment of the UIBC was made by the addition of either small amounts of fresh serum and apotransferrin or of Fe-NTA. The resulting serum and TF-supplemented medium were sterilized by filtration through a 0.22-μm membrane filter (Millipore) and used immediately. Serum and medium supplemented with TF or OP had an iron-binding capacity of 2.5 μg/ml. The base medium was used as a diluent in all experiments requiring dilution of serum, TF, or OP.

Cultures and fungal growth. The wells of flat-bottom, 96-well microtiter dishes (Costar; Cambridge, Mass.) containing 200 μl of medium per well were inoculated with the desired number of spores (viable and nonviable) suspended in 10 μl of sterile deionized water. The cultures were incubated in a tissue culture incubator (VIP CO_2 incubator 420 Lab-line; Melrose Park, Ill.) at 37°C in a humidified atmosphere consisting of approximately 4.5% CO_2 in air. This atmosphere, with minor adjustments of CO_2 content, maintained the pH of the cultures between 7.4 and 7.5. The pH of designated control cultures was monitored continuously within the incubator, using a digital readout pH meter (Beckman model 3560) equipped with a microcombination pH electrode. At 12-h intervals, fine adjustment of the CO_2 flow was made to maintain the culture pH.

Fungal growth was monitored by measuring the optical density of the cultures at 450 nm through the bottom of the microtiter dishes with a Titertek Multiskan plate reader (model 78-504-00; Flow Laboratories, Inc., Rockville, Md.). The readings were corrected for background absorbance by subtracting the absorbance of freshly inoculated but nongrowing control cultures from the experimental cultures. All cultures were set up in quadruplicate. The absorbance data given represent the mean of triplicate or quadruplicate cultures corrected for background absorbance. The variation of absorbance between replicate cultures was ≤7%.

Glucose utilization. Quadruplicate microcultures were inoculated with 10^4 *R. oryzae* spores at various intervals (0, 12, 16, 20, and 24 h from the onset of incubation) and incubated at 37°C in the tissue culture incubator system described above. After 26 h from the onset of incubation, 185 μl of medium was carefully withdrawn with a 12-channel pipette (model 77-889-00; Flow Laboratories, Inc.) and discarded. The volume removed was replaced with 200 μl of warmed fresh base medium supplemented with various concentrations of TF. After 4 h of incubation, the TF-containing medium was removed with a multichannel pipette, the cultures were washed four times with 250 μl of warmed base medium, and 200 μl of warmed fresh base medium supplemented with [*U*-^{14}C]glucose (0.15 μCi/ml; 0.11 mCi/mmol) was added to each culture. After an additional 2 h of incubation, the mycelial content of each well was collected on filter paper (grade 934 AH; Reeve Angel, Clifton, N.J.) with a multiple automated sample harvester by the procedure described by Artis et al. (4). The ^{14}C incorporated by the growing mycelium was determined by liquid scintillation counting.

Fluorescent and immunofluorescent staining of fungi. Midexponential-phase mycelium of *R. oryzae* (36 h) and *T. mentagrophytes* (72 h) was obtained by growing the fungi in 10 ml of nutrient broth cultures maintained at 37°C. A small tuft of mycelium was transferred to 1.0-ml portions of the indicated fluorescein conjugate in 0.15 M PBS (pH 7.4), incubated at 37°C for 1 h, and then washed three times with PBS by centrifugation. The mycelium was mounted in glycerol on a standard microscope slide and examined by incandescent and UV light with a Leitz Dialux 20 EB microscope equipped with a Ploemopak 2.4 fluorescence vertical illuminator.

Fluorescein-conjugated TF and albumin were used at a final concentration of 0.75 mg/ml. Anti-TF and anti-albumin were reacted with the fluoresceinated TF and albumin under conditions of antibody excess, determined beforehand by an optimal proportion titration. No precipitates were visible in any of the staining mixtures.

Fluorescein-conjugated anti-TF and antialbumin IgG were used at a 1:50 dilution prepared in PBS. Mycelia used in sandwich technique experiments were first incubated with 1.0 ml of albumin or TF (0.75 mg/ml) or human serum for 1 h at 37°C. The mycelia were washed three times with PBS by centrifugation and then incubated for 1 h at 37°C with 1.0 ml of the fluoresceinated antibody solution. The mycelia were washed three times with PBS, mounted in glycerol, and examined with the UV microscope. The fluorescent intensity of all preparations was ranked on a scale of 1 to 4, where 4 was the highest intensity.

RESULTS

Effect of spore numbers on fungal growth in medium and noninhibitory human serum. Figure 1 defines the normal kinetics for growth initiated with large (10^4 spores) and small (10^2 spores)

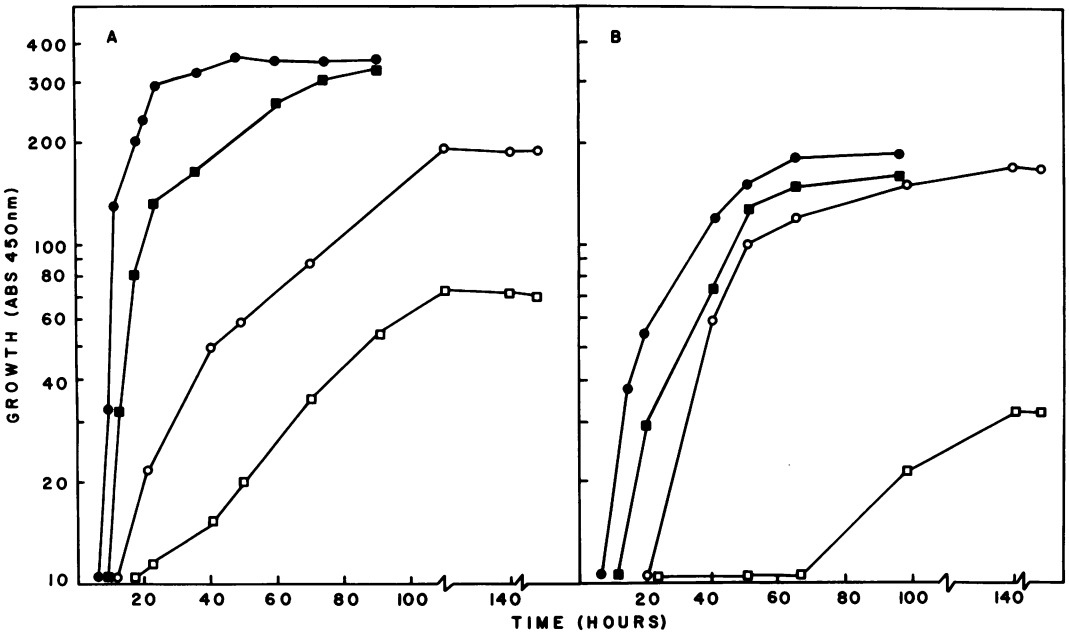

FIG. 1. Effect of inoculum size on growth of *R. oryzae* (solid symbols) and *T. mentagrophytes* (open symbols) in serum (A) and TF-supplemented medium (B) rendered noninhibitory by saturation of the UIBC. Symbols: ● and ○, 10^4 spores; ■ and □, 10^2 spores. ABS, Absorbance.

inocula of *R. oryzae* and *T. mentagrophytes* in microcultures at physiological temperature and pH in human TF-supplemented medium and human serum rendered noninhibitory by the addition of Fe-NTA. Growth kinetics in medium without TF were not different from that in medium supplemented with iron-saturated TF. These results indicate that fully iron-saturated TF does not inhibit the growth of *R. oryzae* or *T. mentagrophytes*.

For both fungi, lag, exponential, and stationary growth phases were clearly definable. *R. oryzae* grew much more rapidly than *T. mentagrophytes*, which is clearly indicated by the greater slope and shorter duration of the exponential growth phase for *R. oryzae*. Both fungi grew to a greater mass in the noninhibitory serum than they did in the noninhibitory TF-supplemented medium. The number of spores used to initiate growth affected both the rate and the final amount of growth achieved. This was particularly evident for *T. mentagrophytes*, where the final mycelial mass was directly proportional to the number of spores in the inoculum. In contrast, *R. oryzae* grew to the same mycelial mass, regardless of the number of spores used to initiate growth.

The length of incubation needed to reach the stationary and midexponential growth phases was determined in each medium for each inoculum used to standardize the time at which growth was monitored in experiments where inhibitory activity was evaluated.

Effect of spore inoculum quantity on inhibitory activity of human serum, human TF, and OP. Human TF and the iron chelator OP were prepared in medium so that their UIBCs were 2.5 μg/ml, which was identical to that of the human serum used. Complete saturation of the iron-binding capacity of these three chelators with Fe-NTA rendered them completely noninhibitory for the growth of *R. oryzae* and *T. mentagrophytes*.

Microcultures containing OP, TF, and human serum were inoculated with 10^1 to 5×10^7 spores and incubated under conditions that maintained the culture pH between 7.4 and 7.5. The inhibitory activity of OP was not affected by increasing the number of spores in the inoculum; Inhibition was complete with the largest inoculum (5×10^7 viable spores). In contrast, the inhibitory activity of TF and human serum was overcome by increasing the number of spores in the inoculum (Table 1). The reversal effect became more pronounced as the cultures approached the stationary growth phase. For both *R. oryzae* and *T. mentagrophytes*, growth inhibition was greater in serum than in the TF-containing medium, and a greater number of viable spores was required to overcome inhibition in serum than was required in TF-containing medium; the reversal of inhibition in serum was

TABLE 1. Effect of number of spores in the inoculum on the inhibitory activity of human serum and TF for *R. oryzae* and *T. mentagrophytes*[a]

No. of spores per culture	Microculture absorbance density (absorbance at 450 nm) during:							
	Stationary phase[b]				Exponential phase[c]			
	Noninhibitory serum	Inhibitory serum	Medium	Medium + TF	Noninhibitory serum	Inhibitory serum	Medium	Medium + TF
R. oryzae								
10^1	—[d]	—	0.153	0.000	—	—	0.076	0.000
10^2	0.370	0.000	0.151	0.000	0.185	0.000	0.083	0.000
10^3	0.381	0.000	0.171	0.076	0.193	0.000	0.086	0.048
10^4	0.376	0.128	0.189	0.119	0.197	0.000	0.095	0.050
10^5	0.380	0.155	0.224	0.215	0.194	0.040	0.112	0.098
T. mentagrophytes								
10^2	0.074	0.000	0.043	0.000	0.037	0.000	0.022	0.000
10^3	0.125	0.000	0.112	0.000	0.063	0.000	0.056	0.000
10^4	0.195	0.010	0.175	0.022	0.098	0.000	0.088	0.000
10^5	0.227	0.201	0.213	0.190	0.114	0.065	0.107	0.052

[a] Microcultures (0.2 ml) were incubated at 37°C within a CO_2 incubator adjusted to maintain the pH between 7.4 and 7.5. Growth was evaluated in situ spectrophotometrically by measuring the culture absorbance density at 450 nm. Each value is the mean absorbance density derived from triplicate or quadruplicate cultures corrected for the absorbance of the medium and inoculum before growth. The range of measurements varied by no more than 7% of the mean.

[b] Stationary-phase growth was measured at the time that accumulated mycelium for the inoculum quantity was maximum.

[c] Exponential-phase growth was measured at the half-maximum growth time.

[d] —, None.

obtained with 10^4 viable *R. oryzae* spores, whereas 10^3 spores reversed inhibition in the TF-containing medium. A comparable reversal of the inhibitory effect with *T. mentagrophytes* required 10^5 spores for serum and 10^4 spores for TF. Although the fungistatic activity of serum and TF was diminished by increasing the quantity of viable spores in the inoculum, maximum growth was never as great as in the noninhibitory control cultures.

When cultures were inoculated with a mixture of viable spores and dead spores (killed by incubation at 80°C for 4 h), the capacity of the inoculum to overcome the inhibitory activity of serum and TF was found to be dependent upon the number of viable spores rather than the total number of spores. A mixture of 10^3 viable and 9.9×10^4 nonviable *R. oryzae* spores did not initiate growth in serum. This inoculum grew slightly in TF-containing medium but no more than did 10^3 spores alone. Increasing the viable spore content of the mixture to 10^4 spores gave it the capacity to grow well in serum and TF-containing medium. The growth, however, was never different from that of the given viable inoculum alone (Table 1). Similar results were obtained with mixtures of viable and nonviable *T. mentagrophytes*. The addition of dead spores to cultures containing OP had no effect on the inhibitory activity of the chelator.

The addition of 10^1 to 10^5 viable spores of either *R. oryzae* or *T. mentagrophytes* to serum and the TF-containing medium did not reduce the iron-binding capacity or increase the SI value when measured 2 h after the addition, suggesting that iron contamination had not occurred.

Effect of dilution on inhibitory activity of serum, TF, and OP. Serum, TF, and OP with the same iron-binding capacity (2.5 μg/ml) were serially diluted with medium, and their inhibitory activity was evaluated against 10^2 to 10^5 viable *R. oryzae* or *T. mentagrophytes* spores. The first dilution permitting growth and the absorbance density at that dilution were determined after 96 h of incubation for various numbers of spores (Table 2). The dilutions at which the fungistatic activity of serum and TF was lost ranged between neat and 1:128 and varied with the number of spores in the inoculum. The fewer the spores, the greater was the dilution required before inhibition was lost. The amount of fungal growth was usually ≥40% of that occurring in the noninhibitory serum and medium at the first dilution where inhibition was overcome. Using the dilution endpoint as a measure of the inhibitory strength of serum and TF, we were not able to determine which fungus was more susceptible to growth inhibition. At inoculum quantities of $\geq 10^4$ viable spores, *T. mentagrophytes* was more susceptible to growth inhibition. At quantities of $\leq 10^3$ spores, *R. oryzae* was more suscep-

TABLE 2. Effect of dilution on capacity of serum, TF, and OP with the same iron-binding capacity to inhibit fungal growth[a]

No. of spores per culture	First dilution permitting growth (absorbance at 450 nm) in:			Growth (absorbance at 450 nm)	
	Serum	Medium + TF	Medium + OP	Non-inhibitory serum	Medium
R. oryzae					
10^2	1:64 (0.160)	1:128 (0.143)	1:8 (0.162)	0.340	0.155
10^3	1:32 (0.151)	1:64 (0.145)	1:8 (0.175)	0.345	0.171
10^4	1:2 (0.290)	Neat (0.131)	1:8 (0.189)	0.357	0.185
10^5	Neat (0.155)	Neat (0.219)	1:8 (0.205)	0.360	0.223
T. mentagrophytes					
10^2	1:16 (0.054)	1:32 (0.040)	1:8 (0.035)	0.062	0.024
10^3	1:8 (0.067)	1:8 (0.067)	1:8 (0.092)	0.101	0.089
10^4	1:4 (0.065)	1:4 (0.123)	1:8 (0.160)	0.150	0.155
10^5	1:2 (0.189)	Neat (0.227)	1:8 (0.210)	0.230	0.220

[a] Microcultures (0.2 ml) were incubated at 37°C for 96 h within a CO_2 incubator adjusted to maintain the pH at 7.4 to 7.5. Growth was evaluated in situ spectrophotometrically at 450 nm. Each value is the mean absorbance density derived from quadruplicate cultures corrected for the absorbance of the medium and inoculum before growth. The range of the measurements varied by no more than 7% of mean.

tible to growth inhibition. In contrast to the growth inhibition mediated by serum and TF, the inhibitory activity of OP was lost at the same dilution (1:8) for both fungi and was not affected by the number of spores in the inoculum.

Effect of preincubation of serum and TF with viable spores on subsequent fungistatic activity. The preincubation of serum or TF-containing media with 5×10^3 viable *R. oryzae* or 10^4 viable *T. mentagrophytes* spores for 72 h at 37°C reduced fungistatic activity (Table 3). This was assessed by determining the highest dilution of serum or TF-containing medium which was still fungistatic. The first dilution in the primary inoculated cultures which permitted the growth of *R. oryzae* in both serum and TF-containing medium was 1:16. This dilution was 1:4 for *T. mentagrophytes*. Dilutions less than these values did not support discernable fungal growth. Immediately after the first incubation, 200 μl

TABLE 3. Reduction of the fungistatic activity of serum and TF for *R. oryzae* and *T. mentagrophytes* after preincubation with viable spores[a]

Dilution	Fungal growth (absorbance at 450 nm)[a] in:			
	Serum		Medium + TF	
	Primary[b] inoculation	Secondary[c] inoculation	Primary inoculation	Secondary inoculation
R. oryzae (5×10^3 spores)				
Neat	0.000	0.000	0.000	0.000
1:2	0.000	0.065	0.000	0.037
1:4	0.000	0.175	0.000	0.162
1:8	0.000	0.323	0.000	0.175
1:16	0.165	0.350	0.075	0.328
1:32	0.327	0.347	0.353	0.318
T. mentagrophytes (10^4 spores)				
Neat	0.000	0.025	0.000	0.160
1:2	0.000	0.127	0.000	0.145
1:4	0.057	0.135	0.110	0.150
1:8	0.134	0.141	0.160	0.147

[a] Growth was evaluated in situ spectrophotometrically after 72 h of incubation at 37°C within a CO_2 incubator. Each value is the mean absorbance density derived from triplicate cultures corrected for the absorbance of the medium and the inoculum before growth.

[b] The primary inoculation cultures contained 250 μl of the indicated serum and TF dilutions.

[c] The secondary inoculation cultures contained 200 μl derived from the primary inoculation after 72 h of incubation.

was carefully withdrawn from each culture so as not to include any fungi and was transferred to a fresh culture well. The microtiter well was examined with an inverted microscope to exclude those secondary cultures where fungus had been carried over. Secondary cultures were reinoculated with the same quantity of viable spores used in the primary culture and reincubated at 37°C for 72 h. After the second incubation, the first dilution which supported fungal growth had decreased by three dilutions from 1:16 to 1:2 for *R. oryzae*. *T. mentagrophytes* grew in undiluted serum and TF which was a reduction of two dilutions. The UIBCs of the transferred serum and TF determined at the neat and 1:2 dilutions were not significantly different from the UIBC at the start of the experiment. These results suggest that the active TF species or a necessary cofactor had been removed by the first incubation. Alternatively, an anti-inhibitory substance may have been secreted into the medium.

Binding of TF to fungal surface. The capacity of TF to interact with the fungal surface was examined directly by observing the binding of fluoresceinated TF and indirectly by fluorescent staining of bound TF with fluoresceinated anti-TF immunoglobulin. The results with fluoresceinated TF are presented in Table 4. Fluoresceinated albumin was used as a specificity control. Fresh exponential-phase mycelia of *R. oryzae* and *T. mentagrophytes* bound fluoresceinated TF and fluoresceinated albumin. Reacting fluoresceinated TF with anti-TF immunoglobulin in antibody excess almost completely inhibited the binding of fluoresceinated TF. Antialbumin immunoglobulin was somewhat less effective in reducing the binding of fluoresceinated albumin. Albumin, however, did not significantly inhibit the interaction of fluoresceinated TF with either fungus. The reaction of *R. oryzae* and *T. mentagrophytes* mycelia which had been preincubated in either TF prepared in PBS or in serum for 20 min at 37°C with fluoresceinated anti-TF immunoglobulin confirmed the capacity to bind TF (Table 5). Interestingly, not all of the mycelium in any given preparation demonstrated the capacity to bind TF, regardless of the method used to assess binding. Representative binding of fluoresceinated TF is depicted in Fig. 2.

TABLE 4. Direct binding of fluoresceinated human TF and human albumin to *R. oryzae* and *T. mentagrophytes*

Conjugate mixture[a]	Intensity of fluorescence[b] of:	
	R. oryzae	*T. mentagrophytes*
Fl-TF + PBS	+3	+4
Fl-TF + anti-TF	±	±
Fl-TF + anti-Alb	2+	2+
Fl-TF + Alb	2+	3+
Fl-Alb + PBS	3+	1+
Fl-Alb + anti-Alb	1+	±
Fl-Alb + anti-TF	2+	1+

[a] The components of the conjugate mixture were combined and incubated for 1 h at 37°C before they were used. Fl-TF, Fluoresceinated TF: Fl-Alb, fluoresceinated Alb; Alb, albumin.

[b] The fluorescent intensity was ranked on a scale of 1 to 4, where 4 was the highest intensity.

TABLE 5. Binding of human TF and human albumin to *R. oryzae* and *T. mentagrophytes* assessed by fluoresceinated anti-TF and anti-albumin immunoglobulin[a]

Pretreatment of fungus	Fluorescein conjugate[b]	Intensity of fluorescence[c] of:	
		R. oryzae	*T. mentagrophytes*
PBS	Anti-TF	−	−
TF	Anti-TF	+3	+4
PBS	Anti-Alb	−	−
TF	Anti-Alb	−	−
Albumin	Anti-Alb	+1	+2
Serum	Anti-TF	+2	+4
Serum	Anti-Alb	+1	+2

[a] The fungi were incubated with the indicated substance for 1 h at 37°C and washed three times before staining with the fluorescein conjugate.

[b] Alb, Albumin.

[c] The fluorescent intensity was ranked on a scale of 1 to 4, where 4 was the highest intensity.

TF inhibition of glucose utilization. *R. oryzae*, which readily adheres to the plastic surface of microculture trays, was grown for lengths of time selected so that the mycelium could be exposed to TF for a short period of time during each of its growth phases (Table 6). When the fungus had reached the indicated growth phase, the medium was replaced with fresh TF-supplemented medium. After 4 h of incubation, the TF was removed by extensive washing and replaced with fresh [*U*-^{14}C]glucose-supplemented medium. Eight hours later, the uptake of [*U*-^{14}C]glucose was determined. The uptake of [*U*-^{14}C]glucose was greatest in late-exponential- and early-stationary-phase cultures, which corresponded with the greater quantity of mycelia in these cultures. A 4-h exposure to TF significantly inhibited [*U*-^{14}C]glucose utilization. Exponential-phase fungi were more sensitive to TF than were preexponential-phase fungi, whereas uptake by stationary-phase fungi was resistant to the effect of TF and occurred only at concentrations of ≥2.5 mg/ml. We found no dose-response effect of TF on the inhibition of [*U*-^{14}C]glucose utilization. These results suggest that the metabolism of glucose by *R. oryzae* during active growth can be significantly altered by a brief exposure to a very small quantity of TF.

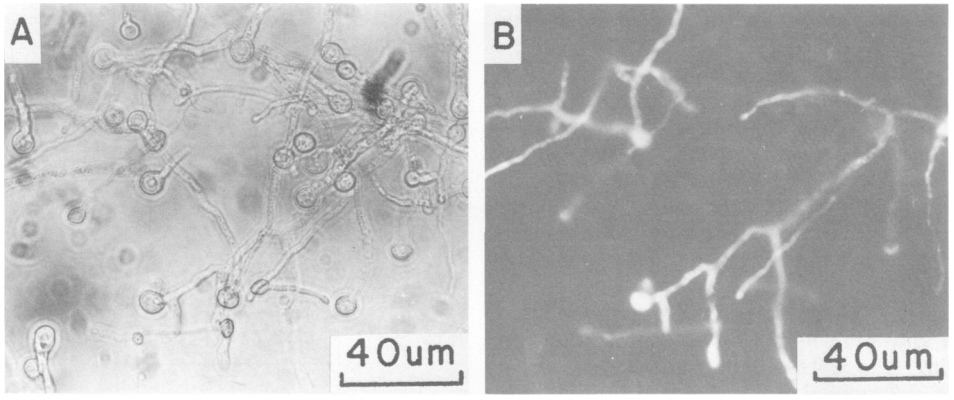

FIG. 2. Differential binding of TF to surface of exponential-phase *R. oryzae*. (A) Light photomicrograph; (B) fluoresceinated TF.

DISCUSSION

The addition of iron compounds to serum or to medium containing TF can disrupt the TF-mediated microbial inhibitory activity (6, 22). Traditionally, this has been interpreted to indicate that TF-mediated inhibition results from the deprivation of nutritionally required iron. Recently, Arnold and co-workers have presented evidence that the antibacterial activity of lactoferrin, an iron-binding glycoprotein similar to TF, may not be attributable to simple iron deprivation (1–3). In this report, we present evidence that the inhibitory activity of TF for the opportunistic fungus *R. oryzae* and the dermatophyte *T. mentagrophytes* may not be mediated by simple iron deprivation either. We also demonstrate that TF has the capacity to bind to the surface of these fungi.

The first evidence that serum or TF-containing medium may not exert their fungistatic effects by simple iron deprivation was derived from the observation that growth inhibition could be overcome by increasing the number of spores added to a culture. If growth inhibition is the result of simple iron deprivation, then one would predict that if too little iron is present to allow the growth of a few spores, then even less iron per spore would be available if additional spores were added. However, it could be argued that increasing the number of spores permitted the growth of a few spores at the expense of many by bringing additional spore-associated iron into the system. To rule out this possibility, we kept the number of spores in the inoculum constant, while varying the ratio of nonviable to viable spores. The fungistatic activity of serum and TF was found to be dependent on the number of viable spores and not the total number of spores, indicating that the introduction of spore-associated iron did not account for the fungistatic activity being lost by increasing the number of spores.

Alternatively, the inhibitory effect of TF may be dependent upon the interaction between spores and a finite number of TF molecules. Consistent with this possibility is the observation that the fungistatic capacity of TF and serum could be diluted out and that the dilution at which the fungistatic activity was lost was

TABLE 6. Effect of human TF on utilization of glucose by *R. oryzae*[a]

Growth phase[b] (h)	^{14}C uptake (cpm)[c] of control cultures (no TF)	% Inhibition of [*U*-^{14}C]glucose uptake with concn of TF of (mg/ml):					
		5.0	2.5	1.25	0.62	0.31	0.15
Preexponential (10)	864 ± 199	32	37	27	30	27	21
Early exponential (14)	1,946 ± 547	50	63	42	42	40	44
Late exponential (18)	6,415 ± 539	76	72	64	63	62	68
Early stationary (22)	6,392 ± 706	26	30	0	7	0	0

[a] Quadruplicate microcultures (200 μl) were inoculated with 10^4 spores and incubated at 37°C within a CO_2 incubator adjusted to maintain the pH at 7.4 to 7.5. All cultures were pulsed with 0.1 μCi of [*U*-^{14}C]glucose for the last 2 h of incubation and harvested with an automated sample harvester.

[b] TF was added 4 h before the pulse time and was removed before pulsing with [*U*-^{14}C]glucose (noncontinuous exposure).

[c] The mean counts per minute plus or minus the standard deviation for control cultures which were not exposed to TF but were subjected to the same manipulations as TF-treated cultures.

dependent on the number of viable spores used to inoculate the cultures (Table 2). Unlike TF or serum, the chelator OP inhibited fungal growth at all spore concentrations tested. The inhibitory activity of OP also diluted out at the same point for both fungi, regardless of the number of spores used to inoculate the cultures. This is consistent with the results that would be predicted if OP inhibited fungal growth by simple iron deprivation. Clearly, these data indicate that OP suppresses fungal growth by a mechanism different from that by which TF suppresses it.

The preexposure of serum or TF to spores or growing mycelia before re-inoculation reduced the growth-inhibiting activity for a second inoculation (Table 3). This could be explained by the removal or destruction of the active inhibitory TF species. Alternatively, this result could be explained by the presence of a secreted siderophore. Similar to the bacteria-derived siderophore enterochelin, a fungus-derived siderophore may be capable of disrupting the inhibitory activity of TF (24).

Recently, we have shown that *R. oryzae* and *T. mentagrophytes* can produce and release small amounts of a hydroxamate-type siderophore when grown in a chemically defined low-iron medium after prolonged culture (11). The presence of an anti-inhibitory factor, such as a siderophore, should be detectable by its capacity to diminish the inhibitory activity of fresh serum or TF. The dilution of fresh serum with serum that had supported the growth of *R. oryzae* did not diminish the inhibitory activity of the fresh serum, suggesting that it did not contain an active anti-inhibitory factor or siderophore (unpublished data). We have also found no chemical evidence for the presence of a siderophore in serum which had supported *R. oryzae* growth. Furthermore, like bacterial siderophore production, fungal siderophore production was suppressed at 37°C. Also, *R. oryzae* and *T. mentagrophytes* secreted a siderophore only after prolonged culturing (weeks). Thus, the probability of a secreted siderophore explaining these results is minimal.

The possibility that the active inhibitory TF species may become associated with the fungal surface and be removed from the medium was supported by our findings that fluoresceinated TF bound to both *R. oryzae* and *T. mentagrophytes* and that surface-bound TF could be detected by immunofluorescence after exposure to serum and TF-supplemented medium (Tables 4 and 5). Despite the adsorption of TF to *R. oryzae* and *T. mentagrophytes*, no detectable decrease in TF in serum or TF-supplemented medium resulted from exposure to these fungi. These data suggest that only a small proportion of the total TF present reacts with the fungi.

TF is known to exist in four distinct species with regard to its state of saturation (16). It is tempting to speculate that only a small subpopulation of TF molecules is active. Perhaps only *N*-terminal or *C*-terminal iron-saturated species are capable of interacting with the fungal surface and inhibiting growth. The remaining TF may be irrelevant.

The above idea is consistent with our finding that a small quantity of TF exerts a significant effect on glucose utilization by *R. oryzae* (Table 6). *R. oryzae* was exposed to apotransferrin for only 4 h. That the observed effect was the result of iron deprivation is hard to accept because of the activity of the very low levels of TF which would theoretically not be sufficient to bind all of the iron in the medium. Alternatively, we speculate that the effect on glucose utilization may have been mediated by an active TF species that remained bound to the fungal surface. This active TF species may be required in only small amounts and may, in fact, be actively formed by interaction with iron or some other transition metal contained within the medium.

The mechanism by which TF may interact with the fungal surface is unknown. Based on knowledge of the interaction of TF with mammalian cell types, it is possible that the carbohydrate portion of the molecule is required (14, 15). Alternatively, we propose the possibility that hydroxamate-type siderophores contained within the fungal cell wall membrane complex may be involved. It has recently been demonstrated that iron-saturated acetohydroxamic acid interacts with apotransferrin and forms a mixed ligand complex (8). The complex does not form with iron-saturated TF. Thus, the binding of TF to the fungal surface could be mediated by the formation of a mixed ligand complex at the fungal surface, and the differential staining with fluoresceinated TF (Fig. 2) may represent the expression of a siderophore on the surface of some mycelia but not others. A requirement for a siderophore is consistent with our observation that both *R. oryzae* and *T. mentagrophytes* have the capacity to synthesize hydroxamic acid.

Based on the results presented in this report, it is reasonable to suggest that TF may mediate its effect on fungi by mechanisms other than simple iron deprivation. We are currently investigating the possibility that this mechanism requires a direct interaction of TF with the fungal surface mediated through a siderophore.

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