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Different Components in Human Serum Inhibit Multiplication of *Cryptococcus neoformans* and Enhance Fluconazole Activity

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The inhibitory effect of human serum on the multiplication of *Cryptococcus neoformans* and the interaction with fluconazole were studied. Compared with cryptococcal multiplication in RPMI 1640 medium alone, 5% human serum in medium inhibited multiplication by 76% ± 6% ($n = 8$). The inhibitory effect of human serum was donor independent heat stable (56°C, 30 min), and not due to albumin or globulin. Bovine and murine sera were not inhibitory at that concentration. A fungistatic concentration of fluconazole (5.0 μg/ml) in medium plus 5% human serum resulted in 40% ± 5% ($n = 8$) killing (reduction of inoculum CFU) in a 24-h assay. Bovine or murine sera did not have the enhancing effect, and this human serum activity was heat stable and donor independent. At 2.5 μg of fluconazole per ml, fungistasis by fluconazole plus human serum was significantly greater than with either alone. Higher concentrations in serum potentiated fluconazole more. At higher fluconazole concentrations (e.g., 20 μg/ml) fluconazole alone could kill, but serum potentiated this. A fluconazole-resistant isolate (MIC, 100 μg/ml) was not killed by fluconazole (5.0 μg/ml) in 5% human serum, but human serum potentiated the partial fluconazole inhibition. When human serum was dialyzed (molecular weight cutoff, 6,000 to 8,000) against phosphate-buffered saline, it lost the ability to synergize with fluconazole for killing *Cryptococcus* organisms but not the capacity to inhibit multiplication. Filtration of serum suggested the filtrate with a molecular weight of <10,000 could interact synergistically with fluconazole for killing but could not inhibit cryptococcal multiplication. These findings indicate that human serum has two components, one (macromolecular) with a unique ability to inhibit *C. neoformans* and a low-molecular-weight component that enhances fluconazole anticryptococcal activity.

In earlier studies we showed that fungistatic concentrations of fluconazole (FCZ) acted synergistically with fungistatic murine macrophages for killing *Cryptococcus neoformans* (2, 3). In subsequent studies with human macrophages (12), we used human serum in the culture system and noted that FCZ concentrations previously found to be fungistatic were now fungicidal. In the present study, we systematically investigated this phenomenon, in light of prior observations (1, 9, 11) that serum alone is inhibitory to *C. neoformans.*

MATERIALS AND METHODS

C. neoformans. *C. neoformans* (CDC 9759) was transferred from storage at 4°C or room temperature and subcultured twice on blood agar plates (BAP) at 35°C before use in experiments. Yeast cells grown on BAP for 48 h at 35°C were collected, washed, counted, and suspended in test media. RPMI 1640 containing penicillin (100 U/ml) and streptomycin (100 μg/ml) was the basic test medium (referred to as RPMI). Fresh frozen human serum, human serum heated (56°C, 30 min) to inactivate complement (referred to as heat-inactivated serum), commercial pooled blood group AB serum (Gibco, Gaithersburg, Md.), fetal bovine serum (FBS), or mouse serum was added to RPMI at 5% (vol/vol) to form other test media. Human gamma globulin or albumin was added to RPMI to a concentration found in 5% human serum. In some experiments, RPMI containing 10 or 20% human serum or 0.165 M morpholinepropanesulfonic acid (MOPS) buffer (a buffer shown optimal for fungal susceptibility testing) (4) was tested.

Susceptibility testing was performed as previously described (8). Resistance was defined, relevant to achievable FCZ concentrations in serum with ordinary human doses (10), as a MIC of >12.5 μg/ml.

Dialysis or filtration of human serum. Serum was dialyzed at 4°C five times against phosphate buffered saline (PBS) with 32-mm-diameter Spectrapor membrane tubing (Spectrum Medical Industries, Inc., Los Angeles, Calif.) with a molecular weight (MW) cutoff of 6,000 to 8,000. The ratio of serum to buffer was 1:25. Serum was also filtered with an Ultrafree-MC unit (MW cutoff, 10,000) (Millipore, Bedford, Mass.).

FCZ. FCZ was prepared in distilled water at 2 mg/ml and stored at 4°C. Dilutions of stock FCZ were made in distilled water so that 0.011 ml of a dilution delivered per microtest plate well culture resulted in a final concentration of 2.5, 5.0, 10, or 20 μg/ml.

Quantitative cultures. Quadruplicate cultures of *C. neoformans* in various test media were set up by the dispensing of 0.1 ml of the inoculum per well of a microtest plate. FCZ was added to appropriate sets of quadruplicate cultures. Inoculum CFU were determined by the plating of a dilution of the inoculum on BAP at time zero. Cultures were incubated at 37°C in 5% CO_2–95% air for 24 h and then harvested with distilled water. Dilutions of the harvested material were plated on BAP, and CFU were counted after incubation for 48 h at 35°C.

Inhibitory and fungicidal activity. The percentage of inhibition of multiplication (24 h) was calculated by the formula [1 − (experimental CFU/control CFU)] × 100. "Control" refers to growth at 24 h without active agents. When the number of experimental CFU is less than the inoculum, this is referred to as 100% fungistasis, and fungicidal activity, i.e., reduction of inoculum (time zero) CFU, was determined by the formula [1 − (experimental CFU/inoculum)] × 100.

Statistics. Student's t test was used to compare differences between groups and to determine statistical significance, which was set at $P < 0.05$. Data are presented as means ± standard deviations. When several groups were compared with a single control, Bonferroni's adjustment to the t test was used (6).

RESULTS

Inhibition of *C. neoformans* multiplication by human serum. Human serum (5%) in RPMI significantly inhibited multiplication of *C. neoformans* (65%, $P < 0.001$) compared with multiplication in RPMI alone in a 24-h assay (Table 1). Heat-inactivated serum in RPMI had a similar effect (data not shown). FBS or murine serum in RPMI did not have a significant inhibitory effect on multiplication of *C. neoformans* (Table 1). Moreover, albumin or gamma globulin in RPMI did not inhibit multiplication of *C. neoformans* (data not shown). In eight experiments, 5% human serum in RPMI inhibited multiplication of *C. neoformans* by 76% ± 6% (Table 2). In three

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TABLE 1. Effect of 5% sera from different species on *C. neoformans* multiplication and FCZ activity

Time (h)	Serum	Concn of FCZ (μg/ml)	*C. neoformans* (CFU ± SD)[a]	% Inhibition	*P*[b]	% Killing	*P*[c]
0	None		430 ± 80				
24	None	0	23,300 ± 4,900	0		0	
24	None	5	690 ± 140	97	<0.001	0	NS[d]
0	Human		445 ± 44				
24	Human	0	8,375 ± 1,100	65	<0.001	0	NS
24	Human	5	305 ± 46	100	<0.001	32	<0.01
0	Mouse		567 ± 43				
24	Mouse	0	21,000 ± 3,160	10	NS	0	NS
24	Mouse	5	1,580 ± 390	93	<0.001	0	NS
0	Bovine		720 ± 88				
24	Bovine	0	29,750 ± 4,850	0	NS	0	NS
24	Bovine	5	685 ± 240	97	<0.001	0	NS

[a] Mean CFU of quadruplicate cultures ± standard deviation (SD) from the mean.
[b] Percentage of inhibition compared with multiplication of *C. neoformans* in RPMI without serum.
[c] Percentage of reduction of zero hour CFU (inoculum CFU).
[d] NS, not significant ($P > 0.05$).

experiments, 10% human serum inhibited multiplication by a mean of 86% (range, 80 to 93%). Experiments with 10% human serum and two other isolates produced inhibition of 86 and 94%.

Effect of 5% human serum on FCZ activity. FCZ at 5 μg/ml was highly fungistatic for *C. neoformans* when tested in RPMI (96%), RPMI plus 5% mouse serum (93%), or RPMI plus 5% FBS (97%) (Table 1). In contrast, FCZ at 5 μg/ml was fungicidal (reduced inoculum CFU by 32%) when tested in RPMI plus 5% human serum (Table 1). In eight experiments, the fungicidal activity of FCZ (5 μg/ml) in RPMI plus 5% human serum was 40% ± 5% (Table 2).

Heat inactivation of the human serum did not affect its ability to result in synergy with FCZ for killing. Neither albumin nor gamma globulin alone produced synergy with FCZ for killing (data not shown), nor could additional buffering capacity alone (MOPS buffer at 0.165 M) substitute for serum.

Human serum (5%) also potentiated inhibition by FCZ at a lower concentration of FCZ, 2.5 μg/ml ($P = 0.02$), compared with the same FCZ concentration in the absence of serum (Table 2, row 2 versus row 5). In the presence of serum, the inhibition by 2.5 μg/ml FCZ was significantly greater ($P < 0.001$) than that caused by 5% serum without FCZ (Table 2, row 4 versus row 5). Thus, the combination of FCZ plus serum resulted in significantly greater inhibition than either alone.

TABLE 2. Effect of human serum on FCZ activity against *C. neoformans* isolate CDC 9759

RPMI treatment		% Inhibition (no. of expt)	*P*[a]	% Killing (no. of expt)	*P*[b]
Serum (%)	FCZ (μg/ml)				
0	0.0	0 ± 0 (6)		0 ± 0 (6)	NS[c]
0	2.5	92 ± 5 (5)	<0.005	0 ± 0 (5)	NS
0	5.0	98 ± 1 (6)	<0.005	0 ± 0 (6)	NS
5	0.0	76 ± 6 (8)	<0.005	0 ± 0 (8)	NS
5	2.5	97 ± 2 (6)	<0.005	0 ± 0 (6)	NS
5	5.0	100 ± 0 (8)	<0.005	40 ± 5 (8)	<0.005

[a] Multiplication in 24 h of *C. neoformans* compared with multiplication in RPMI without human serum or FCZ.
[b] Inoculum CFU (zero hour) compared with CFU at 24 h.
[c] NS, not significant ($P > 0.05$).

TABLE 3. Effect of serum from different donors on synergy with FCZ against *C. neoformans* isolate CDC 9759

Concn of FCZ (μg/ml)	Effect of RPMI with fresh human serum concn of[a]:			
	0%	5% (donor 1)	5% (donor 2)	5% (donor 3)
2.5	88	96	95	96
5.0	96	47[b]	48[b]	31[b]

[a] FHS, fresh human serum. Values represent percentage of fungistasis at 24 h compared with that of the controls cultured in RPMI alone except as noted.
[b] Percentage of killing compared with that of the inoculum at time zero.

The effect of serum on potentiating FCZ appears independent of the source of the serum (Table 3). Serum samples from three healthy donors potentiated the inhibitory activity of 2.5 μg of FCZ per ml ($P < 0.0005$) but were indistinguishable from each other ($P \geq 0.7$). Addition of serum from any of the three donors to 5 μg of FCZ per ml converted fungistasis to killing ($P < 0.0005$), but the effect of individual donors was the same ($P \geq 0.2$). This was corroborated in an additional two experiments (not shown), which also showed that the inhibition of 5% human serum without fluconazole was donor independent.

Microscopic examination of harvested culture material prior to plating revealed that the fungal units in suspension consisted of 89 to 100% single cells whether serum or fluconazole was present or absent. The average number of cells per unit in the four possible combinations (serum, FCZ, both, or neither) ranged from 1.0 to 1.5 randomly with respect to serum or fluconazole; hence, clumping does not explain the reductions in the numbers of CFU.

Effect of human serum concentration on FCZ activity. We found that increasing the concentration of human serum (5, 10, and 20%) in RPMI slightly increased the fungicidal activity of FCZ (Table 4). For example, FCZ at 2.5 μg/ml in RPMI plus 20% human serum could now reduce the inoculum CFU by 17% ($P < 0.05$). In four additional experiments, killing by 2.5 μg of FCZ per ml in only 10% human serum was sometimes seen. Similarly, FCZ at 5 μg/ml in RPMI plus 10 or 20% human serum significantly ($P < 0.05$) reduced more CFU than FCZ at 5 μg/ml in RPMI plus 5% human serum (53 and 63% versus 46%, respectively) (Table 4). The difference in killing between 10 and 20% serum was not significant ($P > 0.05$).

In additional experiments, in 10% human serum, FCZ at 5

TABLE 4. Effect of human serum concentration on fungicidal activity of FCZ for *C. neoformans*

RPMI treatment (% human serum)	Effect of FCZ at concn of[a]:		
	1.0	2.5	5.0
0.0	62	86	98
5.0	90 (0)	98 (0)	100 (46)
10.0	95 (0)	99 (0)	100 (53)
20.0	96 (0)	100 (17)	100 (63)

[a] Percentage of inhibition of multiplication by RPMI–FCZ or RPMI– human serum–FCZ compared with multiplication of *C. neoformans* in RPMI without serum or FCZ in a 24-h assay. Values in parentheses represent percentage of reduction of inoculum CFU (percentage of killing) in a 24-h assay.

TABLE 5. Effect of dialyzed human serum versus whole human serum on FCZ activity against *C. neoformans*

RPMI treatment	*C. neoformans* (CFU ± SD)[a]	% Inhibition	*P*[b]	% Killing[c]	*P*[d]
10% Dialyzed serum					
0 h	1,032 ± 66				
24 h (FCZ added [μg/ml])					
0.0	6,850 ± 1,150	81	<0.005	0	NS[e]
5.0	1,420 ± 110	96	<0.005	0	NS
10.0	1,330 ± 140	96	<0.005	0	NS
10% Whole serum					
0 h	1,077 ± 93				
24 h (FCZ added [μg/ml])					
0.0	5,650 ± 1,500	85	<0.005	0	NS
5.0	427 ± 49	100	<0.005	61	<0.005
10.0	373 ± 41	100	<0.005	66	<0.005

[a] Mean CFU of quadruplicate cultures ± standard deviation (SD) from the mean in 24-h assays.
[b] Comparison with the no. of CFU of *C. neoformans* in 24-h cultures with RPMI (36,000 ± 4,300) without serum or FCZ.
[c] Percentage of reduction of inoculum CFU (time zero CFU).
[d] Inoculum CFU (time zero CFU) compared with CFU in experimental cultures.
[e] NS, not significant ($P > 0.05$).

μg/ml had a fungicidal activity of 49% ± 10% ($n = 4$), and at 10 μg/ml, FCZ had a fungicidal activity of 58% ± 17% ($n = 4$). Activity at 10 μg/ml was significantly different from that at 2.5 μg/ml ($P < 0.05$). At an FCZ concentration of 20 μg/ml (and in some experiments, 10 μg/ml), killing occurred in the absence of serum, but serum (5 or 10%) potentiated the fungicidal effect.

Effect of dialyzing human serum on FCZ fungicidal activity. Human serum that was exhaustively dialyzed against PBS was compared with an undialyzed sample of the same serum for inhibition of *C. neoformans* multiplication and collaboration with FCZ for killing. Dialyzed serum (at a 10% volume) in RPMI inhibited multiplication of *C. neoformans* to an extent (81%) similar to that of 10% whole serum in RPMI (85%) (Table 5). However, dialyzed serum in RPMI could not collaborate with FCZ for killing *C. neoformans* (Table 5). In contrast, whole serum in RPMI plus FCZ was efficient in reducing the inoculum CFU (Table 5). Similar results (but with less inhibition and killing) were obtained with 2.5 μg of FCZ per ml. FCZ could still inhibit the fungus in dialyzed serum ($P < 0.0001$ for all three FCZ concentrations).

Similar results with these concentrations were obtained in a second experiment, as well as in a third experiment in which a membrane with a MW cutoff of 12,000 was used.

Filtration of serum provided preliminary confirmatory evidence for these findings. In an experiment in which 10 μg of FCZ per ml with 10% human serum provided 99 and 93% inhibition, respectively, but no killing, whereas 10 μg of FCZ per ml and 10% human serum produced 45% killing, the filtrate with a MW of <10,000 did not inhibit *C. neoformans* multiplication. However, the filtrate at a volume of 10% combined with 10 μg of FCZ per ml to produce 22% killing ($P < 0.05$).

Commercial pooled serum alone was fungistatic but was less synergistic (no killing), even at a 10% concentration, than fresh or frozen individual donor sera with 5 or 10 μg of FCZ per ml (two experiments).

Effect of susceptibility of the isolate to FCZ on synergy with human serum. Although MICs for most (48 of 77) clinical isolates of *C. neoformans* are ≤12.5 μg/ml by this methodology (8), occasional resistant isolates are encountered. The MICs for isolates CN 92-197 and CDC 9759 were 100 and 6.25 μg/ml, respectively. Resistance in vitro by routine susceptibility methods correlated with less inhibition in this system by FCZ in the presence or absence of serum and at both concentrations of FCZ tested (Table 6). Whereas the inhibition of the resistant isolate was potentiated by serum (FCZ, 2.5 μg/ml, $P < 0.0005$; FCZ, 5 μg/ml, $P < 0.002$), this did not extend to conversion to killing at these concentrations, as occurred with isolate CDC 9759. Two additional susceptible isolates, tested concurrently with CDC 9759, also demonstrated the synergy of 5 μg of FCZ per ml with 10% human serum in killing. The percentages of killing of isolates CDC 9759, CN 94-191, and CN 94-192 were 61, 51, and 27%, respectively. A fifth isolate, CN 93-255, for which the MIC was 12.5 μg/ml, produced results in the presence or absence of serum and at both FCZ concentrations that were all intermediate between those for the susceptible isolate, CDC 9759, and the resistant isolate, CN 92-197, tested concurrently (not shown). With this isolate, potentiation by serum was again significant at both FCZ concentrations ($P \leq 0.002$).

DISCUSSION

We found in a quantitative culture system that human serum, in concentrations as low as 5%, inhibited the multiplication of *C. neoformans* in a 24-h assay. Howard (9) earlier reported, with proteose peptone medium, which is an undefined complex medium (in contrast to RPMI medium), that human serum concentrations of ≥10% inhibited *C. neoformans* growth over periods of ≥120 h. He reported the phenomenon was not due to clumping and occurred at pHs as low as 6 and that the serum component responsible was heat stable and was not an antibody. Baum and Artis (1) studied inhibition of *C. neoformans* by 90% serum at 48 and 120 h compared with that by saline, 5% glucose in water, or undefined complex medium (Sabouraud's) and found inhibition by serum which was much more marked at the later time. They found that the serum factor was present in normal individuals and patients with a variety of conditions, present in plasma, and stable even to heating to 65°C for 30 min. It was unrelated to alternate complement pathway components. Their studies with buffers indicated that serum inhibition was optimal at neutral pH but occurred above pH 7 and below pH 8. Igel and Bolande (11) also studied serum inhibition in Sabouraud's medium and reported that inhibition was not related to agglutination, dependent on cations, due to albumin, or susceptible to heat (56°C, 120 min) or dialysis but was susceptible to phosphate ions,

TABLE 6. Correlation of isolate FCZ sensitivity with synergy of FCZ and serum for killing

RPMI treatment	Effect of treatment on isolate[a]	
	CDC 9759	CN 92-197
0% Fresh human serum + FCZ (μg/ml)		
2.5	88	54
5.0	96	78
5% Fresh human serum + FCZ (μg/ml)		
2.5	97	84
5.0	43[b]	89

[a] Values represent percentage of fungistasis at 24 h compared with that of each isolate cultured in RPMI without fresh human serum except as noted.
[b] Percentage of killing compared with that of the inoculum of each isolate.

trypsin treatment, or serum being heated to 70°C. These studies (1, 9, 11) have demonstrated that serum inhibition of *C. neoformans* is isolate independent. The components in human serum that mediate this inhibitory effect have not been identified, but we also show that low-MW molecules are not necessary for this effect.

Other animal sera did not significantly inhibit cryptococcal multiplication under our conditions. In contrast to our finding with 5% mouse serum, Granger et al. (7) reported that 10% mouse serum (and sera from other species, except for FBS) in defined medium inhibited cryptococcal multiplication as measured by particle counts with an automated counter.

Human serum in RPMI enhanced the anticryptococcal activity of FCZ compared with the activity of medium alone or medium plus sera from other species. FCZ at 5 μg/ml was fungicidal in RPMI plus 5% human serum. To our knowledge, this is the first report of enhanced and fungicidal antifungal activity of FCZ in human serum.

RPMI is buffered with a bicarbonate buffering system. The effect of serum on FCZ was not due to the additional buffering capacity of serum, as demonstrated by the lack of effect on inhibition by FCZ of substituting a more powerful buffer than serum (i.e., MOPS).

It should be emphasized that our observations with serum do not explain our prior data (12) on the synergy between macrophages and FCZ versus *C. neoformans*. In those studies, the effect of FCZ alone was compared with that of macrophages in the same media, which macrophages require, and thus the controls, the FCZ alone, and the macrophage cultures all had the same percentage of serum. The observations with serum synergy appear to be another phenomenon. Taken together, these results indicate there is, first, synergy between human serum and FCZ and, second, synergy between the first combination and macrophages. The summation would have the effect of enhancing FCZ activity under in vivo conditions.

The synergy of serum and FCZ for killing is relatively insensitive to serum concentration (small differences over a fourfold serum range of 5 to 20%) and very sensitive to FCZ concentration, best demonstrated at 5 μg/ml. At half that FCZ concentration, only serum potentiation of inhibition by FCZ was usually seen, and at twice that concentration, killing by drug alone may be seen as well as only potentiation of the killing effect by serum, as described above. However, it should be noted that the direction of interaction is always positive. This sensitivity to FCZ concentration probably explains why synergy to produce killing was not seen with the resistant isolate, because this would likely have required similar studies with higher concentrations of FCZ.

A striking feature of FCZ has been its relative lack of activity in vitro compared with its activity in vivo (13). To this end, in vitro conditions, including medium (14) and use of an 80% inhibition (as opposed to complete inhibition) endpoint (5), have been manipulated in attempts to define conditions under which the FCZ antifungal effect can be better expressed and which might better correlate with in vivo results. Our data would suggest testing in serum, which more closely approximates physiologic conditions, would represent a direction for such attempts. Ordinarily, serum is excluded from in vitro susceptibility testing because antimicrobial drug activity is reduced in vitro by serum because of binding of drug by serum and reduction of free drug available for antimicrobial activity. In the case of FCZ, the level of protein binding is low (10), and any binding effect may be negligible.

We find that low-MW serum components not responsible for the inhibition by serum of cryptococcal multiplication may be necessary for collaboration with FCZ for enhancement of the anticryptococcal effect. Dialyzed serum did not synergize with FCZ for killing, but instability of nondialyzable serum components could also, at least in part, explain this observation. The isolated low-MW components (filtrate) were less synergistic than whole serum, suggesting some loss during the filtration process or instability of the synergizing component(s). Recent data (unpublished) suggest instability is a factor, and the diminished synergistic activity in pooled commercial sera may be related. The specific dialyzable or filterable serum components and the mechanism by which they collaborate with FCZ remain to be determined.

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