REVERSAL OF SERUM FUNGISTASIS BY ADDITION OF IRON*

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This paper deals with the role of the unsaturated iron-binding capacity of serum in its growth inhibitory action on *Candida albicans*.

The fungistatic power of normal human serum has been described and studied by many investigators (1, 2, 3, 4, 5). Roth and coworkers showed that 10% to 20% of serum added to nutrient medium was sufficient to inhibit growth of *Candida albicans*. This inhibition could not be overcome by addition of various carbon and nitrogen sources (6, 7).

In 1946 Schade and Caroline (8) demonstrated the antimicrobial effect of siderophilin‡), the beta-1 iron-binding globulin component of human serum and plasma. By chelating iron, siderophilin renders serum nutritionally deficient for predominantly aerobic microorganisms whose metabolic requirements for iron are high. In normal adults the serum siderophilin is approximately one-third saturated with iron. The unsaturated two-thirds is free to combine with whatever ionic iron may be available in its environment. The normal level of bound serum iron (B.I.) averages 100 mcg%. The mean value for the unbound iron binding capacity (U.I.B.C.) of siderophilin is 200 mcg%. One ml of serum is therefore able to bind 2 micrograms of iron and thus make it unavailable for the growth of microorganisms. Iron-binding capacity varies in different individuals, and each serum must be analyzed individually for the particular iron saturation of its siderophilin component (10).

The experiments to be described here are divided into two main sections. Section I consists of preliminary studies in which serum diluted with broth served as a substrate for demonstrating the effect of iron on serum fungistasis. The second section describes a series of experiments in which both whole serum and eggwhite-trypticase were used as substrates.

SECTION I. REVERSAL OF GROWTH INHIBITION OF CANDIDA ALBICANS IN SERUM-NEOPEPTONE BROTH BY ADDITION OF IRON

Material and Methods

Organism:

A strain of *C. albicans* freshly isolated from a case of cutaneous candidiasis.

Test Substrate:

Final concentration of 0.5% neopeptone, 2% glucose and 14.3% serum in demineralized water.

Iron:

Ferrous ammonium sulfate solution filtered through Corning ultrafine fritted glass filters and the filtrates analyzed for iron content.

Inoculation and Incubation:

Inocula were made from cultures grown on Sabouraud's agar for 18 hours at 37° C and consisted of 2,500 viable units added to a final volume of 7 ml of test substrate. The tubes were incubated at 37° C in air in stationary culture tubes.

Determination of extent of growth:

Densities of growth were read in a Coleman Junior Spectrophotometer at /λ 650 mμ in optically matched culture tubes.

Results

0.5% neopeptone and 2% glucose provides a satisfactory basal growth medium for *C. albicans*. To determine the effect serum would have on the adequacy of this medium for growth, we added to it an amount of serum sufficient to effect a final concentration of 14.3%. Sera from two normal individuals and three sera from patients with cutaneous candidiasis were selected for this study. Using the combined medium, two sets of cultures were established: One, a control,

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‡ While the name "transferrin" proposed for this globulin by Holmberg and Laurell (9) has priority, we prefer the term "siderophilin" because it has less restrictive physiological implications.

and the other, an iron enriched set in which 5 mcg of iron were added to a total volume of 7 ml of the test medium. These 5 mcg of iron were sufficient to provide iron in excess of the unbound iron-binding capacity of the 1 ml of serum per culture. The results obtained are summarized in Table I.

Each of the five sera, when added to the basal medium, effected complete inhibition of growth of *C. albicans* as indicated by the cultural turbidimetric measurements. In every case, however, the mere addition of iron in excess of the iron-chelating power of the serum, overcame the inhibition of growth. It is of particular interest that the addition of iron to the sera from the normal individuals as well as from the candidiasis patients supported growth of *C. albicans* to the same extent.

TABLE I

Reversal of growth inhibition of C. albicans *on addition of iron*[2] *to human serum-neopeptone broth*[1]

Sera	Status of Subject	Optical Density Readings at 20 Hours in Broth Medium with 14.3% Serum	
		Without added iron	With excess iron added 5mcg. Fe/ml serum
P. G.	Normal	0.000	0.307
C. L.	Normal	0.000	0.370
M. S.	Chronic superficial candidiasis	0.000	0.275
M. Y. A.	Chronic superficial candidiasis	0.000	0.369
F. S.	Chronic superficial candidiasis	0.000	0.358

[1] Medium is 14.3% serum, 0.5% neopeptone, 2.0% glucose to a final volume of 7 ml. with an inoculum of 2500 viable units of *C. albicans*.

[2] Iron added as ferrous ammonium sulfate. Amount in excess of that needed to saturate U.I.B.C.

SECTION II. GROWTH OF *CANDIDA ALBICANS* IN SERUM ON ADDITION OF IRON, WITH PRELIMINARY STUDIES IN "LOW"-IRON AND "HIGH"-IRON MEDIA

Materials and Methods

Organism:

C. albicans B311 provided by the courtesy of Dr. H. Hasenclever of the National Institute of Allergy and Infectious Diseases.

Basal "low"-iron medium:

2% de-ironized trypticase (Schade 11), 0.1% dextrose, 10 mcg% biotin plus 5% raw egg white. The conalbumin in raw egg white renders the medium iron-deficient by virtue of its specific iron-binding capacity (Schade and Caroline 12).

Basal serum mix:

Sera:

Serum I. Interstate serum #2430 (bound serum iron 16 mcg%; unbound iron-binding capacity 454 mcg%; percent saturation with iron 3.4).

Serum II. C.L. (bound serum iron 94 mcg%; unbound iron-binding capacity 220 mcg%; % saturation 30).

Dextrose, to a final concentration of 0.1%, and ascorbic acid to 0.0025%, were added to these sera. The sera were equilibrated with 5% CO_2 in 95% air to adjust their pH to the physioligical range of 7.4–7.5.

Iron solutions:

Ferrous ammonium sulfate in 0.6 N HCl with 15 mg ascorbic acid, /100 ml, stable for one month, and assayed to give 100 mcg iron per ml. Radioactive iron solution was prepared by adding to 1.0 ml of this iron solution 0.1 ml of Fe^{59} and Fe^{56} containing 0.28 mcg of iron so that the final total concentration of iron was 91.16 mcg /ml.

Determination of extent of growth:

Densities of suspensions of *C. albicans* were determined in the Beckman DU Spectrophotometer at 650 millimicrons in cuvettes of 1 cm light path. Chamber counts were performed in the usual manner. For the optical density of growth in serum the cells were harvested in Kimble capillary centrifuge tubes (vaccine tubes) and dilutions made in saline as indicated. For radioactive studies the growth was washed five times with saline and the amount of radioactivity in the cells was determined in a gamma ray scintillation counter.

Incubation:

In the studies with egg white, 20 ml of experimental culture were placed in 200 ml sterile Erlenmeyer flasks and the flasks placed in a humidified rotary shaker at 37° C. In the studies with Serum I, 2 ml of serum culture were put in 20 ml Warburg vessels, and for Serum II 10 ml of serum culture were placed in 125 ml Warburg vessels. The cultures were incubated in a water bath at 37° C with shaking at a rate of 130 rpm and an amplitude of 4 cm under 5% CO_2 and 95% air.

Results

Studies with "Low"-Iron and "High"-Iron Egg White Medium:

The de-ironized trypticase, egg white medium is useful as an inexpensive, readily available medium to determine the relative iron demands of organisms with respect to saturation of the naturally occurring iron-chelator, conalbumin, in egg white. So far as is known conalbumin is similar to siderophilin in all of its iron-binding characteristics (Warner and Weber 13, Schade 14). This medium was therefore used to study the growth response of *C. albicans* to different percentages of iron saturation of the conalbumin contained in the medium. For this purpose, iron was added to aliquots of the trypticase-egg white medium in amounts to give 0, 15, 20, 35, 60, 100, and 110% iron-saturation of the conalbumin. The resultant media were then inoculated with *C. albicans* at a concentration of 143 viable units per ml and incubated at 37° C with shaking in air for 17 and 40 hours. The results are given in Table II. As seen in Table II the "low"-iron egg white medium allowed only severely limited growth in 17 hours when additions of iron were insufficient to saturate the iron-binding capacity of the contained iron-binding conalbumin. At 100 and 110% saturation with iron excellent growth of *Candida albicans* resulted. It is deemed likely that much of the growth which did take place in the media containing conalbumin at less than 100% iron-saturation resulted from iron already contained in the cells used for inoculum. At the end of 40 hours the striking difference in growth attained by the "low"-iron and the "high"-iron cultures is maintained.

Studies with Undiluted Human Serum at Different Percentages of Iron-Saturation:

The availability of a serum with an unusually high unbound iron-binding capacity enabled us to study the effect of different degrees of iron saturation on the growth response of *C. albicans* in undiluted serum. 2 ml aliquots of serum in 20 ml Warburg vessels were brought to 25, 50, 75, 100 and 120% iron-saturation of the siderophilin content of this serum by additions of iron. These media were inoculated with 171 viable units/ml of "low"-iron *C. albicans*, diluted in serum. The "low"-iron inocula were obtained from cells grown in 0% iron-saturated 2% de-ironized trypticase, 5% raw egg white medium in the preceding experiment. The results of this experiment are presented in Table III. Complete fungistasis is observed at 25% iron-saturation; at 50 and 75% iron-saturation almost complete fungistasis was

TABLE II

Growth of C. albicans *in a "low"-iron medium*[1] *with conalbumin as iron-chelator and with graded amounts of iron added*

% Saturation[2] with Iron	17 Hour Growth Determination		40 Hour Growth Determination		Final pH
	Optical density 650 mu	Chamber count per ml	Optical density 650 mu	Chamber count per ml	
0	.030	40,000	.198	3,410,000	7.838
15	.022	35,000	.192	3,440,000	7.787
20	.029	70,000	.169	2,560,000	7.787
35	.030	45,000	.198	3,340,000	7.810
60	.024	75,000	.260	3,865,000	7.736
100	.406	3,605,000	4.25	75,600,000	8.574[3]
110	.403	3,065,000	4.51	90,600,000	8.560

[1] Basal Medium 2% Iron-low trypticase, 5% raw egg white, 0.1% dextrose, 0.1 mcg./ml. Biotin. Final volume 20 ml. Inoculum was 143 viable units of *C. albicans* per ml.

[2] % Saturation with iron of the unbound iron-binding capacity of the conalbumin in the egg white present.

[3] Formation of ammonia proven by chemical tests.

TABLE III

Addition of graded amounts of iron to C. albicans *in undiluted serum with high U.I.B.C.*

% Saturation with Iron	Iron Added in mcg/ml Serum	Chamber Counts Per ml at 19 Hours			Optical Density at 650 mu
		Hyphae	Yeasts	Total	
25	1.02	0	0	0	Debris
50	2.15	0	2,000	2,000	.017
75	3.37	0	1,000	1,000	.017
100	4.54	65,000	85,000	150,000	.325
120	5.48	85,000	30,000	115,000	.357

Serum #2430, bound iron 16 mcg%, U.I.B.C. 454 mcg%, % saturation 3.40%.

Final volume of serum in experimental vessels was 2.0 ml.

Inoculum: 171 viable units of *C. albicans* per ml (low-iron inoculum from 0% saturated "low"-iron egg white medium).

TABLE IV

Release of radioactive-iron to Candida albicans *in serum on saturation of the U.I.B.C. with iron*

% Saturation with Iron	Final Concentration of Iron per Flask* (10 ml volume)	Count (Chamber) of Units of *C. albicans* per ml		Mcg Fe 59 taken up by *C. albicans* Total Washed Cell Sediment†	Counts per Minute of Entire Cell Content
		18 hrs	44 hrs		
	mcg				
50	15.7	0	5,000	0.00038	10
90	28.3	40,000	445,000	.0053	99
120	37.68	1,115,000	72,500,000	1.167	47,906

* Total volume in flasks 10 ml of serum (C.L.) uniform small inoculum of iron-low cells of *C. albicans* (U.I.B.C. = 220 mcg% B. 1. mcg%).

† Entire growth in flask collected in sediment, washed five times and then radioactivity counted in scintillation counter.

seen. At 100% and 120% iron-saturation of the siderophilin, growth of *C. albicans* in serum flourished. It is of interest to note that excess of iron over that needed to saturate the iron-binding demands of the siderophilin did not result in increased growth of the fungus.

Study of C. albicans in Serum with Radioactive Iron to Determine the Uptake of Iron by the Organism:

To determine the total amount of iron taken up by a culture of *C. albicans* grown in a serum medium at different percentage iron-saturations and to check on the conclusions drawn from the previous results (Table III) we performed the following experiment. A normal serum, with 30% saturation of the iron-binding capacity, was utilized and through additions of a mixture of Fe^{56} and Fe^{59} was brought to 50%, 90%, and 120% iron-saturation. 10 ml volumes of serum were used in 125 ml volume Warburg vessels in order to provide enough growth for the anticipated radioactivity measurements. The inoculum was uniformly 171 viable units/ml of "low"-iron *C. albicans* diluted 1:1000 in serum C.L. The results obtained are summarized in Table IV. The growth results are in accord with the previously reported experiments. Luxuriant growth of *C. albicans* occurred following saturation of the U.I.B.C., with minimal growth at a level just below the level of saturation, while no growth occurred with iron-low inoculum at the 50% saturation level. The difference in uptake of radioactive iron by the accumulated culture of *C. albicans* in the iron-saturated medium, 1,167 mcg, is in striking contrast to the negligible amount, 0.0053 mcg, taken up at the 90% iron-saturation level. The severely limited growth of the fungus at iron levels below saturation of the U.I.B.C. is reflected by the absolute difference in iron uptake.

Discussion

It has been shown that inhibition of *Candida albicans* in serum results from the removal of available iron through competitive physiological chelation by siderophilin. The cells of *Candida albicans* to obtain iron sufficient for their growth and normal metabolism, must compete with the iron-free molecules of this naturally occurring iron-chelator in serum. Obviously they fail in this competition. The inhibitory action of serum is therefore non-specific and may be applied to those microorganisms which are predominantly aerobic and have a high nutritional requirement for iron.

The antimicrobial action of conalbumin through iron-chelation has been known for bacteria and yeast since 1944 (Schade and Caroline (12)) and was confirmed by Feeney for bacteria (15) and by Silva and Buckley (16) in an investigation of over 50 pathogenic fungi. A similar growth inhibitory action of serum was reported by Schade and Caroline in 1946 (8). The present study has demonstrated that, upon saturation of the siderophilin of the undiluted serum with iron employed as a growth medium for *C. albicans*, prompt and vigorous growth of the otherwise inhibited fungus occurs.

Roth (6) reported a lowered inhibitory capacity of cord blood against *C. albicans* as compared with the corresponding maternal serum. Sturgeon

(17, 18) has shown the almost complete saturation with iron of the iron-binding capacity of the cord blood and the serum of the newborn during the first two weeks of life. Our results, showing the inhibitory effect of the unsaturated portion of the siderophilin, serve to explain Roth's *in vitro* findings of lowered inhibitory capacity since sera with a high degree of iron saturation would require less additional iron to allow growth. Whether the low resistance of the newborn to *Candida albicans* is related to the high degree of iron saturation of neonatal serum remains to be investigated.

Summary

1. It has been shown that saturation of the iron-binding constituent, siderophilin, of the serum results in growth of the inhibited *C. albicans* in serum neopeptone broth.

2. Additions of iron, sufficient to saturate the unbound iron-binding capacity of the conalbumin in a trypticase-egg white medium are required to allow growth of *C. albicans* which is otherwise severely restricted in such a medium without iron.

3. Undiluted human serum with a demonstrable unbound iron-binding capacity does not support growth of *Candida albicans*. When this capacity for binding additional iron is exceeded by added iron vigorous growth ensues.

4. The absolute amounts of iron taken up by cultures of *Candida albicans* grown in undiluted serum are governed by the percentage iron saturation of the contained siderophilin.

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