The inhibitory effect of serum on the growth of *Torulopsis glabrata*

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Summary. Normal human plasma and serum were found to inhibit the growth of *Torulopsis glabrata* and, to a lesser extent, other yeasts. The factor responsible for the inhibition of *T. glabrata* was not dialysable, was heat stable at 56° C for up to 4 h and could be partly removed by absorption with viable *T. glabrata* but not *Candida albicans*. It was fungistatic at low concentrations and fungicidal at high concentrations, stable up to 4 years between –20° C and –70° C, but for only a few weeks at 4° C. Studies with Cohn fractions of serum showed that the inhibitory components were in either the alpha or beta globulin fraction or both. The combined effects of transferrin and IgM accounted for about 70% of the total inhibition observed. We were unable to identify the component responsible for the residual inhibition of growth. The inhibitory effect was totally neutralised by tetracyclines, quinolones, sulphamethoxazole and by very low concentrations of polyenes, imidazoles and 5-fluorocytosine.

Introduction

Transferrin was first identified as an iron binding serum protein (Schade and Caroline, 1946) and it inhibits many species of fungi, including yeasts, by iron-chelation that can be demonstrated *in vitro* and is reversible by the addition of high concentrations of iron to serum-containing media (Caroline *et al.*, 1964; Summers and Hasenclever, 1964; Esterly *et al.*, 1967; King *et al.*, 1975; Otto and Howard, 1976). Moreover, the elevated serum iron concentrations that follow multiple blood transfusions can also contribute to the loss of natural immunity to infection with these organisms by neutralising the inhibitory effect of transferrin (Caroline *et al.*, 1969).

Inhibition of *T. glabrata* by normal serum has been reported previously (Summers and Hasenclever, 1964; Howard and Otto, 1967; Otto and Howard, 1976). We have also observed that *T. glabrata* was inhibited in media containing 10% human serum (Petrou and Rogers, 1985); and we found that growth was restored in the presence of some antibiotics and subinhibitory concentrations of antifungal drugs. These findings led us to investigate whether or not transferrin is the sole serum factor inhibitory to clinically important yeasts, with particular reference to *T. glabrata* which is especially susceptible to the effects of serum (Otto and Howard, 1976). We also attempted

to characterise the components of the inhibitory factor and the possible mechanism by which drugs neutralise its effects.

Materials and methods

Organisms

Thirty two yeasts from patients in receipt of or awaiting bone marrow transplantation were employed; they were stored in 10% glycerol at –70° C as the Westminster Children Collection (WCC). Three strains from the National Collection of Pathogenic Fungi, Colindale (NCPF)—*T. glabrata* no. 3240, *Saccharomyces cerevisiae* no. 3178 and *C. tropicalis* no. 3111—were also used. The yeasts were identified with the API 20C auxanogram system (API Laboratory Products Ltd, Farnborough, Hants, GU14 7QH) and grouped according to their susceptibility to human serum (table I).

Plasma and serum

Plasma was from healthy blood transfusion donors, and serum was from donors on their first visit to an antenatal clinic as well as from healthy volunteers. The antenatal sera gave results identical to sera from healthy volunteers and both groups will be referred to as normal sera. Other normal sera that had been stored at –20° C or –70° C for up to 4 years, and plasma or serum that had been kept at 4° C for up to 6 months, were also used. The inhibitory activity of sera with different immunoglobulin concentrations was studied with three cord sera (substantially deficient in IgM), 10 from patients with

Table I. Distribution of yeasts according to their susceptibility to human serum incorporated into YNB agar medium

Group	Percentage of serum needed for inhibition of growth*	Organisms	Number of strains tested
1	2.5	<i>T. glabrata</i>	12
2	20.0	<i>S. cerevisiae</i>	3
		<i>Cr. neoformans</i>	1
3	30.0	<i>C. pseudotropicalis</i>	2
		<i>C. parapsilosis</i>	2
		<i>Rhodotorula rubra</i>	2
		<i>Cr. laurentii</i>	1
4	50.0	<i>T. maris</i>	1
		<i>C. krusei</i>	2
		<i>T. candida</i>	1
5	No inhibition	<i>C. albicans</i>	4
		<i>C. tropicalis</i>	2
		<i>C. guilliermondii</i>	2

* Inhibition so that no colonies were visible to the unaided eye after incubation for 48 h at 37° C.

IgG myeloma paraprotein and normal IgA and IgM, 10 with IgM myeloma paraprotein and normal IgG and IgA, one substantially deficient of IgG, IgA and IgM (all < 0.01 g/L) but with a normal transferrin concentration, and one serum substantially deficient of IgA (< 0.01 g/L) but normal IgG, IgM and transferrin levels. Normal range refers to levels of immunoglobulin within the range acceptable in our immunology laboratory, according to the age of the donor, and normal transferrin concentrations were between 2 and 4 g/L. As no differences were found between plasma and serum, only the results with serum are given.

Serum proteins

Human transferrin, pure substance (Sigma Chemical Co., St Louis, MO, USA); human gammaglobulin Cohn fraction II (Sigma); human α and β globulin Cohn fraction IV 1 (Sigma); and human serum albumin (Flow Laboratories, Uxbridge), were dissolved in phosphate buffered saline (PBS; NaCl 0.8% w/v, K_2HPO_4 0.12% w/v, KH_2PO_4 0.03% w/v, pH 7.3 $\pm$ 0.1) to a concentration equivalent to that in normal serum. The final concentrations of serum proteins in stock solutions were determined as described by Whicher and Perry (1984) with nephelometry (Beckman ICS System, Beckman, High Wycombe).

Drugs

Those used were either pure substance or intravenous preparations and were dissolved according to the manufacturers' instructions. They included antibacterial, antifungal and other drugs (table II). Each drug was tested at concentrations of 0.1, 0.5, 1, 5, 10, 25, 50 and

Table II. The drugs tested and their effect on the inhibitory factor in 10% serum

Drugs which neutralise IF	Minimum concentration needed, mg/L	Drugs with no effect (at 100 mg/L)
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Quinolones:		Aminoglycosides:
Ciprofloxacin	10	Amikacin
Enoxacin	10	Gentamicin
Nalidixic acid	5	Netilmicin
Norfloxacin	10	Sissomicin
		Streptomycin
		Tobramycin
Tetracyclines:		Cephalosporins:
Doxycycline	10	Cephalexin
Minocycline	5	Cephadrine
Tetracycline-HCl	5	Cefuroxime
		Cefsulodin
		Cefotaxime
		Latamoxef
		Ceftazidime
Polyenes:		Penicillins:
Amphotericin B	0.01	Benzylpenicillin
Meparticin	0.01	Ampicillin
Nystatin	0.01	Carbenicillin
Particin	0.01	Temocillin
		Ticarcillin
		Mezlocillin
		Azlocillin
		Piperacillin
Imidazoles:		Other drugs:
Bifonazole	0.06	Chloramphenicol
Clotrimazole	0.06	Colistin
Econazole	0.06	Erythromycin
Isoconazole	0.06	Fusidic acid
Ketoconazole	0.06	Rifampicin
Miconazole	0.06	Cyclosporin A
		Hydrocortisone
		Methotrexate
		Methylprednisolone
Other drugs:		
Sulphamethoxazole	50	
5-fluorocytosine	0.03	

100 mg/L with the exception that the antifungal drugs were used in subinhibitory concentrations.

Heat inactivation

Volumes of pooled serum were placed at 56° C and triplicate samples removed at various time intervals up to 4 h. The samples were rapidly brought to room temperature before use. Serum was heated to 56° C for 30 min to inactivate complement.

Dialysis

Samples of serum (6–10 ml) were placed individually in pre-treated dialysis tubing which had a molecular

weight exclusion limit of approximately 10 000 and were dialysed against 4 L of PBS for 36 h at 4° C, the PBS being changed twice. After dialysis the sera were heat-inactivated and tested for inhibitory activity.

Absorption of the inhibitory factor

Serum (inactivated and non-inactivated) was incubated at 37° C and 4° C for 1, 4 and 24 h with an inoculum of 10⁶ cfu/ml of *T. glabrata* or *C. albicans*. After incubation the yeast cells were removed either by centrifugation at 2500 g for 20 min or by membrane filtration (Flow Filtration System).

Selective depletion of transferrin and IgM from serum

Transferrin and IgM were individually absorbed from serum with anti-human transferrin and anti-IgM antibodies attached to sepharose particles. The affinity matrix was prepared from cyanogen bromide-activated Sepharose 4B and rabbit antisera, by the method of King *et al.*, (1975). All the separations were performed in 0.1 M Tris buffer, pH 7.4 or pH 8.0, containing 0.5 M NaCl.

Growth medium

Yeast Nitrogen Base (YNB) (Wickerham, 1951) with added glucose and L-asparagine, was used both in its commercial form (Difco Laboratories, Detroit, MI) as well as being prepared from individual components to exclude essential elements such as iron (Fe) magnesium (Mg), copper (Cu) or manganese (Mn). The medium was prepared as a 10-fold concentrate and the pH adjusted to 4.8, 6.0, 7.2 or 8.0 before filter sterilisation. Media solidified with 2% agar included YNB, Mueller-Hinton Agar (MHA; Oxoid), Brain Heart Infusion Agar (BHIA; Oxoid) and Sabouraud's Dextrose Agar (SDA; Oxoid).

Liquid cultures

These were grown at 37° C in an orbital shaker incubator (New Brunswick Scientific, Edison, NJ, USA) at 230 rpm, to a final concentration of *T. glabrata* WCC strain no. 083 10⁵ cfu/ml. The cultures included: (a) YNB at the four pH values; (b) YNB, pH 7.2, without Mg, Fe, Cu or Mn; (c) YNB, pH 7.2, with serum (non-inactivated, and with the inhibitory factor absorbed) 10% or 20%; (d) YNB, pH 7.2, with added serum proteins; and (e) cultures in 100% serum. Decimal-dilution and plate-count techniques were used to estimate the viable counts at 24 and 48 h with Triton-X-phosphate buffer (pH 7.2, BDH Chemicals Ltd, Poole) as diluent and SDA plates.

Experiments on solid media

YNB with and without any of the four metals, was added to sterile water agar at 56° C. Serum or serum proteins were added as necessary to ensure a range of

concentrations. MHA, BHIA and SDA were also used with and without 10% or 20% serum. After pouring triplicate samples, the plates were dried and inoculated with a Denley multipoint inoculator (A 400, Denley Instruments Ltd, Sussex) which delivered 1 µl of an inoculum of 10⁵ cfu/ml. The results were recorded after incubation for 2 days at 37° C and scored as + for full growth, ± partial growth and – for no growth.

Radial diffusion assay of the inhibitory factor

Melted YNB agar (60 ml), cooled to 45° C, was seeded with an overnight culture of *T. glabrata* WCC strain no. 083, to yield a final cell concentration of 2 × 10⁵ cfu/ml, and poured into a 100 mm square petri dish. Wells of 8 mm diameter for a 0.2-ml capacity and 5 mm diameter for one of 0.1 ml were cut. Serum, serum dilutions, or serum proteins were added to each well. After overnight incubation at 37° C the zone diameters were measured. With the exception of cord sera, all samples were tested in triplicate.

The effect of ferric iron on the medium and the inhibitory factor

Ferric iron 1 M, in the form of either ferric chloride (FC), ferric ammonium citrate (FAC) or ferric nitrate (FN) was added to iron-free Wickerham's YNB, pH 7.2, to achieve iron concentrations of 1.23 (as in YNB, Difco), 10, 25, 50, 100, 250, 500 and 1000 µM. During the addition of iron, the pH was tested with an EIL pH meter model 7010 (Electronic Instruments Ltd, Chertsey, Surrey), and the medium was used at the final pH. For FC-containing liquid medium, the pH was also adjusted to pH 7.2 by adding measured amounts of 1 M NaOH. For solid media, a fixed volume of NaOH was added to bring the pH of the FC-containing medium to 7.2. The final pH of the plates was then checked with a glass flathead electrode in conjunction with a calomel reference electrode. Serum or transferrin was added to liquid and solid media to give a concentration of 10% when required.

Serum and transferrin titration

Either serum or transferrin (100% serum-equivalent) was added to sterile YNB agar, pH 7.2, at 56° C to obtain concentrations of 0, 0.1, 0.5, 1, 2.5, 5, 10, 20, 30, 40 and 50% (final concentration of agar was 2%). After drying, the plates were inoculated, incubated as before and the results recorded after 1 and 2 days.

Indirect tests of the effect of the inhibitory factor on the medium components

The dialysis tubing, which was boiled twice for 10 min in sterile deionised distilled water, was cut open and placed on agar medium to cover either all or half of a dry 100 mm square plate. The plates included YNB, pH 7.2, or YNB, pH 7.2, with either serum or serum proteins.

The plates with and without the membrane were either left uncovered or overlaid with a very thin film of 1 or 2% water agar. The two areas of the half-covered plates were inoculated with the 12 strains of *T. glabrata* and the effect on growth on the two sides was compared. The plates which were completely covered by the membrane were inoculated with all the yeasts and growth compared with that on plates without the membrane after incubation for 2 days at 37° C.

The effect of drugs on the inhibitory factor

Agar mixtures containing YNB, pH 7.2, or YNB, pH 7.2, with 10% serum were distributed in 19.5-ml portions in sterile 25-ml glass bottles. Each bottle received 0.5 ml of an appropriate drug dilution to yield the concentration required, whereas the controls received 0.5 ml of sterile drug solvent at the highest concentration used with the drug. After drying, the plates were inoculated, incubated and the results recorded as before.

Results

Growth media and medium pH

The four media tested supported the growth of all the yeast strains (tables III and IV). The more acidic the medium the faster the culture entered the stationary phase, and the viable counts were slightly higher than in media with basic pH (table III). Wickerham (1951) medium was employed because its defined chemical composition facilitated use with omission of essential elements. Mg was found to be the only metal needed for growth by many strains at concentrations higher than those found in impurities; Fe, Cu and Mn were needed at concentrations only slightly higher than in impurities for full growth (tables V and IV).

Table III. Effect of pH on the growth of *T. glabrata* in the presence or absence of serum

YNB medium		Viable count, cfu (SD)/ml at	
pH	inactivated serum concentration (%)	24 h	48 h
4.8	0	2.3 (1.9) × 10 ⁹	5.0 (1.4) × 10 ¹⁰
	10	2.9 (1.1) × 10 ⁸	6.4 (1.6) × 10 ⁹
6.0	0	1.9 (0.6) × 10 ⁹	3.8 (1.2) × 10 ¹⁰
	10	8.4 (0.9) × 10 ⁶	2.7 (0.5) × 10 ⁸
7.2	0	1.7 (0.4) × 10 ⁹	1.3 (1.3) × 10 ¹⁰
	10	2.5 (1.3) × 10 ⁶	2.7 (1.9) × 10 ⁶
8.0	0	4.0 (1.2) × 10 ⁸	4.2 (0.8) × 10 ⁹
	10	2.1 (1.4) × 10 ⁶	6.4 (1.8) × 10 ⁶

The results are expressed as mean (± SD).

Effect of normal and patients' sera

As shown in table I, *T. glabrata* was inhibited by 2.5% normal serum, and the yeasts of groups 2, 3 and 4 by concentrations between 20 and 50%, whereas group-5 yeasts were not inhibited. Transferrin alone only inhibited *T. glabrata* at a concentration of 40% serum-equivalent or greater and the yeasts of groups 2 and 3 at concentrations higher than 50% with no inhibition of the group 4 and 5 organisms. In liquid cultures of *T. glabrata*, some growth was observed in the presence of 10 or 20% serum, but after 48 h it remained 10³–10⁴ cfu/ml below the serum-free control cultures; transferrin, however, showed an initial inhibitory action but after 48 h the viable count was only 10-fold below the controls (tables III and VII). There was inhibition of growth in inactivated serum, whereas some fungicidal action was observed with the non-inactivated samples; however, after incubation for 1 week at 37° C, both sets of sera exhibited killing of *T. glabrata* with final counts reduced to 10⁴ cfu/ml below the inoculum. By contrast, transferrin was not fungicidal at 100% serum-equivalent concentration. Sera stored for up to 4 years at –20° or –70° C retained their inhibitory properties, whereas those stored at 4° C exhibited a gradual loss of inhibitory activity after storage for a few weeks. Sera from patients with IgG paraprotein and from the patient without detectable IgA gave results similar to those of normal controls when tested in the radial diffusion assay (table VI). The patient's serum without any detectable globulin but normal transferrin and the sera with IgM paraproteins showed inhibition equivalent to 40–50% of that found with normal serum whereas the three cord sera showed a very low inhibitory activity, equivalent to about 10% of that of normal serum (table VI). Cohn fraction II showed no inhibitory activity when tested at 20% serum-equivalent (table VII). When the concentration of Cohn fraction IV 1 was adjusted to give an IgM concentration equivalent to 10% serum (IgG 10%, IgA 60% and transferrin 5% serum-equivalent), partial inhibition was observed on solid media while in the radial diffusion assay (IgM 100% serum-equivalent) the inhibition was about 80% of that of the control serum.

Heat inactivation, dialysis and absorption

The serum inhibitory factor was completely stable at 56° C for up to 2 h, although partial inactivation was obtained beyond 2 h and this remained unchanged up to 4 h. Non-inactivated

Table IV. The effect of medium, pH and metal depletion on yeast growth in the presence or absence of inactivated serum

Medium	pH	Growth (+), partial growth (±) or no growth (−) of yeasts in the given percentage of serum											
		Group 1			Group 2			Group 3			Group 4		
		0	10	20	0	10	20	0	10	20	0	10	20
YNB	7.2	+	−	−	+	+	−	+	+	+	+	+	+
YNB (− Fe)	7.2	+	−	−	±	±	−	+	+	+	+	+	+
YNB (− Mg)	7.2	±	−	−	±	±	−	+	+	+	+	+	+
YNB (− Cu)	7.2	+	−	−	+	+	−	+	+	+	+	+	+
YNB (− Mn)	7.2	+	−	−	+	+	−	+	+	+	+	+	+
YNB	4.8	+	+	+	+	+	+	+	+	+	+	+	+
Sabouraud's	7.2	+	+	±	+	+	+	+	+	+	+	+	+
Sabouraud's	5.6	+	+	+	+	+	+	+	+	+	+	+	+
Muller-Hinton	7.3	+	−	−	+	+	±	+	+	+	+	+	+
Brain Heart Infusion	7.3	+	−	−	+	+	±	+	+	+	+	+	+
Water Agar	7.2	−	−	−	−	−	−	−	+	±	±	+	+

Table V. Effect of metal depletion on the growth of *T. glabrata* in YNB medium

Metal depleted	Medium pH	Viable count, cfu (SD)/ml at	
		24 h	48 h
Iron	7.2	$2.0 (0.6) \times 10^8$	$1.2 (0.3) \times 10^9$
	4.8	$4.5 (1.5) \times 10^8$	$2.3 (1.2) \times 10^{10}$
Magnesium	7.2	$2.7 (1.9) \times 10^5$	$2.0 (1.1) \times 10^4$
	4.8	$1.6 (1.2) \times 10^6$	$4.0 (1.3) \times 10^5$
Manganese	7.2	$6.1 (1.6) \times 10^7$	$6.0 (1.4) \times 10^8$
	4.8	$8.9 (1.1) \times 10^7$	$7.1 (1.5) \times 10^8$
Copper	7.2	$1.0 (0.8) \times 10^8$	$8.2 (1.2) \times 10^8$
	4.8	$2.5 (1.5) \times 10^8$	$9.3 (1.0) \times 10^8$

Results with controls are shown in table III.

serum was more inhibitory than inactivated serum (tables VII and VIII). The inhibitory factor was also non-dialysable (table VIII), showing that it has a mol. wt > 10 000. Liquid cultures containing sera absorbed at either 4° or 37° C gave viable counts 10-fold below the control but approximately 100-fold above the cultures incorporating normal serum (table VII). On solid media very little difference was found between the effects of absorbed and normal sera (table VIII). The inhibitory factor was not absorbed by *C. albicans* when incubated in the same way as for *T. glabrata*.

Sera depleted of transferrin and IgM

The dilution factors that resulted from depleting the serum of transferrin and IgM were 17 and 14

Table VI. Inhibition of *T. glabrata* WCC083 by serum

Serum	Number tested	Mean zone diameter, mm (SD)	Percentage inhibition†
Normal: Neat	20	24.4(0.3)	100
1 in 1.25	10	23.5(0.2)	80
1 in 2	10	22.5(0.1)	50
1 in 5	10	21.0(0.3)	20
1 in 10	10	19.5(0.1)	10
1 in 15	10	19.1(0.2)	7
1 in 20	10	18.2(0.3)	5
IgG paraprotein	10	23.8(0.2)	84
IgM paraprotein	10	22.3(0.3)	40
IgG, IgM, IgA deficient	1	22.9(0.2)	55
IgA deficient	1	24.2(0.2)	94
Cord	3	19.3(0.4)	8
IgM depleted (1:14)*	2	18.2(0.1)	5(70‡)
Transferrin depleted (1:17)*	2	17.5(0.1)	3.5(60‡)

*Final dilution of serum after depletion. ‡ Undiluted serum-equivalent.

† To determine the percentage inhibition obtained with abnormal sera the log % serum inhibition of normal serum and serum dilutions in PBS was plotted against the mean zone diameter of inhibition. Neat serum was taken as 100% inhibition. The graph was analysed statistically using regression line analysis ($r=0.996$).

respectively and were estimated from the final concentrations of other proteins. Final concentrations of <0.001 and <0.01 g/L for transferrin and IgM, respectively, were obtained. When inhibition by the depleted sera was compared with that caused by the control diluted serum, the contribution of

Table VII. Effect of serum and serum proteins on *T. glabrata* in liquid culture

YNB medium, pH 7.2, with addition of	Viable count, cfu (SD)/ml at	
	24 h	48 h
10% serum (In)	$2.5(1.3) \times 10^6$	$2.7(1.9) \times 10^6$
20% serum (In)	$2.1(0.5) \times 10^6$	$6.2(0.6) \times 10^5$
100% serum (In)	$1.2(1.0) \times 10^6$	$4.2(0.9) \times 10^5$
10% serum (NIn)	$1.4(0.7) \times 10^6$	$6.9(1.8) \times 10^5$
100% serum (NIn)	$1.1(0.5) \times 10^5$	$7.7(1.7) \times 10^4$
10% serum (In, A)	$4.8(1.8) \times 10^8$	$2.2(0.9) \times 10^8$
10% serum (NIn, A)	$3.3(1.7) \times 10^8$	$1.7(0.5) \times 10^8$
*10% transferrin	$8.3(1.3) \times 10^6$	$5.6(1.8) \times 10^8$
*20% transferrin	$3.6(1.1) \times 10^6$	$2.0(0.8) \times 10^8$
*20% Cohn fraction II	$1.5(0.7) \times 10^9$	$1.1(0.7) \times 10^{10}$
*20% Cohn fraction IV 1	$4.5(1.5) \times 10^8$	$1.5(1.1) \times 10^9$

In = Inactivated; NIn = Non-inactivated; A = Absorbed.

* Serum-equivalent percentage concentrations.

transferrin towards inhibition was estimated to be approximately 40%, that of IgM about 30%, and the remaining 30% inhibition was attributed to an unidentified component(s) (table VI).

Effect of ferric iron on the inhibitory factor

Fig. 1 shows the effect of ferric iron on the pH of the YNB medium. After incubation for 48 h at 37° C (data not shown), the pH value of the cultures with 10% serum and no added iron was reduced from 7.2 to 6.5, whereas for those cultures supplemented with iron concentrations above 10 µM, the pH value was further reduced to 4.8 and to as low as 2.5 at higher concentrations of iron. For serum-free cultures not supplemented with iron, the lowest

Fig. 1 Effect of ferric chloride on the pH of the medium. (a) YNB, Oh, 25° C, (b) YNB + 10% serum, Oh, 25° C, (c) YNB, 24 h, 37° C, (d) YNB + 10% serum, 24 h, 37° C.

pH value recorded after 48 h was 3.5. When iron was added to the serum-containing medium, but the medium was not readjusted to the starting pH, the growth observed was never equivalent to that of the serum-free controls. When the pH was readjusted to 7.2 the viable count was very close to that in normal serum (fig. 2). In the absence of the

Table VIII. Effect of serum in YNB agar on the growth of yeasts at 37° C after incubation for 48 h

YNB pH			Serum treatment	Number of sera	Number of sera showing growth (+), partial growth (±) and no growth (–)											
					Group 1			Group 2			Group 3			Group 4		
					+	±	–	+	±	–	+	±	–	+	±	–
7.2	Inactivated	400	0	12	388	200	80	120	328	40	32	396	4	0		
7.2	Non-inactivated	100	0	0	100	12	18	70	25	30	45	75	22	3		
7.2	Dialysed (In)	10	0	0	10	5	2	3	10	0	0	10	0	0		
7.2	Absorbed (4° C, In)	20	0	0	20	12	4	4	17	2	1	19	1	0		
7.2	Absorbed (37° C, In)	20	0	2	18	11	4	5	17	3	0	20	0	0		
7.2	Absorbed (4° C, NIn)	20	0	0	20	2	4	14	6	6	8	19	1	0		
7.2	Absorbed (37° C, NIn)	20	0	1	19	12	4	4	17	2	1	19	1	0		
4.8	Inactivated	40	25	13	2	34	2	4	38	2	0	40	0	0		
4.8	Non-inactivated	40	23	13	4	32	5	3	38	2	0	40	0	0		

inoculum, FN acted as FC, whereas FAC reduced the pH value to a lesser extent than either of the other compounds. In the inoculated media the effects of the three ferric compounds at concentrations $> 250 \mu\text{M}$ were similar; they restored growth of *T. glabrata* in the presence of serum.

Indirect testing of the properties of the inhibitory factor

When the medium incorporating serum was placed under the dialysis membrane and the organism inoculated either directly on to the membrane or on to a film of water agar on top of the membrane, none of the yeasts tested was fully inhibited and the reduction in the growth of *T. glabrata* was negligible. Full growth was also found when the individual serum proteins were added to medium under the membrane. These findings suggest that no essential growth element was completely bound by proteins under the membrane.

Effect of drugs on the inhibitory factor

Table II shows the drugs that had no effect on the inhibitory factor and those that reversed inhibition. The concentrations shown are those needed to restore full growth to a level comparable with that found in media without added serum. In the radial diffusion assay the drugs used at the concentrations shown in table II reduced the activity of the inhibitory factor by $> 80\%$.

Discussion

Our studies indicate that fresh normal human serum inhibits the growth of *T. glabrata* and also, but to a lesser extent, that of *Cryptococcus neoformans*, *S. cerevisiae* and other yeasts through a more complicated mechanism than has been reported previously. Transferrin appears to account for approximately 40% of the total inhibitory effect and other metal-chelating proteins were not implicated; other serum components that were non-dialysable, heat stable and which could be partly absorbed by live cells of *T. glabrata*, were responsible for the residual inhibition of growth. These components were found to be sensitive to pH changes, being partially inactivated between pH 7.6 and 8.0, or 6.9 and 6.0, with complete inactivation at and below pH 5.0. Fig. 1 demonstrates the marked pH-value reduction that follows the addition of ferric iron to YNB media; ferric iron hydrolyses to form complexes, the hydrolysis being equivalent to acid dissociation of H_2O with the

Fig. 2. Effect of ferric chloride on the viable count of *T. glabrata* in YNB, pH 7.2, + 10% serum after incubation for 48 h at 37° C; (○) pH left unchanged. (●) pH readjusted to 7.2. S-F = serum-free culture, In = inoculum.

production of hydrogen ions (Cotton and Wilkinson, 1972). Furthermore, fig. 2 shows that if the effect of iron on pH is not taken into account, then transferrin could be considered to be the only inhibitor in human serum. However, by readjustment of the medium pH to 7.2, an inhibitory effect was still observed. Inhibition could be demonstrated in sera stored at -20°C or -70°C for up to 4 years but there was gradual reduction in inhibition when sera were stored at 4°C for more than a few weeks.

By testing the various serum fractions and patients' sera, it was found that none of the components of the inhibitory factor was present in the purified albumin or γ -globulin fractions of serum. The inhibitory factor consisted of a combination of transferrin, IgM and probably some other protein present in Cohn fraction IV 1. These findings were confirmed by testing sera depleted of transferrin or IgM. The poor inhibition obtained with the cord sera was further support for the role of transferrin and IgM since the transferrin in cord blood and newborn blood is nearly fully saturated with iron (Caroline *et al.*, 1969) and the IgM level is very low or undetectable (Hobbs, 1969).

Various factors predispose to fungal overgrowth, as reviewed by Seelig (1966a, b; 1968) and Warnock (1980), and our findings suggest that some antimicrobial agents such as tetracyclines, quinolones, sulphamethoxazole and subinhibitory concentrations of antifungal agents may also contribute by protecting *T. glabrata* from inhibitory serum components. This may be either by direct attachment on to the inhibitory factor, reaction with the inhibitory factor's substrate, such as iron, or attachment to the organism's receptors for the inhibitory factor. The binding of drugs to serum proteins is believed to be due mainly to an interaction between the drug and serum albumin. However, other serum proteins, including enzymes, have been shown to interact with some drugs, e.g., tetracycline (Kornguth and Kunin, 1976). Transferrin and IgM have not previously been shown to interact with any of the drugs which we found to neutralise the inhibitory factor. Tetracycline has

been shown to chelate iron (Greenberger, 1973), and this effect might account for the neutralisation of transferrin activity if the organism is able to remove iron from tetracycline. The reversal of inhibition by IgM may be either by a direct binding to the drug or by the action of the drug on the surface of the organism, which will disturb the configuration of the membrane, or cell wall, in such a manner that the binding of IgM on to the organism is inhibited. The third component of the inhibitory factor, which we could not characterise, was responsible for 30% of the inhibition, and as it exhibited similar properties to IgM, we conclude that it may be another serum protein.

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