CANDIDA ALBICANS AND THE FUNGICIDAL

ACTIVITY OF THE BLOOD

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ABSTRACT

The growth of C. albicans by blastospore production in Wickerham Fluid and Sabouraud Broth, and as hyphae in human serum was investigated. The growth of T. glabrata by blastospore production occurred more slowly in serum than in broth. It was considered that the conversion of C. albicans from exponential multiplication by blastospore production to hyphal growth was due to an inhibitory action of the serum or a reduced availability of nutrients.

A technique for producing synchronous cultures of C. albicans by sedimentation through a sucrose density gradient was developed. Synchronous growth of blastospores was studied and it was found that inoculation of synchronous blastospores into human serum gave synchronous initiation of hyphal production and a uniform linear growth of the hyphae.

The survival of C. albicans strains Hasenclever 207/53A and 792/53B and WF 668A was studied in 1 ml. lots of blood. With inocula of small numbers of blastospores about 80% were killed, but the percentage survival increased as the numbers of blastospores in the inoculum were increased. It was found that killing was very much reduced when the hyphal length of the propagules exceeded 200 μ .

Microscopic observation showed 100% of a synchronous

blastospore suspension inoculated into serum produced hyphae. It was concluded therefore that serum was not fungicidal for C. albicans.

After 2 days incubation, the dry weight of blastospores from Sabouraud Broth was more than 3 times the dry weight of mycelium grown in plasma under identical conditions. The blastospores contained a higher percentage of protein, carbohydrate, and nucleic acids but significantly less fat than the mycelium.

Human polymorphonuclear leucocytes (PMN) were observed to ingest up to 8 blastospores per cell with evidence of intracellular death. Hyphal outgrowth from the PMN was very rarely observed.

Phagocytic killing of C. albicans was enhanced by plasma, and it was shown that this was due to the chemotactic activity of complement.

The chemotactic migration of PMN through the pores of millipore membranes was studied by means of modified Boyden Chambers. In a standard interval of time, more PMN migrated towards a standard suspension of blastospores than to a similar suspension of mycelium. This migration ceased when the complement was inactivated. Cell-wall and mannan enriched preparations of C. albicans were highly stimulatory for PMN chemotaxis. E. coli caused the migration of more PMN than

equivalent weights of C. albicans.

Lysosomal cationic protein extracted from PMN was candidacidal in vitro.

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SECTION 2

GENERAL INTRODUCTION

Interest in the disease candidosis began with the classical description of thrush by Langenbeck (1839). Gruby (1842) described a fungus causing "le vrai Muguet des enfants" and Robin (1853) named this fungus Oidium albicans. The species was transferred to the genus Candida by Berkhout (1923).

Hurley and Winner (1966) reported that not only C. albicans, but also C. tropicalis, C. stellatoidea, C. pseudotropicalis and C. krusei are capable of causing candidosis.

C. albicans is the most important pathogenic species of the genus. It grows as spherical or oval blastospores 2.5 - 4.0 μ in diameter, which reproduce by budding, or as non-branching hyphae which give rise to blastospores by budding at the nodes. Both forms are gram-positive and non-capsulate. It is anascosporogenous and resting chlamydospores 7 - 17 μ in diameter are formed in some circumstances. A detailed description of the species is given in the Appendix, p.156. It has been suggested that members of the Candida genus may be Basidiomycetes (Van der Walt, 1970).

Hasenclever and Mitchell (1961) described two serotypes

of C. albicans. Stallybrass (1964) reported that 75% of the strains of C. albicans isolated in Southern England belonged to serotype A.

Chemically, the Candida cell wall is composed mainly of mannan and glucan with small amounts of chitin (Bartnicki-Garcia, 1968). Kemp and Solotorovsky (1964) reported that some Candida antigens were present both in the wall and the cytoplasmic components of the cell, but antigens 1 to 3 were solely wall components and antigen 5 was cytoplasmic. The role of mannan or wall antigen and cytoplasmic antigen is discussed further in relation to the chemotaxis of leucocytes in Section 8 of this thesis.

The intravenous injection of 2,000 cells of C. albicans per gram of body weight is fatal to the rabbit (Winner, 1956). 80,000 organisms per gram of body weight was fatal to the guinea-pig when given intravenously but only gave a non-fatal, self-limiting disease when given intradermally or intraperitoneally (Winner, 1960).

A mouse given an injection of less than 2×10^6 cells of C. albicans died in 6 - 60 days with lesions confined to the kidneys. Injection of more than 2×10^6 cells caused death in 1 - 10 days with lesions in the heart, brain and kidneys (Hurley and Winner, 1963).

Young (1958) reported that hyphae were formed 1 hour after

injection of 4×10^7 C. albicans blastospores into the mouse peritoneum. Polymorphonuclear leucocytes (PMN) appeared in peritoneal smears after 3 - 4 hours and the numbers of yeast cells decreased sharply. Blood cultures demonstrated that C. albicans did not persist beyond 2 days in the blood but kidney cultures were positive for up to 6 months, the mycelial phase being considered to be the infective form.

Louria, Brayton and Finkel (1963) inoculated mice intravenously with 6 strains of C. albicans and observed that the more virulent strains grew out of the leucocytes. Candida hyphae grew in the renal tubular lumen and the inflammatory response of the kidney tissue occurred 4 hours later than in other tissues.

Stanley and Hurley (1969) observed that the hyphae of C. albicans grew out of mouse macrophages in tissue culture. Albrecht (1969), on the other hand, reported that C. albicans was eradicated by PMN in experimental candidosis in the rabbit.

Observations on the killing of C. albicans by polymorphonuclear leucocytes are described in Section 6.

There is no correlation between the possession of detectable antibodies and immunity to infection by C. albicans. Low titres of agglutinins to C. albicans have been reported in 31.6% (Winner, 1955), 46.6% (Andersen, 1968) and 96% (Esterly, 1968) of the normal population. These agglutinins may be

due to past or present latent infection or to the occurrence of the organism in the alimentary tract.

C. albicans is carried as a commensal in the normal microbiota by about half the population (Barlow and Chattaway, 1969; Davies and Leese, Personal communication).

Bennett (1844) suggested that systemic candidosis was a complication of a debilitated state due to other diseases and today it is accepted that the organism becomes an opportunist pathogen when immunity is reduced by debility or immunosuppressive therapies.

Certain immunological defects predispose to Candida infection (Chilgren, Quie, Meuwissen and Hong, 1967; Buckley, Lucas, Hattler, Zmitjewski and Amos, 1968; Landau, 1968; Chilgren, Hong and Quie, 1968; Hermans, Ulrich and Markowitz, 1969; Chilgren, Meuwissen, Quie, Good and Hong, 1969; Oh, Rodey, Good, Chilgren and Quie, 1969; Canales, Middlemas, Louro and South, 1969).

Infections with C. albicans have been associated with therapy with steroids (Smolin and Okumoto, 1968; Nema, 1968; Williamson, 1968; Folb and Trounce, 1970), with broad-spectrum antibiotics (Seelig, 1966; Black, 1968; McKendrick, 1968; Shastri, 1969), with oral contraceptives (Porter and Lyle, 1966; Catterall, 1971), and with tranquillizers (Pollack, Buck and Kalnins, 1964; Kane and Anderson, 1964).

Candidosis is a frequent complication seen in patients debilitated by other diseases, including leukemia (Bodey, 1966; Viola, 1967; Preisler, Hasenclever, Levitan and Henderson, 1969; Caroline, Rosner and Kozinn, 1969; Bodey, De Jongh, Isassi and Freireich, 1969), Addison's disease (Alteras, Cojocar and Balanescu, 1969), Di George's syndrome (August, Rosen, Filler, Janeway, Markowski and Kay, 1968), pernicious anaemia (Blizzard and Gibbs, 1968), cancer (Sokolova, Karasik and Klechinkov, 1966), endocrine disorders (Louria, Shannon, Johnson, Caroline, Okas and Taschdjian, 1967), and allergies (Dobias, 1966).

Candida can cause post-operative complications (Conway, Kothari, Lockey and Yacoub, 1968; Hairston and Lee, 1969) and burn-wound infections (Gayford, 1969). A case of congenital candidosis was reported by Rhatigan (1968).

In the review of Winner (1969) it was suggested that the transition from commensalism to parasitism may depend on the site affected. C. albicans on the mucous membrane or skin may well be a commensal, but if it gets into the blood stream it may establish itself as a parasite. Different organs favour the growth of Candida to different extents.

A number of workers have demonstrated that fresh blood in vitro kills bacteria inoculated into it (Wright and Colebrook, 1921; Mackie and Finkelstein, 1931; Miles and Misra, 1938).

Since no attempt at measuring the candidacidal activity of whole blood appeared to have been undertaken it was decided that this should be investigated, see Section 4.

The bactericidal and bacteristatic effects of human plasma and serum are well known. A number of reports describe the antifungal activities of serum, and several of these (Roth, Boyd, Sagami and Blank, 1959; Roth and Goldstein, 1961; Louria and Brayton, 1964a; Esterly, Brammer and Crounse, 1967; Hendry and Bakerspigel, 1969) have claimed that serum kills or inhibits the growth of C. albicans. Other workers (Chilgren, Hong and Quie, 1968; Lehrer and Cline, 1969a) suggest that these results are due to the growth of hyphae and clumping of mycelium causing a drop in the viable counts.

In view of these reports, it was decided to investigate the possibility that normal human serum neither killed nor inhibited the development of C. albicans. This literature is further reviewed and experiments and observations on synchronous cultures of C. albicans in serum and plasma are described in Section 5.

An investigation into the chemical and physical factors involved in the tubulation of Candida in serum and its components was undertaken. The chemical composition of blastospores and mycelium was examined to compare the protein, carbohydrate, RNA, DNA and fat contents of cells of the two growth phases. These investigations are described in Section 5.

The phagocytosis of C. albicans by PMN was described in 1959 by Wang and Schwarz. However, Louria and Brayton (1964b) reported that following phagocytosis, the Candida cells within the phagosome developed hyphae and grew out of the PMN.

It has been suggested that the phenomenon of filamentation hinders phagocytosis (Hill and Gebhardt, 1956; Young, 1958).

Taschdjian, Kozinn, Fink, Cuesta, Caroline and Kantrowitz (1969) commenting on macrophage activation and the phagocytosis of Candida, reported that hyphae were never observed to form inside human macrophages. However, Stanley and Hurley (1969) observed the outgrowth of hyphae of C. albicans from mouse peritoneal macrophages in tissue culture.

Chilgren, Oh, Hong and Quie (1969) investigated the killing of C. albicans by PMN and found that 40 - 55% of the C. albicans inoculated were killed. Lehrer and Cline (1969a) reported that $29.0 \pm 7.4\%$ of ingested C. albicans were killed in 1 hour.

In the present studies, experiments were undertaken to investigate the role of the PMN in the killing of Candida (Sections 6 and 7), whether filamentation inhibited killing (Sections 4, 6 and 7) and the role of the plasma (Section 5).

The chemotaxis of PMN towards microbes is a recognition mechanism linked to their phagocytic activities. Chemotaxis of PMN towards bacteria has been extensively studied by a number of workers, including Keller and Sorkin (1968), who

reviewed the literature. PMN chemotaxis towards C. albicans was studied in vitro in this study using the Boyden Chamber (Boyden, 1962) and is described in Section 8.

Zeya and Spitznagel (1966b) have shown that polycationic proteins in the phagosome kill ingested microbes. Lysosomal cationic protein from normal PMN was prepared and tested for candidacidal activity and is described in Section 9.

In 1968, when the studies to be described began, there was controversy concerning the roles of serum and phagocytosis in the killing of C. albicans. The experiments performed were designed to elucidate this and to investigate PMN chemotaxis towards C. albicans antigens, to determine the effect of complement on this chemotaxis and to examine the effect of serum on growth and form of C. albicans.

SECTION 3

GROWTH AND FORM IN CANDIDA ALBICANS

Nowadays, in the identification of yeasts from clinical specimens, much use is made of the tubulation test (Taschdjian, Burchall and Kozinn, 1960). In this test, a loopful of cells is inoculated into serum and incubated at 37°. After 2-3 hours, characteristic germ-tubes emerge and, if incubation is prolonged, these develop into long lengths of hyphae.

Since C. albicans is a dimorphic fungus which in broth multiplies asexually by budding (blastospore production), but in serum or plasma produces a predominantly hyphal type of growth, it was decided to study the kinetics of growth in the two forms. Furthermore, if synchronously dividing cells could be produced when required it offered the possibility of using precise standard inocula of blastospores or hyphae of known length for studies on the destruction of Candida in human blood.

A microbe inoculated into culture medium has a characteristic growth comprising a log phase, exponential growth phase and maximum stationary phase (cited Stanier, Doudoroff and Adelberg, 1966). The studies described in this section give basic information on the random and synchronous

growth of C. albicans blastospores, and the rate of hyphal elongation.

In an inoculum removed from agar culture to broth, there are cells in varying stages of development, some are dormant, others are actively budding. The growth which then occurs is described as random. A synchronous culture is a population of cells in which the majority are at the same stage of the growth cycle at the same time.

The synchronous culture gives a magnified image of the functions of the single cells of which it is made up and thus a single cell can be studied at the population level and small changes discerned more easily.

Study of synchronous cultures has allowed understanding of the life-cycle in S. cerevisiae. Bud formation precedes nuclear division (Delamater, 1950). The DNA content then doubles and RNA synthesis increases. RNA synthesis ceases when the bud breaks off, only to be resumed after an appreciable delay (Williamson and Scopes, 1960).

A variety of techniques have been used to produce synchronous cultures of yeasts. Ogur, Minciker and McClary (1953) obtained a rough degree of synchrony by removing maximum stationary phase yeasts from their exhausted medium and transferring them to a fresh, rich medium, with the production of a resumed multiplication.

Beam, Mortimer, Wolfe and Tobias (1954) observed that starved cultures of yeast were roughly synchronous.

Campbell (1957) failed to obtain a synchronous culture of yeasts by either alternation of low and higher temperatures or favourable and unfavourable pH.

Spoerl and Looney (1959) obtained a synchronous population of Saccharomyces cerevisiae with X-irradiation as a means of inhibiting cell division.

In these reports of synchronous growth of Saccharomyces, the complication of mycelial function and the production of pseudohyphae does not occur, since Saccharomyces multiplies only by blastospore production.

In the only report on the synchronous culture of C. albicans, Dabrowa, Landau and Newcomer (1967) used a technique in which a starvation medium was alternated with a rich medium for growth.

Because C. albicans produces mycelium in sera, T. glabrata which never produces mycelium was also used as a test organism for comparative purposes in the following investigations.

METHODS

To measure random growth

A small inoculum of washed cells of Candida albicans strain 207/53A or Torulopsis glabrata from an overnight

culture in Glucose Nutrient Broth was introduced into 10 ml. of medium in a screw-capped glass universal bottle of 25 ml. capacity. The suspensions were then incubated at 37° on a gyrotory shaker.

At intervals over a 36 hour period of incubation, samples were withdrawn, decimal dilutions prepared and cell clumps disrupted by ultrasonic vibration in an MSE Ultrasonic Disintegrator for 30 seconds with an amplitude of 5 μ peak to peak. 0.1 ml. volumes of the dilutions were spread over Sabouraud dextrose agar plates and colony counts made after 2 days incubation at 37°.

The production of a synchronous culture by the linear sucrose density gradient

The apparatus used to produce the linear sucrose density gradient was modified from the design of Britten and Roberts (1960) and made in our own laboratories from stainless steel to withstand repeated autoclaving.

The apparatus (Fig. 1) consists of two vertical stainless steel cylinders of 2 cm. diameter, connected by rubber tubing of 0.3 cm. internal diameter fitted with a gate clamp. A similar rubber tube, terminating with a hypodermic needle descends from the right-hand cylinder into a glass test-tube. A small magnetic flea in the right-hand cylinder is operated

Fig. 1. The linear sucrose density gradient apparatus.

by a magnetic stirrer below.

2% and 12% w./v. solutions of commercial sucrose were sterilized by Seitz-filtration.

Method. The gate-clamp is closed and, using sterile technique, 8 ml. lots of 2% and 12% sucrose solution are introduced into the left-hand and right-hand cylinders respectively. The magnetic stirrer is then switched on and the gate-clamp opened.

The 12% sucrose solution begins to flow down the rubber tube, through the hypodermic needle and runs down the side of the glass tube. As this happens, 2% sucrose moves to the right-hand cylinder to take its place. The continuous mixing results in a lowering of the sucrose concentration until finally only about 2% sucrose leaves the needle. The glass tube then contains a linear sucrose density gradient with 12% sucrose at the base passing up to 2% sucrose at the top.

An overnight culture of C. albicans is centrifuged, resuspended in water, and 2 ml. of the suspension is gently placed on top of the gradient and carefully stirred with the tip of a Pasteur pipette to prevent the formation of "streamers".

The density gradient is then allowed to stand for an hour at room temperature. The larger cells, hyphae and aggregates settle to form a relatively opaque zone towards the bottom and this grades upwards to become translucent. When approximately

0.5 ml. is removed from this uppermost zone it contains only young newly formed blastospores. The 0.5 ml. is then diluted to a desired optical density and decimal dilutions prepared. In the experiments to be described optical densities were measured by means of a Hilger "Spekker" absorptiometer.

The estimation of synchronous growth

1. Quantitative observations in Wickerham Fluid

The method was as described on p. 22.

2. Visual observations

A synchronous culture of C. albicans strain 207/53A was prepared. The cells were inoculated into Wickerham Fluid and placed on a gyrotory shaker at 37° and into fresh human plasma and incubated without shaking at 37°.

Samples were withdrawn from the broth at intervals and the number of "budding" and "non-budding" cells counted. A cell at any stage of bud formation was considered "budding".

Samples were similarly withdrawn from the plasma and an eye-piece graticule used to measure hyphal lengths.

The preparation of spheroplasts

C. albicans spheroplasts were prepared by a modification of the technique of Schlenk and Dainko (1965).

An overnight culture of C. albicans was centrifuged, washed and resuspended in 1 M mannitol.

0.1 ml. Snail Digestive Juice from Helix pomatia (Koch-Light Laboratories Ltd.) was added to 0.2 ml. 1 M mannitol and centrifuged at 1,000 g. for 30 minutes. 0.2 ml. of the resulting supernatant PLUS 0.8 ml. of washed C. albicans in 1 M mannitol was placed on a gyrotory shaker at 37° for 5 hours. The cells were then centrifuged down at 250 g. for 15 minutes.

RESULTS

The results of experiments on the growth of C. albicans and T. glabrata in random and synchronous culture are shown in Tables 1, 2, 3, 4 and 5 and Figures 2, 3, 4 and 5.

Examination of spheroplasts formed

Phase contrast examination of the cells under high power (x630) showed that in about 40% of the cells a major part of their walls were digested.

These spheroplasts deviated from the usual oval yeast shape. Some were completely spherical, others were basically oval with convex bulges of protoplast emerging, but the majority had become elongate and hyphal-like.

Wall digestion was seldom complete around the whole cell and it was never possible to obtain spheroplast conversion of

Table 1. Random growth of C. albicans in Sabouraud Broth and Wickerham Fluid.

INCUBATION TIME IN HOURS	SABOURAUD BROTH	WICKERHAM BROTH
	LOG ₁₀ MEAN CELLS/ML	LOG ₁₀ MEAN CELLS/ML
0	1.95	1.98
1		2.06
2	2.07	2.00
3		2.16
4	2.66	2.32
5		2.56
6	3.36	2.72
7		2.84
8	3.62	
9		3.19
10		3.41
11		3.61
12		3.77
13		3.82
16	6.06	
17		4.38
20	7.14	
24	7.28	5.78
32	7.27	
36		6.13

Table 2. Random growth of T. glabrata in Sabouraud Broth and Human Plasma.

INCUBATION TIME IN HOURS	SABOURAUD BROTH	HUMAN PLASMA
	LOG ₁₀ MEAN CELLS/ML	LOG ₁₀ MEAN CELLS/ML
0	3.02	3.08
2	3.16	3.10
4	3.60	3.24
6	3.92	3.75
8	4.45	3.83
16½	6.73	5.04
20	7.97	5.45
23½	8.17	5.18
31	8.32	5.00

Table 3. The synchronous culture of C. albicans in Wickerham Fluid.

TIME IN HOURS	LOG ₁₀ MEAN CELLS/ML
0	1.61
$\frac{1}{2}$	1.56
$\frac{1}{2}$	1.53
1	1.62
$1\frac{1}{2}$	1.65
2	1.60
$2\frac{1}{2}$	1.54
3	1.95
$3\frac{1}{2}$	1.93
4	1.99
$4\frac{1}{2}$	2.23
5	2.27
6	2.23
7	2.51
8	2.63
9	2.61
10	2.93

Table 4. Visual observations on synchronous growth of C. albicans in Wickerham Fluid.

TIME IN HOURS	% BUDDING	% NOT BUDDING
0	4	96
$\frac{1}{2}$	6	94
1	4	96
2	93	7
$2\frac{1}{2}$	90	10
3	8	92
$3\frac{1}{2}$	22	78
4	96	4
$4\frac{1}{2}$	95	5
5	3	97
$5\frac{1}{2}$	81	19
6	90	10
$6\frac{1}{2}$	70	30
7	30	70
8	65	35
$8\frac{1}{2}$	78	22
9	85	15
$9\frac{1}{2}$	70	30
10	60	40
11	75	25
12	78	22

Table 5. The rate of hyphal elongation in C. albicans.

TIME IN HOURS	MEAN HYPHAL LENGTH IN μ
0	0
1	10.5 $\pm$ 2.8
1 $\frac{1}{2}$	19.7 $\pm$ 4.4
3	38.0 $\pm$ 5.6
4 $\frac{1}{2}$	68.2 $\pm$ 13.8
5	93.3 $\pm$ 18.9
5 $\frac{1}{2}$	104.1 $\pm$ 16.9
6	126.4 $\pm$ 17.2
8 $\frac{1}{2}$	223.6 $\pm$ 58.4

The rate of hyphal growth was determined from a synchronous culture of blastospores inoculated into plasma at 37°. Measurements were discontinued after 8 $\frac{1}{2}$ hours because by this time the hyphae were too long to be conveniently measured.

Fig. 2. Random growth of C. albicans in Sabouraud Broth and Wickerham Fluid.

Fig. 3. Random growth of *T. glabrata* in Sabouraud Broth and Human Serum.

Fig. 4. The synchronous culture of C. albicans in Wickerham Fluid.

Fig. 5. The rate of hyphal elongation in C. albicans.

all the cells so treated.

Different strains of C. albicans were tried and appeared to have different susceptibilities to wall digestion.

Spheroplasts in plasma and serum

The spheroplasts so formed were placed in human plasma and sera at 37°C and observed microscopically by phase contrast microscopy.

Initially, the spheroplasts showed gross distortion of form while apparently growing in the plasma. After several hours it appeared that wall formation was controlling form. Subsequent growth was similar to that of normal hyphae.

Those yeasts in the population which appeared to have intact cell walls produced normal hyphae in the plasma.

DISCUSSION

C. albicans produced classical growth curves in broth (Tables 2 and 3). Growth was poorer in the chemically-defined Wickerham fluid than in the nutritionally richer Sabouraud Broth (Table 1 and Fig. 2).

In order to investigate relative rates of development in serum and broth, the growth of Torulopsis glabrata was studied. C. albicans could not be used for this because it produces hyphae in serum. T. glabrata, however, grows in serum by budding alone.

In serum after 24 hours, the numbers of T. glabrata increased from 10^3 to 10^5 , in broth from 10^3 to 10^8 (Fig. 3). The slow development in serum may be due to an inhibitory action or a lessened availability of nutrients, and this may account for the fact that C. albicans has a predominantly hyphal growth in serum.

The linear sucrose density gradient technique proved to be a convenient and rapid method of producing a population of similar-sized, synchronous, young blastospores without buds. The technique had the advantage of using healthy growing cells from an exponential culture. The cells were not subjected to trauma and a criticism of other techniques for inducing synchrony - that the cells may be in a state of physiological shock or recovery in lag phase - does not apply.

Microscopical examination of the 0.5 ml. of suspension removed from the density gradient revealed a uniform population of similar-sized, synchronous, newly-formed blastospores uncontaminated by hyphae or budding cells. A good degree of synchronization as evidenced by the stepwise manner of growth was obtained for about four generations (see Fig. 4).

When synchronous blastospores were inoculated into human plasma, the initiation and growth of hyphal elements was synchronous. After a lag phase of around 4 hours, hyphal growth was linear (Fig. 5).

After different periods of incubation, when 10 hyphal elements were measured they were found to be of similar length (Table 5) and this was maintained throughout the period of 8½ hours of observations. The technique of introducing synchronous blastospores into plasma or serum affords a method of producing hyphae of a predetermined length for experiments on the phagocytosis of C. albicans.

When spheroplasts of C. albicans were prepared only about 40% of the cell population underwent transformation and it was not possible to separate these spheroplasts from intact blastospores. The spheroplasts formed rapidly reverted to normal cells by wall formation.

SUMMARY

C. albicans showed classical growth curves in broth. The growth rate was slower in the chemically-defined Wickerham fluid than in the Sabouraud Broth. T. glabrata in serum multiplied more slowly than in broth. It was suggested that this might be due to an inhibitory action of the serum or a reduced availability of nutrients which might account for the conversion of C. albicans from blastospore to mycelial growth.

A rapid and convenient method for obtaining synchronous cultures of C. albicans using a linear sucrose density gradient is presented. A good degree of synchrony was obtained over

four generations.

Synchronous populations of blastospores inoculated into human plasma showed a synchronous initiation and linear growth of hyphae. This technique enabled the production of synchronous populations of cells bearing hyphae of known length, which can be used as uniform inocula in experiments on phagocytosis.

Spheroplasts of C. albicans were obtained using Snail Gut Enzyme to digest away the cell walls. However, only about 40% of the cells were transformed to spheroplasts and these reverted rapidly to normal cells by regrowth of the cell wall.

SECTION 4

CANDIDA ALBICANS AND THE FUNGICIDAL

ACTIVITY OF BLOOD

INTRODUCTION

Ostrowsky (1896), cited from Stanley and Hurley (1969), commented that leucocytosis does not occur in the experimental disease, and lymphadenopathy, hepatomegaly, or splenomegaly are not features of spontaneous or induced candidosis. These observations suggest that neither the fixed nor the wandering cells of the reticulo-endothelial system are active participants in host defence in candidosis.

The role of the phagocytic cell in the engulfment and destruction of bacteria was first recognised by Metchnikoff (1892). At that time the relative importance of cellular and humoral factors in innate resistance to infection was the subject of controversy. Wright and Colebrook (1921) suggested the increased amount of specific antibody developed during infection reinforced phagocytosis, and concluded that blood had 5 possible effects on bacteria:-

1. Bacteria clump - spontaneous or specific agglutination.
2. Bacteria are killed - bactericidal power.
3. Bacteria are dissolved - bacteriolytic power.
4. Bacteria are altered to render them more easily

phagocytosed - opsonic effect.

5. Bacteria are altered to render them more easily digested after engulfment - protryptic effect.

Wright and Colebrook considered that the bactericidal power of the blood was best expressed as the largest number of microbes exterminated by a given volume. However, this suggestion ignores the phenomenon of "percentage killing" in which a fraction of the microbes survive.

Other workers, including Mackie and Finkelstein (1931), Miles and Misra (1938), have reported on the numbers of bacteria killed by blood in vitro.

Miles and Misra (1938) in their study of the survival of Staphylococcus aureus in a standard volume of blood produced a reliable quantitative measure of the bactericidal power of the blood.

However, no attempt at measuring the candidacidal capacity of the blood appears to have been made.

The purpose of this study was to investigate quantitatively the candidacidal capacity of blood from a range of normal volunteers and diabetic patients.

METHODS

Collection and preparation of blood samples

A 0.1 ml. volume of Ringer-Locke solution (cited Ruch and Fulton, 1960) containing 50 i.u. heparin was placed in a 25 ml. screw-capped container and 3 ml. of sterile liquid paraffin added. Blood was withdrawn from the antecubital vein with a plastic syringe and immediately 10 ml. was gently injected beneath the liquid paraffin in the container and the cap replaced.

A series of 1 ml. lots of blood was then removed and each injected beneath 3 ml. of liquid paraffin in a dry, detergent-free, glass universal container.

Throughout the study to be described, a layer of liquid paraffin was used as a barrier to gaseous exchange between the blood and the atmosphere. Bond (1919) reported the use of liquid paraffin as a dressing on wounds favoured phagocytosis, and addition of liquid paraffin to blood in vitro seemed to protect the phagocytes from disintegration and stimulated them to increased activity. In four preliminary experiments where survival ranged from 10-19% when liquid paraffin was used, when the blood was exposed to the air of the tube survival ranged from 15-35%. With the liquid paraffin seal, the venous colour of the blood was maintained during the 5 hour period of incubation, whereas that in the controls became bright red.

Preparation of Candida inocula

A synchronous population of C. albicans blastospores from an overnight Sabouraud Broth culture was prepared by the linear density gradient technique (p. 24).

The strains used were 207/53A (Hasenclever serotype A), 792/53B (Hasenclever serotype B) and WF 668A (serotype not known),

A. Blastospore inocula

The cells were adjusted to a desired Optical Density in Ringer-Locke solution using a Hilger "Spekker" absorptiometer and decimal dilutions prepared.

Viable counts were made on Sabouraud dextrose agar to estimate the number of blastospores in the suspensions.

B. Blastospores bearing hyphal lengths ranging from 16.9 to 360 μ

The synchronous cells were inoculated into human plasma, and incubated at 37° for varying periods of time so that uniform suspensions with respect to hyphal length were produced.

Hyphal lengths were accurately measured by microscopy using a micrometer eye-piece. The approximate rate of hyphal elongation was already known from previous observations (Fig. 5).

The cells were washed three times and then adjusted to a desired Optical Density in Ringer-Locke solution and decimal dilutions prepared. Viable counts were made to estimate the numbers of cells in the suspensions.

Experimental method

A 0.1 ml volume of the suspension was carefully inoculated into the 1 ml. of blood and incubated for 5 hours at 37° without shaking.

The 5 hour period of the present experiments was adapted from a preliminary study which showed the maximum kill had been obtained by 5 hours. Further incubation led to misleading results due to the multiplication of survivors.

Following incubation, 9 ml. of sterile water was added to each universal container. Most of the liquid paraffin seal was then withdrawn from the surface, the suspension subjected to ultrasonic vibration at 5 μ peak-to-peak amplitude for 30 seconds using an MSE Ultrasonic Disintegrator. This effects a disruption of the PMN and breaks up the clumped Candida into single cells. A viable count was then undertaken to calculate the percentage survival.

To obtain the viable count, the suspension was poured into a 6 inch diameter Petri dish and the universal container then filled with Sabouraud dextrose medium containing 6% agar. This

was shaken and poured into the petri dish and the procedure repeated. The dish was then rotated to mix the 50 ml. of Sabouraud agar with the 10 ml. blood and Candida suspension. The universal container was then refilled with Sabouraud agar, the cap replaced and the container incubated. This ensured that any Candida remaining in the glass Universal container in which the experiment was performed would develop into colonies and could be counted. The petri dish and tube were incubated for 48 hours at 37° and then counted.

In those experiments where a survival of 10² or more was expected, decimal dilutions of the sonicated suspension were prepared and 1 ml. samples planted on Sabouraud dextrose agar.

The survival rate of C. albicans in blood was calculated from the plate counts by the formula:-

$$\% \text{ survival} = \frac{\text{no. cells surviving} \times 100}{\text{no. cells inoculated}}$$

RESULTS

The results of series A experiments with blastospores are given in Tables 6-10 and Figs. 6-11 and of series B experiments with hyphae in Table 11 and Fig. 12.

The survival values were compared with the log₁₀ counts of the inocula. Lines were fitted and probability bounds obtained by means of a line regression programme on an IBM 1900 computer

Table 6. Survival of C. albicans strain 207/53A in blood from normal volunteers.

INOCULA									SURVIVORS								
DECIMAL DILUTION	PLATE COUNTS						NO. OF PLATES	MEAN INOCULUM	DECIMAL DILUTION	PLATE COUNTS						TOTAL CELLS SURVIVING	% SURVIVAL
	1	2	3	4	5	6				1	2	3	4	5	6		
0	12	20	24	24	26	28	6	22	0	1	3	5	10	11	16	46	34.8
										1	1	4	5	5	11	27	20.5
										0	0	2	3	4	4	13	9.8
$\frac{1}{10}$	2	2	2	4	5	6	6	35	0	2	4	4	4	7	12	33	15.7
										5	8	10	14	19	28	84	40.0
										3	4	7	10	11	12	47	22.4
$\frac{1}{10}$	3	4	5	7	7	12	6	63	0	13	14	20	21	24	42	134	35.4
										12	15	15	17	18	21	98	25.9
										13	21	22	25	38	43	162	42.9
$\frac{1}{10}$	8	9	12	12	13	15	6	115	0	22	26	27	28	30	34	167	24.2
										37	40	40	46	49	51	263	38.1
										10	11	15	17	20	28	101	14.6
$\frac{1}{100}$	4	5	7	8	14	15	6	883	$\frac{1}{10}$	14	18	18	18	23	41	1320	24.9
										21	32	39	40	42	49	2230	42.1
										30	39	43	45	45	49	2510	47.4
$\frac{1}{100}$	32	35	39	41	48	51	6	4100	$\frac{1}{100}$	8	12	17	18	19	22	9600	39.0
										9	14	16	18	23	24	10400	42.3
										14	20	21	22	24	26	12700	51.6
$\frac{1}{10,000}$	8	9	11	21	22	25	6	160000	$\frac{1}{10,000}$	2	4	5	7	9	9	360000	37.5
										3	5	7	8	11	15	490000	51.0
										7	8	9	11	12	15	620000	64.4
$\frac{1}{100,000}$	7	8	9	12	14	16	6	1100000	$\frac{1}{100,000}$	1	3	4	6	8	9	3100000	46.9
										2	4	6	8	9	10	3900000	59.1

Table 7. Survival of C. albicans strain 207/53A in blood from patients with diabetes.

INOCULA									SURVIVORS								
DECIMAL DILUTION	PLATE COUNTS						NO. OF PLATES	MEAN INOCULUM	DECIMAL DILUTION	PLATE COUNTS						TOTAL CELLS SURVIVING	% SURVIVAL
	1	2	3	4	5	6				1	2	3	4	5	6		
0	8	10	11	11	12	15	6	11	0	0	1	2	4	4	7	18	27.3
										1	1	3	4	5	5	19	28.8
										1	2	3	5	6	7	24	36.4
0	61	66	74	78			4	70	0	12	14	14	15			55	19.6
										8	13	14	18			53	18.9
										11	18	20	22			71	25.4
$\frac{1}{10}$	4	7	9	9			4	73	0	19	24	24	30			97	33.2
										15	15	16	17			63	21.6
										25	29	30	31			115	39.4
0	72	85	96	124			4	94	0	46	50	50	54			200	53.2
										43	52	55	57			207	55.1
0	78	86	111	121			4	99	0	20	24	26	27			97	24.5
										26	29	30	32			117	29.5
										53	56	57	57			223	56.3
$\frac{1}{10}$	4	12	15	15	18	28	6	153	0	36	41	43	44	48	61	273	29.7
										40	44	56	59	62	63	324	35.3
$\frac{1}{100}$	23	24	26	28	32	37	6	2833	$\frac{1}{100}$	15	16	16	17	20	38	12200	71.8
										4	8	10	13	13	16	6400	37.7
										6	7	8	8	9	11	4900	28.8
$\frac{1}{1,000}$	6	7	9	12	14	15	6	10500	$\frac{1}{1,000}$	2	3	7	8	9	10	39000	61.9
										4	6	7	8	8	9	42000	66.7
										2	2	4	9	12	14	43000	68.3
$\frac{1}{10,000}$	14	19	23	28	28	32	6	240000	$\frac{1}{10,000}$	6	7	12	19	20	24	880000	61.1
										4	9	11	17	22	34	970000	67.4
										3	12	14	19	27	31	1060000	73.6

Table 8. Survival of C. albicans strain 792/53B in blood from normal volunteers.

INOCULA									SURVIVORS								
DECIMAL DILUTION	PLATE COUNTS						NO. OF PLATES	MEAN INOCULUM	DECIMAL DILUTION	PLATE COUNTS						TOTAL CELLS SURVIVING	% SURVIVAL
	1	2	3	4	5	6				1	2	3	4	5	6		
0	8	8	9	14	14	19	6	12	0	0	0	0	2	3	4	9	12.5
										0	1	2	3	5	6	17	23.6
										0	0	2	3	5	10	20	27.8
$\frac{1}{10}$	1	3	4	4	6	6	6	40	0	11	12	12	13	14	21	83	34.6
										10	11	12	14	22	23	92	38.3
										10	19	21	24	25	48	147	61.3
$\frac{1}{10}$	1	4	5	5	5	6	6	43	0	7	10	14	15	15	17	78	30.2
										10	11	12	14	19	22	88	34.1
										17	18	18	20	22	30	125	48.4
$\frac{1}{10}$	5	8	9	12	12	16	6	103	0	17	19	22	26	27	28	139	22.5
										22	23	28	31	37	39	180	29.1
										21	26	31	36	38	42	194	31.4
$\frac{1}{10}$	18	19	19	26	27	29	6	230	$\frac{1}{10}$	3	4	5	7	9	11	390	28.3
										4	6	8	8	9	11	460	33.3
										4	7	9	10	16	18	640	46.4
$\frac{1}{1,000}$	2	2	3	7	8	9	6	5167	$\frac{1}{100}$	13	15	17	19	24	31	11900	38.4
										17	22	24	28	30	32	15300	49.4
$\frac{1}{10,000}$	21	29	33	34	37	38	6	320000	$\frac{1}{10,000}$	8	13	15	22	27	29	1140000	59.4
										16	16	19	23	23	25	1220000	63.5
										13	19	24	26	28	31	1410000	73.4

Table 9. Survival of C. albicans strain 792/53B in blood from patients with diabetes.

INOCULA									SURVIVORS								
DECIMAL DILUTION	PLATE COUNTS						NO. OF PLATES	MEAN INOCULUM	DECIMAL DILUTION	PLATE COUNTS						TOTAL CELLS SURVIVING	% SURVIVAL
	1	2	3	4	5	6				1	2	3	4	5	6		
0	10	10	11	13	14	14	6	12	0	1	2	4	4	6	11	28	38.9
										1	2	3	4	7	9	26	36.1
$\frac{1}{10}$	2	5	5	7	7		5	52	0	24	29	35	35	42	44	209	67.0
										12	31	37	42	44	45	211	67.6
										12	14	18	24	31	34	133	42.6
$\frac{1}{10}$	4	6	7	8	8	9	6	70	$\frac{1}{10}$	0	1	2	3	4	5	150	35.7
										1	1	2	4	5	6	190	45.2
$\frac{1}{10}$	4	9	9	9	12	12	6	92	0	12	18	20	22	31	37	140	25.4
										20	28	28	32	37	44	189	34.2
										18	22	26	29	31	38	164	29.7
$\frac{1}{10}$	10	11	13	13	14	16	6	128	0	49	53	61	65	70	82	380	49.5
										35	47	60	61	62	64	329	42.8
										66	70	86	95	102	104	523	68.1
$\frac{1}{100}$	8	9	12	15	17	26	6	1450	$\frac{1}{100}$	2	4	4	7	9	11	3700	42.5
										1	3	6	9	10	17	4600	52.9
										3	4	6	9	12	13	4700	54.0
$\frac{1}{100}$	39	42	50	63	66	78	6	5633	$\frac{1}{100}$	20	22	22	28	31	39	16200	47.9
										15	26	28	31	35	44	17900	53.0
										20	26	31	38	42	53	21000	62.1
$\frac{1}{100,000}$	4	7	9	13	15	16	6	1066667	$\frac{1}{100,000}$	3	5	5	7	8	12	4000000	62.5
										2	3	6	7	11	15	4400000	68.7
										5	6	7	9	10	13	5000000	78.1

Table 10. Survival of C. albicans strain WF 668A in blood from normal volunteers.

INOCULA									SURVIVORS								
DECIMAL DILUTION	PLATE COUNTS						NO. OF PLATES	MEAN INOCULUM	DECIMAL DILUTION	PLATE COUNTS						TOTAL CELLS SURVIVING	% SURVIVAL
	1	2	3	4	5	6				1	2	3	4	5	6		
0	7	8	9	14	17		5	11	0	2	3	4				9	27.3
										0	2	6				8	24.2
										4	4	4				12	36.4
0	9	12	14	14	16		5	13	0	1	2	3				6	15.4
0	8	12	12	15	23		5	14	0	0	2	3				5	11.9
0	3	22	23				3	16	0	0	0	0				0	0
										0	0	0				0	0
0	14	15	17	18	20		5	17	0	1	2	2				5	9.8
0	14	14	19	24	30		5	20	0	2	3	4				9	15.0
										1	2	3				6	10.0
										0	1	2				3	5.0
0	15	17	23	32	34		5	24	0	1	3	3	3	4		14	11.7
										1	3	3	4	6	6	23	16.0
0	20	25	29	35			4	27	0	0	2	5	6	7		20	14.1
0	35	35	38	45	47		5	40	0	2	4	4	5	5	11	31	12.9
0	36	37	37	39	53		5	40	0	4	10	11	15	20		60	30.0
0	36	48	49	50	58		5	48	0	12	14	16				42	29.2
										8	12	14	26	35		95	39.6
0	51	52	57	57	61		5	56	0	19	20	20				59	35.1
										6	9	11				26	15.5
										4	8	11				23	13.7
										12	13	13				38	22.6
0	73	79	85				3	79	0	21	26	27				74	31.2
										5	11	15	28	36		95	24.1
0	64	92	99				3	85	0	8	14	17				39	15.3
0	70	94	112				3	92	0	0	19	21				40	14.5
0	78	97	98	111	120		5	101	0	6	17	22	22	28		95	18.8
0	85	120	154	162	179		5	140	0	46	50	53				149	35.5
										53	56	79				188	44.7
										53	55	55				163	38.8
$\frac{1}{10}$	23	39	47				3	363	0	21	51	62				134	12.3
										90	125	130				345	31.7

Table 10 continued.

INOCULA									SURVIVORS								
DECIMAL DILUTION	PLATE COUNTS						NO. OF PLATES	MEAN INOCULUM	DECIMAL DILUTION	PLATE COUNTS						TOTAL CELLS SURVIVING	% SURVIVAL
	1	2	3	4	5	6				1	2	3	4	5	6		
$\frac{1}{10}$	55	58	68				3	603	$\frac{1}{10}$	19	20	24				630	34.7
										21	25	27				730	40.2
$\frac{1}{10}$	120	90	126				3	1120		374	260	380				1014	30.2
										220	270	270				760	22.6
$\frac{1}{100}$	71	74	76				3	7367	$\frac{1}{100}$	29	30	42				10100	45.6
$\frac{1}{100}$	78	80	103				3	8700	$\frac{1}{100}$	21	52	56				12900	49.4
$\frac{1}{100}$	81	90	120				3	9700	$\frac{1}{100}$	30	31	50				11100	38.1
$\frac{1}{1,000}$	14	15	19	22			4	17500	$\frac{1}{1,000}$	7	9	10	19			45000	64.3
$\frac{1}{1,000}$	18	19	22	23	25		5	35666	$\frac{1}{1,000}$	4	6	7	10	12		39000	36.5
$\frac{1}{1,000,000}$	1	2	3				3	2×10^6	$\frac{1}{100,000}$	4	9	11	12	14		5×10^6	50.0
$\frac{1}{100,000}$	30	32	34				3	3.2×10^6	$\frac{1}{100,000}$	15	18	18	25	40		11.6×10^6	72.5
$\frac{1}{1,000,000}$	1	5	6				3	4×10^6	$\frac{1}{100,000}$	18	22	25	35	35		13.5×10^6	67.5

Table 11. Survival of *C. albicans* strain 207/53A blastospores with hyphae in blood from normal volunteers.

MEAN LENGTH OF HYPHAE IN μ	INOCULUM							SURVIVORS							
	PLATE COUNTS						MEAN	PLATE COUNTS						MEAN	% SURVIVAL
	1	2	3	4	5	6		1	2	3	4	5	6		
16.9	36	37	42	47	48		42.0	4	8	12	14	17		11.0	26.2
								3	7	8	8	9		7.0	16.7
24.1	29	37	38	39	43	47	38.8	5	6	10				7.0	18.0
								8	15	17				13.3	34.3
								12	13	13				12.7	32.7
27.4	9	10	12	12	19	22	14.0	1	3	3				2.3	16.4
								2	2	3				2.3	16.4
37.9	41	45	47	48	50	53	47.3	4	6	7	9	9	10	7.5	15.9
								8	8	8	9	14	15	10.3	21.8
								10	14	18	23	24	26	19.2	40.6
81.9	59	66	68	69	72	77	68.5	17	18	19	20	23	26	20.5	29.9
								4	5	6	8	14	14	8.5	12.4
105.5	19	19	24	25	25	30	23.7	1	1	1	4	5	6	3.0	12.7
								2	3	3	4	4	5	3.5	14.8
								2	3	4	4	7	9	4.8	20.3
110.6	17	24	30	31	36	40	29.7	6	8	11	11	12	13	10.2	34.3
								3	5	5	8	9	10	6.7	22.6
								2	4	7	8	8	12	6.8	22.9
149.2	44	52	63				53.0	11	17	18				15.3	28.9
								10	14	16				13.3	25.1
187.4	30	36	37	39	49	49	40.0	3	7	11	16	18	20	12.5	31.3
								2	6	8	10	14	18	9.7	24.3
								5	11	12	15	16	19	13.0	32.5
250.0	27	28	29	34	38		31.2	8	12	14	15	16		13.0	41.7
								12	13	15	17	18		15.0	48.5
								13	14	16	19	22		16.8	53.8
360.0	26	32	33	35	38		32.8	16	17	17	19	21		18.0	54.9
								16	18	24	27	27		22.4	68.3
								19	23	25	26	31		24.8	75.6

Fig. 6. Survival of C. albicans strain 207/53A in blood from normal volunteers.

Fig. 7. Survival of C. albicans strain 207/53A in blood from patients with diabetes.

Fig. 8. Survival of C. albicans strain 792/53B in blood from normal volunteers.

Fig. 9. Survival of C. albicans strain 792/538 in blood from patients with diabetes.

Fig. 10. Survival of C. albicans strain WF 668A in blood from normal volunteers.

Fig. 11. Composite diagram of survival of C. albicans strains 207/53A, 792/53B and WF 668A in blood from normal volunteers and patients with diabetes.

Fig. 12. Survival of C. albicans strain 207/53A blastospores with hyphae in blood from normal volunteers.

at Queen Mary College. This provided a summary of the observations.

The 95% confidence limits were calculated by the distance D from the mean:

$$D = t_{n-2, 0.05} \times S_r$$

where S_r is the root of the mean square of the residual.

The standard deviation was calculated as $\frac{D}{2}$ from the line A.

DISCUSSION

C. albicans occurs in the normal microbiota of about half the population (Davies and Leese, personal communication). Superficial infections with C. albicans, such as mucocutaneous candidiasis, are fairly common but systemic candidiasis is rare and probably only affects persons who are immunosuppressed or debilitated by other diseases (Winner and Hurley, 1964). It must therefore be concluded that Candida is killed in the blood of normal healthy subjects.

No previous work could be found which reported on the killing of C. albicans in blood. The killing of the two Candida serotypes, Hasenclever A and B, in blood samples from normal subjects and patients with Diabetes mellitus was studied. It has been shown, Tables 6-10 and Figures 6-11, that only a percentage of the inocula were killed in these experiments.

With an inoculum of less than 10 viable cells of C. albicans, variation and errors induced in the sampling technique did not permit statistical evaluation. With between 10 and 100 cells, and 100 and 1000 cells, the predicted mean kill was approximately 80 and 70% respectively.

These experiments on the candidacidal power of 1 ml. blood during 5 hours differ from the in vivo situation in which the Candida is exposed to a constant supply of fresh blood. In a stagnant ml. of blood the activities of the leucocyte pool may decline with its half-life and should any serum factors be effective these may become exhausted. The role of serum and polymorphonuclear leucocytes are the subjects of investigations in Sections 5, 6 and 7.

In bacterial septicaemia, viable counts are usually less than 10 cells per ml. of blood sampled.

From the experiments described it may be concluded that the blood has adequate candidacidal activity. No differences were observed between the blood from normal and diabetic patients and no difference was detected in the disposal of the two serotypes.

SUMMARY

The survival rates of C. albicans strains Hasenclever 207/53A and 792/53B and WF 668A were measured in bloods from normal volunteers and patients with Diabetes mellitus. No

detectable differences in the survival between three strains were discovered. In this respect blood from diabetics appeared to have the same activity as bloods from normal subjects. Percentage survival increased with increasing size of the inoculum. The rate of survival of propagules with hyphae up to 200 μ in length was within the same range as survival of blastospores, but killing of mycelial elements of greater length than this was much reduced.

S E C T I O N 5

C. ALBICANS AND SERUM

INTRODUCTION

The antibacterial qualities of cell-free human serum were described by Nuttal (1888).

The inhibition of dermatophyte fungi by human serum has been reported by Ayres and Anderson (1934), Memmesheimer, McNall and Sternberg (1962), Owens, Shacklette and Baker (1965) and Lorincz, Priestley and Jacobs (1958).

Roth, Boyd, Sagami and Blank (1959) inoculated C. albicans into a culture fluid containing 10% human serum and into the culture fluid alone. The "growth rates" in the two fluids were measured turbidimetrically. Since the "growth rate" in the fluid containing serum was less than that in the control fluid, they considered that serum inhibited the growth of C. albicans.

Using a similar technique, Roth and Goldstein (1961) confirmed the above result by reporting that serum concentrations as low as 5% were highly active at inhibiting the in vitro growth of C. albicans.

Baum and Artis (1961) cultured C. albicans for 48 hours in Sabouraud Broth and Sabouraud Broth with 50% serum added. Since viable counts disclosed no difference, they concluded

serum had "no influence" on C. albicans.

Caroline, Taschdjian, Kozinn and Schade (1964) incubated C. albicans for 18 hr. at 37° in a neopeptone-glucose medium to which had been added 14.3% serum. They reported that sera from normal subjects and patients with cutaneous candidosis completely inhibited the growth of *Candida* as indicated by cultural turbidimetric methods.

Louria and Brayton (1964a), inoculated 10⁷ C. albicans cells into 1 ml. lots of serum and incubated the suspension on a gyrotory shaker at 37°. Their examination of pour plates made after 6 and 24 hours showed a drop in colony count which led them to conclude that serum was lethal to C. albicans.

The growth of C. albicans, as measured by determination of light transmission, was inhibited by the addition of 10-50% serum from horse, dog and buffalo calf to Sabouraud Broth, compared with growth in Sabouraud Broth diluted with 10-50% water (Srivastava, Kannan, Kutty and Vora, 1965).

Chilgren, Hong and Quie (1968), however, reported that such a decrease in colony counts could be wholly explained by the phenomenon that *Candida* cells clump in serum. Disruption of the C. albicans clumps by gentle homogenization in their experience returned the colony count to the original number and there was no evidence that C. albicans was killed in serum.

Hendry and Bakerspigel (1969), using measurements of Optical Density and dry weight, reported that C. albicans was inhibited by low concentrations of human serum in a neopeptone-glucose broth, but this inhibition was reversed if

FeCl_3 was added.

Lehrer and Cline (1969a) considered that the drop in colony count which they observed during the first four hours when C. albicans was incubated in serum was due to hyphal growth and the consequent formation of mycelial masses.

In view of the findings of Chilgren et al. (1968) and Lehrer and Cline (1969a) which appeared to refute the conclusions of the earlier workers, the following studies were undertaken to confirm that normal human serum neither killed nor inhibited the development of C. albicans. A secondary aim of these investigations was to study the effect of serum and its components on the growth, form and chemical composition of C. albicans.

Serum and components in relation to
tubulation in *C. albicans*

The preparation of synchronous *C. albicans* blastospores

Synchronous cultures of *C. albicans* blastospores, Hasenclever strains 207/53A or 792/53B from overnight Sabouraud Broth cultures were prepared as p. 24. The synchronous blastospores were suspended in water to give an Optical Density of 0.30 in the Hilger "Spekker" absorptiometer.

The preparation of serum, plasma, modified plasma or other substrate

The serum used was from time-expired human blood and seitz-filtered using a Carlson-Ford HPS 10 filter pad.

The serum was diluted by means of a standard volume delivery pipette with Wickerham Broth or water as diluent.

When required, anaerobic conditions were obtained by placing an uncapped bijou bottle containing serum and *Candida* inoculum in an anaerobe jar with a cold catalyst (Baird & Tatlock Ltd.). The jar was evacuated, refilled with hydrogen and allowed to stand for 24 hours at 3° so that serum and *Candida* were in equilibrium with the anaerobic atmosphere of the jar before the jar was transferred to an incubator at 37° for 5 hours. This ensured that any growth of *C. albicans* in the serum occurred under strictly anaerobic conditions.

The treatment of serum with pancreatin

A sample of serum was placed in a water-bath of boiling water for 30 minutes.

Boiled and unboiled samples of serum were incubated with 0.01 g/ml. pancreatin for 16 hours at 37° on a gyrotory shaker and then dialysed against water for 24 hours at 3°.

The preparation of plasma protein enriched fractions

Albumin extraction

The technique used was that of Vallance-Owen, Dennes and Campbell (1958).

1. 10 ml. plasma was treated with 10 ml. of 10% (w/v) trichloroacetic acid (TCA). The mixture was centrifuged at 750 r.p.m. for 20 mins. The supernatant was discarded.
2. The precipitate was washed with 10 ml. 5% TCA, recentrifuged and the supernatant discarded.
3. The precipitate was then extracted with 3 volumes of 1% TCA in 96% ethanol. This was centrifuged and the supernatant transferred to a $\frac{1}{2}$ " dialysis tube.
4. The residual precipitate was re-extracted with 10 ml. of 1% TCA in 96% ethanol. After centrifugation the supernatant was added to that previously placed in the dialysis tube.
5. The extract was dialysed against running tap water for

24 hr. and then against two changes of distilled water at 3°C for 48 hr.

6. The dialysed extract was freeze-dried to a white powder.

The albumin contained a contaminating trace of insulin.

γ Globulin precipitation

The technique was that of Kekwick, R.A. (1940).

1. 1.8 g. sodium sulphate was dissolved in 10 ml. serum.
2. The mixture was allowed to stand for 1½ hr. at 37°C.
3. The mixture was centrifuged for ½ hr. at 750 r.p.m. and the clear supernatant removed.
4. The volume of the precipitate was read off on the graduated side of the centrifuge tube.
5. The tube was made up to 4 ml. with distilled water.
6. Each tube was made up to 12% sodium sulphate by adding $(4 \times 0.12) - (\text{Vol. of ppt.} \times 0.18)$ g. sodium sulphate.
7. This mixture was allowed to stand for 1 hr. at room temperature.
8. Steps 2 to 7 were then repeated.
9. The precipitate volume was again read off.
10. Each tube was made up to 2 ml. with water.
11. Each tube was then made up to 12% sodium sulphate by adding $(2 \times 0.12) - (\text{Vol. of ppt.} \times 0.12)$ g. sodium sulphate.

12. The mixture was allowed to stand at room temperature for 1 hr. and then centrifuged.
13. The precipitate was freeze-dried.

The freeze-dried proteins were then dissolved in water, albumin at the rate of 40 mg/ml. and γ globulin at the rate of 25 mg/ml.

EXPERIMENTAL METHODS

1 ml. lots of serum, modified serum or other substrate were placed in bijoux bottles and 0.1 ml. of the synchronous *Candida* suspension added. The bottles were incubated, without shaking, for 5 hours at 37° unless otherwise stated.

Loopfuls of cells were then removed and spread over a glass microscope slide. Under the phase contrast microscope 100 cells were examined for hyphal production and the lengths of 10 of these hyphae measured with an eye-piece graticule. The results are presented as the % cells with hyphal and the mean length of hyphal in μ in Table 12.

RESULTS AND DISCUSSION

In human serum and plasma not only was *C. albicans* not killed, but microscopic examination showed all the synchronous blastospores commenced hyphal development at the same time. The rate of hyphal elongation was measured at

Table 12. Tubulation of C. albicans after 5 hrs. at 37° in Serum, Plasma, Treated Plasma and other fluids.

SUBSTRATE	% CELLS WITH HYPHAE	MEAN HYPHAL LENGTH IN μ
Serum	100	105 $\pm$ 16
Plasma	100	102 $\pm$ 20
Serum heated 56° for $\frac{1}{2}$ hr.	100	109 $\pm$ 46
Serum from diabetic patient	100	140 $\pm$ 38
Serum treated with Pancreatin	100	91 $\pm$ 28
Serum boiled then treated Pancreatin	96	26 $\pm$ 38
Serum + water 50:50	100	98 $\pm$ 17
25 Serum:Water 75	100	85 $\pm$ 22
12.5 Serum:Water 87.5	100	69 $\pm$ 26
6.25 Serum:Water 93.75	45	62 $\pm$ 31
3.125 Serum:Water 96.875	12	36 $\pm$ 24
Serum + Wickerham fluid 50:50	100	163 $\pm$ 36
Wickerham fluid	1	27
Albumin	2	18 $\pm$ 4
γ Globulin	95	44 $\pm$ 23
Serum with anaerobic incubation	2	7 $\pm$ 1
Tubulations in serum incubated at 23°	2	32 $\pm$ 6
Tubulation in serum incubated at 3°	0	0

intervals (see Fig. 5) up until $8\frac{1}{2}$ hours when measurement became impracticable. These results, together with those given in Table 12, refute the contentions of Roth et al. (1959), Roth and Goldstein (1961), Louria and Brayton (1964a) and Hendry and Bakerspigel (1969) that serum killed or inhibited the growth of C. albicans. The conclusions of Chilgren et al. (1968) and Lehrer and Cline (1969a) that any decrease in the number of colonies grown from a culture of C. albicans in serum could be explained by clumping of the cells and mycelial formation are supported.

It may be seen (Table 12) that 100% of all the synchronous blastospores showed tubulation in serum, plasma, serum with complement inactivated, serum diluted to 12.5% with water or 50% with Wickerham Fluid.

The rate of hyphal elongation in serum from patients with uncontrolled Diabetes mellitus was significantly greater than that of normal serum ($t = 4.074$ and $P < 0.01$), a result (Table 12) attributed to the high glucose concentration of the diabetic serum. Fasting blood glucose levels are 68-96 mg/100 ml. in normal persons and 130 mg/100 ml. in diabetics (Long, King and Sperry, 1961).

Heating the serum at 56° for 30 minutes to destroy the complement system had no effect on the tubulation of C. albicans ($t = 0.550$ and $P > 0.05$).

No hyphal development occurred at 3° and 23°.

Only 2% of the cells produced hyphae in serum cultured anaerobically, which shows that some oxygen is needed for hyphal growth.

Although serum was diluted to 6½% with water before any reduction in the percentage of cells producing hyphae occurred, hyphal lengths decreased with increased dilution of the serum. Dilution of the serum by 50% with Wickerham Fluid significantly increased the hyphal growth rate ($t = 4.060$ and $P < 0.01$) and this again was considered to be due to the additional nutrients. Hyphae are only rarely formed in Wickerham Fluid without added serum.

Treatment of serum with pancreatin had no significant effect on the percentage of blastospores producing hyphae, nor their lengths. However, when the serum was first boiled, and then treated with pancreatin before inoculation with C. albicans and hyphal growth was significantly reduced ($t = 9.739$ and $P < 0.01$) 95% of the blastospores inoculated into a solution of 25 mg/ml. γ globulin produced hyphae. The hyphae were short probably due to the lack of readily available nutrients in the solution. However, this protein did seem important to the formation of hyphae. Serum normally contains 2.3 - 3.5 g γ globulin per 100 ml. 40 mg/ml. albumin produced very few hyphae and seems unimportant to the formation of hyphae. Serum normally contains 3.9 - 4.6 g albumin per 100 ml.

Analysis of the Blastospore and Mycelial

Phase of *C. albicans*

C. albicans in glucose rich, serum free, medium grows rapidly by budding off blastospores. In serum growth commences by tubulation and mycelial development predominates.

Preparation of *C. albicans* blastospores and mycelium for chemical analysis

0.1 ml. lots of the *C. albicans* synchronous blastospore suspensions were inoculated into 15 ml. amounts of serum and Sabouraud Broth in chemically-clean, screw-capped glass universal containers of 25 ml. capacity. These were incubated for 2 days at 37° on a gyrotory shaker.

The cells were then centrifuged and washed three times with water prior to freeze-drying.

The freeze-dried pellet of blastospores was a smaller, darker, more compact mass than the freeze-dried mycelium which was pure white and of a foamy consistency (Fig. 13).

The pellets were broken up with a clean scalpel and 1 mg. samples prepared and dissolved in 3 ml. of 0.3 N potassium hydroxide and rotated at 37° for 16 hours. The insoluble residues were removed by centrifugation and discarded.

The clear supernatant was subjected to quantitative chemical analysis for protein, carbohydrate, RNA and DNA. The

Fig. 13. The freeze-dried pellets of blastospores (B) and mycelium (M).

fat concentration was determined from freeze-dried whole cells.

The techniques involved were as follows:-

Protein estimation

Two solutions were prepared. The first was 2% sodium carbonate in 0.1 N sodium hydroxide. The second was equal parts of 1% copper sulphate and 2% sodium tartrate in water. Before use 50 parts of the first were added to 1 part of the second solution.

0.5 ml. supernatant was incubated at room temperature for 10 minutes with 5 ml. of the freshly mixed solution.

0.5 ml. solutions of Bovine Serum Albumin containing 500 µg. per ml. and 250 µg. per ml. were similarly treated in order to prepare a standard curve.

1 ml. of 50% Folin's solution was pipetted into each tube and mixed instantly. After $\frac{1}{2}$ hr. the optical density was read at 750 mµ. in a 3 ml. cuvette against a blank of water treated as above. The optical densities were read in a Unicam SP500 spectrophotometer.

Carbohydrate estimation

Standard solutions of glucose containing 250 µg. and 125 µg. per ml. were prepared.

1 ml. of 5% aqueous phenol was added to 1 ml. samples

of each test solution. 5 ml. concentrated sulphuric acid was run in quickly and incubated at room temperature for 20 minutes. Solutions were read at 490 mμ in the spectrophotometer.

RNA estimation

Standard solutions of RNA containing 25 μg. and 12.5 μg. per ml. were prepared.

1 ml. of test solution was incubated with 1 ml. of 0.1% orcinol and 0.1% ferric chloride in concentrated hydrochloric acid in a 100°C bath for 20 minutes.

The tubes were then plunged into ice and 2 ml. water added to each. The optical densities were read at 660 mμ.

DNA estimation

Standard solutions of DNA containing 25 μg. and 12.5 μg. per ml. were prepared.

A solution of 1.5 g. diphenylamine in 100 ml. acetic acid was prepared and 1.5 ml. of concentrated sulphuric acid added and the whole stored in the dark. Before use 0.1 ml. of aqueous acetaldehyde (16 mg/ml.) was added to 20 ml. solution. 4 drops of perchloric acid were then added. 2 ml. of this reagent was added to 1 ml. samples of the test solutions. The tubes were incubated at 30°C for 16 hr. with constant rotation. The optical densities were read at 600 mμ.

Fat estimation

An accurately weighed sample of freeze-dried cell material was wrapped in a filter paper and introduced into a pyrex glass Soxhlet apparatus. The basal bulb of the apparatus was weighed. 15 ml. of a 2 parts chloroform:1 part ethanol mixture was placed in the bulb and the bulb placed in a sand bath. A Liebig Condenser was fitted above the Soxhlet.

The sand bath was heated by a 100w. electric light. The vapour from the lower bulb ascended and extracted the fat from the cell material and distilled back into the bulb.

Following 12 hours extraction, the remaining liquid was evaporated from the bulb and the bulb was then reweighed. The difference between this weight and the original weight of the bulb is the weight of fat extracted.

RESULTS AND DISCUSSION

Following freeze-drying, the weight of blastospores grown in Sabouraud Broth was over 3 times the weight of mycelium grown in serum (Table 13). This confirms the earlier observation that plasma is a poorer growth medium than Broth (Fig. 3).

The blastospore material was richer in protein, carbohydrate, RNA, and DNA but poorer in fats than the mycelial material. This result reflects the lower rate of metabolism

Table 13. Chemical analysis of C. albicans blastospores and mycelium.

INVESTIGATION	BLASTOSPORES		MYCELIUM	
	SEROTYPE A	SEROTYPE B	SEROTYPE A	SEROTYPE B
Mean weight of freeze-dried material	0.1007g.	0.1194g.	0.0326g.	0.0330g.
Protein %	46.75	50.03	23.98	28.72
Carbohydrate %	41.20	39.03	28.80	29.42
RNA %	10.37	8.01	6.85	6.37
DNA %	0.760	1.197	0.144	0.129
Fat %	2.18	1.96	30.40	27.80

and growth of the cells in serum compared with Broth. The presence of a greater amount of fat in the mycelium is probably related to the large amount of fat present in serum.

SUMMARY

C. albicans is not killed or inhibited by human serum. Microscopic observations revealed that all synchronous blastospores inoculated into serum grew as hyphae. The growth rate of the hyphae in serum from a diabetic patient and in normal serum diluted 50% with Wickerham Broth was enhanced due to the additional nutrients.

Hyphal growth did not occur in serum incubated at 3° or 23°, nor in serum incubated anaerobically at 37°.

Dilution of serum with water decreased the growth rate of the hyphae, but the serum had to be diluted to 6% before the % of blastospores producing hyphae dropped from 100%.

Hyphae were produced by solutions of γ globulin, but not by albumin, extracted from human plasma. Hyphal growth was reduced by boiling the serum for 30 minutes and then digesting it overnight with pancreatin.

After 2 days incubation, the dry weight of blastospores from Sabouraud Broth was more than 3 times the dry weight of mycelium grown in serum under identical conditions. The blastospores contained a higher percentage of protein,

carbohydrate, RNA and DNA, but much less fat than the mycelium. Serum is clearly a much less satisfactory growth substrate for C. albicans than Sabouraud Broth.

S E C T I O N 6

THE PHAGOCYTOSIS OF C. ALBICANS BY PMN

INTRODUCTION

Visual observations on the phagocytosis of C. albicans by human PMN in vitro were described by Wang and Schwarz (1959). In their study, a loopful of buffy-coat and a loopful of C. albicans were placed beneath a cover glass on a microscope slide, and they observed that ingestion occurred rapidly at room temperature. Louria and Brayton (1964b), however, reported that ingested Candida cells formed hyphae and grew out of PMN after 4 hours incubation at 37°. Various authors, including Hill and Gebhardt (1956) and Young (1958) have argued that filamentation in vivo may constitute a significant hinderance to phagocytosis and this may partially explain the failure of body defences to cope with the invading Candida.

This present study was undertaken to observe and describe the phagocytosis of C. albicans by human PMN in plasma.

METHODS

Preparation of PMN

50 i.u. heparin in 0.1 ml Ringer-Locke solution was placed in a 25 ml. screw-capped plastic container and 3 ml. liquid

paraffin added. 10 ml. blood was withdrawn from the antecubital vein and gently injected beneath the liquid paraffin in the container. The liquid paraffin acted as a gaseous seal. The blood was then allowed to stand for one hour at room temperature.

One drop of the buffy coat was then removed with a siliconized glass tube and placed on a glass microscope slide. A drop of liquid paraffin was placed over this and the slide incubated at 37° for 20 minutes in a damp chamber.

Preparation of Candida inoculum

The standard inoculum was of twice-washed blastospores of C. albicans strain WF 668A from an overnight shaken Sabouraud Broth culture, resuspended in Ringer-Locke to give the required Optical Density in a Hilger "Spekker" Absorptiometer.

Opsonised blastospores were prepared by shaking the blastospores in the plasma from the blood being studied for 20 minutes at 37°.

Blastospores bearing hyphae were prepared by incubating a population of synchronous blastospores, prepared by the linear sucrose density gradient technique, in the plasma of the blood being studied for 1 hr. or 5 hr. at 37° without shaking, and hyphae of mean lengths 12 μ and 70 μ were thus produced.

C. albicans spheroplasts were produced from blastospores using snail gut enzyme (p. 25). The spheroplasts were then resuspended in Ringer-Locke solution.

The experimental system

Erythrocytes and platelets were washed from the slide with the plasma of the blood being studied at 37°, so that the PMN were left adhering to the glass surface. One drop of C. albicans strain WF 668A in Ringer-Locke solution, previously warmed to 37°, was added to the preparation. A drop of liquid paraffin was then added and a 22 x 50 mm coverslip applied to the preparation which was then viewed under phase contrast using green light. The microscope was housed in a thermostatically-controlled 37° cabinet.

OBSERVATIONS

The PMN were initially rounded up but soon showed active streaming of intracellular granules, followed by whole cell amoeboid movement over the surface of the slide.

The mobile PMN crawled purposefully towards *Candida* blastospores and, after making physical contact, flowed around the yeast, engulfing it. Occasionally, egestion of the *Candida* appeared to occur, although it was never certain that ingestion had really occurred in these cases.

After engulfing a yeast cell, the PMN rested for a short time on the glass surface, then resumed crawling and the yeast was dragged along in the tail region of the PMN.

Following phagocytosis, the PMN continued to engulf further Candida cells. Up to eight blastospores could be engulfed successfully. A local excess of yeasts resulted in the PMN attempting to phagocytose yeasts beyond their capacity, resulting in rupture of the PMN cell membrane and release of previously phagocytosed yeasts. Released yeasts were later re-phagocytosed by other PMN.

The preparation of PMN remained mobile for several hours under the conditions described. Preparations to which Candida was not added showed a longer period of active crawling than in preparations with Candida present.

Towards the end of the period of motility the PMN tended to come to rest in groups of 3-20 cells around rafts of erythrocytes remaining in the preparation. The density of granulation of the PMN cytoplasm was now noticeably less than before phagocytosis. The Candida blastospore showed increasing vacuolization but no damage to the cell wall could be detected. Under an interference microscope the yeast showed a green colouration immediately after phagocytosis, which became bright blue after 10 hours in the phagosome.

Occasionally, Candida blastospores in the phagosome

developed a bud or hypha, and this hypha appeared to grow out of the polymorph in three cells out of the thousands examined at 37° during the 24 hour periods of these observations.

Preparations incubated for 24 hours at 37° showed PMN containing Candida blastospores which were still clearly visible. Some PMN showed no signs of having engulfed Candida. A number of Candida blastospores were seen outside PMN.

Opsonised blastospores

The phagocytosis of opsonised Candida showed no detectable difference from that of non-opsonised Candida.

Blastospores bearing hyphae

The blastospores bearing hyphae of mean length 12 μ were rapidly engulfed by the PMN and hyphal growth out of the PMN was not observed.

Masses of PMN migrated to surround the blastospores with 70 μ hyphae and remained tightly in contact. Hyphal growth then appeared to cease.

Spheroplasts

Spheroplasts appeared to be phagocytosed in the same manner as blastospores. After 24 hrs. incubation the phagocytosed spheroplasts showed obvious destruction within the PMN.

DISCUSSION

Normal blood contains 5,000-9,000 leucocytes per cubic millimetre, of which 56% are PMN. The PMN are primarily responsible for the phagocytosis of invading microbes. More than half of the PMN occur as a reservoir, the marginate granulocyte pool, and the circulating PMN have an intravascular life of approximately 9 hours (cited Guyton, 1961).

In this study, it was observed that PMN in plasma crawled over a glass surface, apparently under a chemotactic stimulus, towards *Candida* cells which were ingested on contact. PMN were seen to ingest up to eight blastospores, pausing for a few minutes after each cell was ingested before resuming motility. PMN often brought about their own destruction by attempting to ingest too many blastospores. The presence of *Candida* in the PMN preparation shortened the period of PMN motility on a glass surface.

Visual evidence of the destruction of *Candida* inside the PMN phagosomes was obtained and the yeast was only rarely seen to bud or produce hyphae inside the PMN. This observation is at variance with those of Louria and Brayton (1964b), and Stanley and Hurley (1969) who studied the phagocytosis of *C. albicans* in human blood and murine macrophages respectively.

Following engulfment, *Candida* was sometimes spontaneously egested by the PMN, a similar phenomenon with bacteria was noted

by Wilson (1953).

Not all of the PMN attempted to phagocytose *Candida* and some *Candida* cells escaped phagocytosis. It is possible that only a part of the PMN population are capable of phagocytosing *Candida*.

The settling down of PMN following phagocytosis suggests the direction of metabolic energy towards the destruction of the *Candida*. The degranulation which accompanies this is indicative of the transference of lysosomal enzymes into the phagosome (Hirsch and Cohn, 1960). The fusion of the lysosomal membranes with the membranes of the phagosome and the subsequent expulsion of contents into the phagosome has been demonstrated with the electron microscope (Zucker-Franklin and Hirsch, 1964).

The final clumping of PMN may increase the chances of rephagocytosis of any egested *Candida*.

Opsonisation of *Candida* with homologous plasma before inoculation into the PMN preparation was not observed to enhance phagocytosis in this system. It would seem therefore that opsonising antibody is less important than a supply of complement which plays a role in chemotaxis of PMN (Section 8).

The phagocytosis of *Candida* with short hyphae appeared to be as effective as the phagocytosis of blastospores. The PMN dealt with hyphae too long to be successfully engulfed by an individual, by aggregating around the hypha and killing it.

Lysosomal enzymes which pass out into the extracellular medium from PMN during phagocytosis have been demonstrated by Baehner, Karnovsky and Karnovsky (1969) and the extracellular release of histamine, muramidase and cathepsins has been shown by Crowder, Martin and White (1969). It is therefore likely that the longer hyphal elements are killed by extracellular digestion by the PMN aggregate.

Spheroplasts were phagocytosed by PMN, the absence of the cell wall appeared to result in more rapid intracellular digestion than in the case of whole blastospores.

SUMMARY

The phagocytosis of C. albicans by human PMN in plasma was observed by phase contrast microscopy. Purposeful movement of the PMN towards Candida suggested chemotaxis. The PMN engulfed the Candida when physical contact was made, but a few blastospores were seen to be spontaneously egested.

The PMN rested shortly after each ingestion, each phagocytic cell could ingest up to 8 blastospores successfully, but some ingested more and then ruptured.

The PMN later came to rest in groups and the intracellular digestion of the Candida was observed. Hyphal outgrowth reported by other workers was observed only very rarely in this system. PMN phagocytosed Candida with hyphae of 12 μ mean

length in the same manner as blastospores. PMN formed aggregates around Candida with hyphae of 70 μ mean length and appeared to kill them by extracellular digestion.

SECTION 7

THE ROLE OF PLASMA AND COMPLEMENT IN THE CANDIDACIDAL ACTION OF PMN

INTRODUCTION

Following the description of the importance of phagocytosis in defence against infection by Metchnikoff (1892), there was much discussion concerning the role of serum in relation to particle ingestion by leucocytes (Weir, 1970).

Wang and Schwarz (1959) observed phagocytosis of C. albicans by human PMN in vitro but did not investigate whether or not the Candida cells were killed.

Brogan (1964) reported that the spores of Aspergillus fumigatus were phagocytosed by human PMN in the virtual absence of serum.

Louria and Brayton (1964b) inoculated 10^7 Candida into 2 ml. samples of blood and observed rapid phagocytosis. After 4 hours of incubation, and staining with trypan blue, they observed that 2-10% of the PMN were not viable and Candida hyphae were growing out of the PMN. They concluded that PMN did not kill Candida.

Chilgren, Oh, Hong and Quie (1969), who used a vital staining technique, found that PMN from normal subjects in normal

serum killed 40-55% C. albicans, but in serum from patients with chronic mucocutaneous candidosis the PMN killed only 10-20% C. albicans. They postulated that the serum of such patients contained an anti-clumping factor for Candida and an inhibitor of PMN phagocytic killing.

Stanley and Hurley (1969) showed that although C. albicans was rapidly phagocytosed by macrophages from the mouse peritoneum the hyphae grew out and killed the cell by rupturing the macrophage membrane. They concluded that the reticulo-endothelial system did not protect the host in candidosis but aided the dissemination of the yeast by acting as vectors.

Lehrer and Cline (1969a), by means of a differential staining technique, reported that in 38 studies, human PMN killed $29.0\% \pm 7.4\%$ of the ingested C. albicans in 1 hour.

The following experiments were undertaken to investigate the role of plasma and the complement system in the killing of Candida by PMN.

METHODS

Preparation of PMN from blood

A plastic syringe was used to collect 20 ml. blood from the antecubital vein and immediately injected beneath 3 ml. sterile liquid paraffin in a 25 ml. screw-capped plastic tube containing 150 i.u. heparin in 0.1 ml. Ringer-Locke solution.

DEXTRAN SEDIMENTATION TECHNIQUE (modified from Skoog and Beck, 1956).

The blood was centrifuged and some of the plasma removed from the top and stored at 3° for later use.

5 ml. of 6% w/v dextran solution in 0.9% sodium chloride ("Dextraven 110") was added to the blood and gently mixed and the PMN allowed to settle at 23° for 1½ hr. The supernatant was removed, centrifuged and the residual PMN washed twice and resuspended in Ringer-Locke solution.

Experimental method

1 ml. aliquots of the PMN suspensions were introduced into clean, dry, detergent-free glass universal containers with a little liquid paraffin.

0.1 ml. of a decimal dilution of a synchronous culture of C. albicans blastospores of determined Optical Density was added to each sample. The containers were then incubated for 5 hr. at 37°.

Viable count

Following incubation, 9 ml. water was added to each container and most of the liquid paraffin removed. Each sample was subjected to ultrasonic vibration for 30 seconds at a 5 µ peak-to-peak amplitude in an MSE Ultrasonic Disintegrator.

This treatment disrupted the PMN and released the Candida as single cells. The whole sample was then pour-plated in a 6-inch diameter petri-dish with 50 ml. Sabouraud medium with double-strength agar. Colony counts were recorded after incubation for 48 hours.

Inactivation of complement and the
phagocytosis of *C. albicans* by PMN

Substances prepared for inactivating complement
Whole Candida cells

C. albicans strain 207/53A was grown on Sabouraud dextrose agar for 4 days at 37°. The surface growth was removed and the suspension heated at 60° for ½ hour to kill the cells. The dead cells were then freeze-dried.

Zymosan

Zymosan (Koch-Light Laboratories Ltd.) was boiled for 30 minutes and then washed several times with sterile distilled water as recommended by Mollison (1967).

Cell wall and cell sap preparations

C. albicans grown and prepared as above was subjected to disintegration in a Braun Homogenizer with Ballotini No. 14 glass beads for four runs each of one minute length. The suspension

was then passed through a sintered glass filter to remove the glass beads, and then centrifuged at 15,000 r.p.m. for 30 minutes. The supernatant, i.e. cell sap enriched preparation, was withdrawn from the residue, i.e. cell wall enriched preparation and the two fractions freeze-dried.

Treatment of plasma

Known weights of the above preparations were added to 1 ml volumes of plasma and placed on a rotary shaker for 30 minutes at 37°. The suspension was then centrifuged at 1,500 r.p.m. and the plasma withdrawn.

Phagocytosis of Candida in the absence of plasma

Preparations of PMN which had been washed twice in Ringer-Locke solution were resuspended at a concentration of 1×10^7 per ml. in:-

- (a) Homologous plasma.
- (b) TCl99 buffered with 4.4% sodium bicarbonate but without added plasma.
- (c) Ringer-Locke solution without added plasma.

RESULTS

The results are shown in Table 14.

Table 14. Phagocytosis of C. albicans by PMN in plasma, TC199 and Ringer-Locke Solution.

MEDIUM IN WHICH PMN SUSPENDED	INOCULUM						NUMBERS SURVIVING						
	PLATE COUNTS					MEAN	PLATE COUNTS					TOTAL	% SURVIVAL
	1	2	3	4	5		1	2	3	4	5		
PLASMA	12	16	19	22	25	18.8	0	1	2	2	3	8	8.5
	26	28	29	35	42	32.0	1	2	3	6	7	19	11.9
	38	40	44	45	53	44.0	0	2	3	3	7	15	6.8
TC 199	12	16	19	22	25	18.8	5	9	24	24	27	89	94.7
	26	28	29	35	42	32.0	22	24	28	31	33	138	86.3
	38	40	44	45	53	44.0	32	36	36	42	51	197	89.5
RINGER-LOCKE	12	16	19	22	25	18.8	3	14	17	17	19	70	74.5
	26	28	29	35	42	32.0	16	18	21	22	28	105	65.6
	38	40	44	45	53	44.0	27	29	32	35	36	159	72.3

Effect of complement and its inactivation in respect to the candidacidal activity of PMN

PMN which had been washed twice in Ringer-Locke solution were resuspended at a concentration of 1×10^7 per ml. in plasma which had been treated as follows:

Preparation

1	Control plasma
2	Plasma heated 56° for 30 minutes.
3	Plasma treated with 50 mg/ml. whole dead <i>Candida</i> cells.
4	Plasma treated with 3 mg/ml. zymosan.
5	Plasma treated with cell wall enriched preparation.
6	Plasma treated with cell sap enriched preparation.

The results are given in Table 15.

DISCUSSION

In recent years, there have been conflicting reports on the action of phagocytic leucocytes against *C. albicans*. Chilgren, Oh, Hong and Quie (1969) and Lehrer and Cline (1969a) reported that *C. albicans* was killed by PMN in serum. Louria and Brayton (1964b) observed hyphal outgrowth from PMN and Stanley and Hurley (1969) also report a similar observation with macrophages, concluding that phagocytic cells of the

Table 15. Complement and the phagocytosis of C. albicans by PMN.

INOCULUM							SURVIVORS							
PLATE COUNTS					MEAN	NO. OF PLATES	PREPARATION	PLATE COUNTS					TOTAL CELLS SURVIVED	% SURVIVAL
1	2	3	4	5				1	2	3	4	5		
3	4	4	6	9	5.2	5	1	0	0	0	0	3	3	11.6
							2	2	3	3	3	4	15	57.7
							3	0	1	4	4	4	13	50.0
							4	3	3	3	4	5	18	69.2
							5	1	1	2	6	8	18	69.2
							6	0	0	0	0	4	4	15.4
15	19	24	26	31	23.0	5	1	0	0	0	0	3	3	2.6
							2	8	11	14	16	19	68	59.1
							3	4	6	12	17	18	56	48.7
							4	9	13	13	14	16	65	56.5
							5	5	9	15	15	21	65	56.5
							6	0	1	2	2	5	10	8.7
46	49	53	57	61	53.2	5	1	0	0	0	8	11	19	7.1
							2	21	24	29	29	36	139	52.3
							3	25	28	31	33	33	150	56.4
							4	22	26	29	30	34	141	53.0
							5	31	32	32	37	42	174	65.4
							6	4	7	9	11	11	42	15.8
81	93	97	98	101	94.0	5	1	7	9	21	24	34	95	20.2
							2	58	61	64	65	69	317	67.4
							3	31	36	40	40	47	194	41.3
							4	29	38	44	49	56	216	46.0
							5	32	35	38	46	68	219	46.6
							6	15	17	18	26	27	102	21.7

reticulo-endothelial system actually promoted infection by acting as vectors in the dissemination of *Candida*.

The present investigation confirms the observations of Chilgren et al. (1969) and Lehrer and Cline (1969a) that *C. albicans* is killed by PMN suspended in their own plasma.

Using *C. albicans* inocula of similar-sized synchronous blastospores, it was demonstrated that there was a low percentage of survival when the PMN were suspended in plasma. A high percentage survival was observed when the PMN were suspended in buffered TC199 or Ringer-Locke solution and no plasma added (Table 14). It has been demonstrated that plasma is necessary for the chemotaxis of PMN towards *Candida* (Section 8). Brogan (1966) found that human PMN could engulf rice particles in the absence of serum, so it seems unlikely that the loss of candidacidal activity in the absence of plasma is due to impaired engulfment. In the absence of plasma some blastospores were killed. Moreover, it has been shown (Table 12) that plasma or serum per se is not lethal to *C. albicans*. It must be concluded that the greater survival of *C. albicans* in a suspension of PMN without plasma is related to an absence of chemotaxis.

Candida survival values were highest with plasma heated 56° for $\frac{1}{2}$ hour (Table 15), a treatment which inactivates C'1 and C'2 and damages C'4 (Mollison, 1967). PMN in plasma

treated with zymosan showed a similar decrease in candidacidal activity. Zymosan has little effect on C'1, C'4 and C'2 but inactivates the 'later' components of complement (McNall, 1957). Whole dead Candida cells and cell wall enriched preparations had a similar effect and the fact that yeast cells inactivate complement was first described by Dungern (1900), and Pillemer and Ecker (1941) reported that fresh yeast cells inactivated C'3. Since zymosan is a complex of insoluble polysaccharides from the cell wall of Saccharomyces cerevisiae, treatments 4 and 5 (Table 15) are basically the same.

Treatment of the plasma with a C. albicans cell sap enriched fraction led to an only slightly increased survival rate. This fraction exerts very little effect on the complement system.

In these experiments the PMN received chemical and physical trauma as a consequence of which their ability to kill Candida may have been impaired. Killing in PMN suspended in TC199 and Ringer-Locke solution may have been facilitated by a small amount of plasma remaining on the PMN after washing.

Approximately 20% of the Candida inoculum survived the candidacidal activity of the PMN in plasma. In vivo the constant supply of fresh PMN and plasma probably results in a total kill.

In conclusion, these experiments demonstrate that the

killing of C. albicans by PMN is enhanced by the presence of plasma, and this appears to be due to the role of complement in the positive chemotaxis of PMN towards the Candida cells. This is the subject of further investigation in Section 8.

SUMMARY

Human PMN kills C. albicans in the presence of plasma, and a greater percentage survival occurs in the absence of plasma. Inactivation of the plasma complement by heating, treating with whole dead Candida cells, zymosan or a cell wall enriched preparation greatly increased the percentage survival. It was concluded that plasma enhances the phagocytic killing of C. albicans by PMN because of the chemotactic activity of complement.

S E C T I O N 8

CANDIDA ALBICANS AND THE CHEMOTAXIS
OF POLYMORPHONUCLEAR LEUCOCYTES (PMN)

INTRODUCTION

Pfeffer (1884), first used the term chemotaxis to describe the directional movements of cells in response to the presence of chemical substances. Positive chemotaxis is the movement of a cell towards the stimulating substance. It has long been held that chemotaxis is an integral mechanism in the accumulation of leucocytes in the inflammatory process (Leber, 1888).

Although the chemotaxis of PMN towards bacteria has been studied by a number of workers, including McCutcheon (1946), Harris (1954), Keller and Sorkin (1965, 1967), stimulation by C. albicans does not appear to have been studied. Keller and Sorkin (1968), in a review article, describe chemotaxis as a recognition mechanism for motile cells which is linked to their migratory activity.

In 1962, Boyden developed an efficient in vitro technique for the demonstration of leucocyte chemotaxis. The "Boyden Chamber" consists of two compartments separated by a filter membrane which allows leucocytes to move through from the upper to the lower chamber.

In the present study, having observed chemotaxis towards *Candida* (p. 83) it was decided to investigate the phenomenon in greater detail. A modified Boyden Chamber was used in a series of experiments on the chemotactic stimulation of PMN by *C. albicans* to investigate the antigens concerned, and to determine whether the antigenic stimulation of PMN by blastospores differed from that of mycelium.

METHODS

The modified Boyden Chamber

The Boyden Chambers used in this study were made from Perspex in our own laboratories. The design followed a simplification by Hurley (1963) of the original chamber described by Boyden (1962) and is shown in Fig. 14.

The chemotaxis of PMN towards a microbe is investigated by filling the lower chamber with an appropriate suspension of the microbe and the upper chamber with a suspension of PMN. The PMN settle on to the upper surface of the membrane and in response to a chemotactic stimulus migrate through the pores of the membrane. After an appropriate time interval the membrane is removed and after fixation, staining and mounting the number of PMN which migrated through a known area of the membrane is counted under the microscope.

THE BOYDEN CUP

Fig. 14. The modified Boyden Chamber.

Preparation of PMN

For each experiment, 10 ml. blood was withdrawn from the antecubital vein and immediately injected beneath 3 ml. sterile liquid paraffin in a 25 ml. screw-capped plastic tube containing 100 i.u. of heparin in 0.1 ml. of Ringer-Locke solution. The blood was gently centrifuged and 2 ml. plasma withdrawn.

5 ml. of a 6% w/v dextran solution in 0.9% sodium chloride (Dextraven 110, Fisons Pharmaceuticals Ltd.) was then added, gently mixed and the leucocytes allowed to sediment at room temperature (23°) for 1½ hours. The PMN enriched supernatant with a few platelets and erythrocytes was removed with a wide-bore siliconized pipette, placed under liquid paraffin in a similar container and centrifuged at 500 rpm for 20 minutes. The dextran solution was then withdrawn with a pipette and the residual PMN resuspended in Ringer-Locke solution. Decimal dilutions of this suspension were prepared, treated with saponin to disrupt erythrocytes, and the numbers of PMN counted using a Coulter Counter. The suspension was then diluted to an appropriate volume so that, after the addition of 10% v./v. plasma, the resulting concentration of PMN was 2×10^6 per ml.

Preparation of the Boyden Chamber

Prior to use, the chambers were cleaned by washing in 3%

lysol, rinsing well and air-drying at 37°.

Approximately 0.5 ml. of the microbe suspension in Ringer-Locke solution plus 10% plasma was added to the lower chamber of the apparatus using a Pasteur pipette, care being taken to ensure that the meniscus was level with the shoulder.

The membrane filters employed were Millipore SSWPO 1300 having a pore size of 3 μ m (Millipore Filter Corp., Bedford, Mass., U.S.A.). The membrane was lowered vertically to the shoulder using Millipore forceps, care being taken to avoid trapping air bubbles. The lower surface of the membrane should be fully in contact with the meniscus and there must be no contamination of its upper surface.

The outside of the upper chamber was smeared with silicone grease to make an air-tight joint and fitted into place.

0.5 ml. of a PMN suspension containing 10^6 cells was then placed in the upper chamber and a drop of sterile liquid paraffin added as a seal. The chambers were then incubated at 37° for 3 hours.

Following incubation, the upper section of each apparatus was removed and the membrane removed with a pin and stuck on to a cork. The staining procedure was as follows:-

Fixing and Staining of PMN on Millipore Membrane

1. 5 secs. Saline
2. 5 secs. 100% Methanol

3. 2 secs. Distilled water
4. 10 mins. Ehrlich's Haematoxylin
5. 2 secs. Distilled water
6. 1 min. 1% Acid (HCl) Alcohol
7. 2 secs. Distilled water
8. 2 mins. Blueing solution (10 g. MgSO_4 and 1 g. NaHCO_3 in 500 ml. distilled water)
9. 2 secs. Distilled water
10. 1 min. 70% alcohol
11. 1 min. 95% alcohol
12. 1 min. 100% alcohol
13. 1 min. 100% alcohol
14. 30 mins. Xylene
15. Mount on microscope slide in Dammar Xylene.

Preparation of microbial suspensions

Viable microbial suspensions were from shaken cultures incubated at 37° unless otherwise stated. Blastospores of C. albicans were from overnight cultures in Sabouraud Broth, but mycelium was from unshaken culture grown for 3 days at 37° in human plasma from time-expired blood, or the medium B of Chattaway (Chattaway, Holmes and Barlow, 1968). Bacteria were from overnight culture in glucose nutrient broth. Torulopsis glabrata was grown in Sabouraud Broth.

All microbial suspensions were washed twice in water, adjusted to an Optical Density of 0.30 in Ringer-Locke solution and from this decimal dilutions were prepared for use in the Boyden Chambers.

Preparation of antigens

In addition to an investigation of the antigenic stimulus of viable cells, cells which were killed by boiling for 30 minutes were also used.

Antigen fractions from C. albicans blastospores were prepared from cultures grown on Sabouraud agar. Colonies were scraped off the agar and suspensions prepared without washing.

Candida cell sap.

A protein-enriched antigen was prepared from cells ruptured by freezing and thawing followed by ultrasonic treatment for 5 minutes at a 5 μ peak-to-peak amplitude. Following this treatment no intact cells were detected by microscopic examination and no growth occurred when samples were cultured.

The suspension was centrifuged at 800 g. for 30 minutes. The supernatant was removed and used as the cell sap antigen.

The chemotactic stimulus of this fraction was compared with that of the residue after centrifugation, i.e. the cell wall enriched fraction.

Candida polysaccharide antigen

A polysaccharide-enriched fraction was prepared from freeze-dried C. albicans strain 207/53A made up to 10 mg/ml. in Ringer-Locke solution. The suspension was boiled for 30 minutes, cooled, reboiled for a further 30 minutes and dialysed against saline for 16 hours at 3°. A similar suspension of freeze-dried Candida which was simply dialysed at 3° to remove small molecules was used as a control.

Cell wall antigen

This was prepared by a tryptic digest of boiled blastospores and mycelium which were ruptured by ultrasonic vibration as described. Following digestion, suspensions were dialysed overnight and then made up to an Optical Density of 0.30 in Ringer-Locke solution as in previous experiments.

Candida mannan antigen

C. albicans from Sabouraud agar was suspended in phosphate buffer pH7 and autoclaved for 2 hours at 10 lbs. per square inch as recommended by Peat, Whelan and Edwards (1961). This technique utilizes the fact that of the polysaccharides of the cell wall, the mannan is soluble and the glucan insoluble. Examination of the supernatant in a Unicam SP 8005 Spectrophotometer showed not only a peak for polysaccharides at

200-225 nm as was expected, but a secondary peak at 255-265 nm indicative of nucleic acid.

1 mg. DNase was added to the supernatant after centrifugation and incubated on a shaker for 12 hr. at 37° and the suspension then dialysed against 3 changes of distilled water for 48 hr. at 3°. The absence of nucleic acids and protein was then verified with the Spectrophotometer.

The solution was freeze-dried. For use, the material was dissolved in Ringer-Locke solution at the rate of 1 mg/ml. and decimal dilutions prepared.

Candida blastospore control

A 1/100 dilution of an Optical Density 0.30 suspension of C. albicans strain 207/53A in Ringer-Locke solution was used as a control inoculum in most of the experiments to ensure that the activity of the PMN was normal.

Chemotactic stimulation of PMN by C. albicans

To investigate the migration of PMN towards C. albicans, blastospore and mycelial suspensions were prepared and used in the Boyden Chambers as described. Decimal dilutions of an Optical Density 0.30 suspension were made to determine the numbers of blastospores or mycelial fragments necessary to produce a PMN migration of approximately 100 cells per

microscope field. A viable count was made for each blastospore suspension but not for mycelium since the numbers of colonies which developed would be unrelated to the mass of mycelium. The results are given in Tables 16 and 17.

Differences in the numbers of PMN migrating towards blastospores of the Hasenclever serotypes A and B were examined with the Student's t test and found to be insignificant. With inocula of 1/10 (7×10^5), 1/100 (7×10^4) and 1/1000 (7×10^3) values for t were 1.466, 2.643 and 2.384, and $P > 0.4 < 0.2$, $> 0.1 < 0.05$ and $> 0.1 < 0.5$ respectively.

Relative stimulation of PMN by blastospores and mycelium

A comparison of the numbers of PMN migrating towards blastospores with those migrating towards mycelial fragments was undertaken. It may be seen (Tables 16 and 17) that with 1/10 and 1/100 dilutions of Optical Density 0.30 suspensions, the mycelium attracted only half as many PMN. Examination of this data with the Student t test showed these results to be highly significant, t being 11.663, 12.547 and 6.279, and $P < 0.001$ in every case.

Preparation of Candida mycelium by growth in human plasma is open to the criticism that plasma antigen or antibody may be bound to the surface of the mycelium. In Torulopsis glabrata, unlike C. albicans, growth in human plasma is by

Table 16. Numbers of PMN migrated towards blastospores of C. albicans.

BLASTOSPORE INOCULUM		NUMBER OF PMN MIGRATED					
VIABLE COUNT COLONIES/ML.	DILUTION OF OD 0.30 SUSPENSION	NUMBERS COUNTED AS MIGRATED IN 5 FIELDS OF THE MICROSCOPE					MEAN MIGRATION
		1	2	3	4	5	
<u>A. STRAIN 207/53A</u>							
720,000	$\frac{1}{10}$	120	130	137	142	156	137.0
72,000	$\frac{1}{100}$	96	107	111	128	132	114.8
7,200	$\frac{1}{1,000}$	41	42	44	53	63	48.6
720	$\frac{1}{10,000}$	8	9	14	16	17	12.8
72	$\frac{1}{100,000}$	0	0	1	3	3	1.4
<u>B. STRAIN 792/53B</u>							
760,000	$\frac{1}{10}$	110	115	128	135	142	126.0
76,000	$\frac{1}{100}$	89	93	102	104	112	100.0
7,600	$\frac{1}{1,000}$	29	30	36	40	42	35.4
760	$\frac{1}{10,000}$	4	4	5	8	9	6.0
76	$\frac{1}{100,000}$	0	0	0	0	0	0

Table 17. Numbers of PMN migrated towards mycelium of C. albicans strain 207/53A.

INOCULUM DILUTION OF O.D. = 0.30 MYCELIAL SUSPENSION *	NUMBER OF PMN MIGRATED					
	NUMBERS COUNTED AS MIGRATED IN 5 FIELDS OF THE MICROSCOPE					MEAN MIGRATION
0	82	83	107	124	146	108.4
$\frac{1}{10}$	62	66	70	74	78	70.0
$\frac{1}{100}$	34	38	41	42	45	40.0
$\frac{1}{1,000}$	10	11	12	13	24	14.0
$\frac{1}{10,000}$	5	5	6	8	12	7.2
$\frac{1}{100,000}$	2	3	4	4	8	4.2

* Mycelial suspension from C. albicans grown
in plasma.

blastospore production alone. T. glabrata was therefore used to test the possibility that plasma antibody might be bound to the yeast cells and thereby affect PMN stimulation.

In addition to inoculating Boyden Chambers with blastospores of T. glabrata grown in plasma or in broth, broth-grown blastospores of C. albicans were used as a control to ensure standardization of the technique. The results are given in Table 18, which shows similar numbers of PMN migrated when the blastospores of T. glabrata had been grown in either broth or plasma.

In the control (Table 18) the numbers of PMN migrating towards C. albicans were similar to those in the previous test (Table 16).

Smaller numbers of PMN migrated towards blastospores of T. glabrata than towards a suspension of similarly grown C. albicans with the same Optical Density. In this experiment the PMN were all from the same subject and the difference in chemotactic stimulus was statistically significant, $t = 4.89$ and $P < 0.001$. Since the numbers of PMN migrating towards C. albicans in this experiment were similar to those in Table 16, the numbers of PMN which migrated towards T. glabrata (Table 18) were compared with those that migrated to blastospores of C. albicans strains 207/53A and 792/53B.

The greater numbers of PMN migrating towards C. albicans

Table 18. Comparative numbers of PMN migrating towards blastospores of T. glabrata grown in human plasma and Sabouraud Broth respectively.

INOCULUM O.D. 0.30 SUSPENSION DILUTED $\frac{1}{100}$	NUMBERS OF PMN MIGRATING					
	NUMBERS COUNTED AS MIGRATED IN 5 FIELDS OF THE MICROSCOPE					MEAN MIGRATION
	1	2	3	4	5	
BLASTOSPORES GROWN IN BROTH	72	74	79	81	87	71.6
	57	63	76	86	94	
	52	53	59	62	73	
BLASTOSPORES GROWN IN PLASMA	59	61	64	72	74	71.1
	63	65	68	96	104	
	59	62	65	71	83	
<u>CONTROL</u> <u>C. ALBICANS:</u> BLASTOSPORES GROWN IN BROTH	86	102	104	109	112	102.6

compared to T. glabrata observed by comparison of Table 16 with Table 18 was statistically significant. In unpaired t tests of T. glabrata against C. albicans serotypes A and B, t was 6.261 and 4.546 respectively and $P < 0.001$ in both cases.

The greater ability of C. albicans to stimulate chemotaxis of PMN may be related to the fact that since C. albicans is a much more frequently carried commensal than T. glabrata, the PMN may have greater sensitivity due to a better developed immunological memory for the Candida.

Growth of the blastospores of T. glabrata in broth or plasma had no statistically significant effect on the numbers of PMN migrating, t being 0.116 and $P > 0.500$. It may therefore be concluded that any plasma antibody or antigen bound to the surface of the blastospores grown in plasma had no effect upon the migration of PMN. Moreover, it would seem likely that any plasma factors bound to the mycelium of C. albicans would be similarly ineffective.

To investigate this further C. albicans mycelium was prepared in the medium B of Chattaway et al. (1968) and incubated without shaking for 3 days at 30° . The yeast grew as a film of mycelium at the surface of the broth and was relatively uncontaminated with blastospores. Although free from any plasma antigen or antibody, the growth was washed twice and freeze-dried and then used as a mycelial antigen in a Boyden Chamber

experiment. The results are given in Table 19. Again the standard C. albicans blastospores suspension used as a control to ensure the PMN behaved in a normal manner.

Fewer PMN migrated towards broth-grown mycelium than towards an equal weight of broth-grown blastospores (see Table 19). At concentrations of 1 mg., 100 µg., 10 µg., 1 µg. and 0.1 µg. per ml. t was 5.766, 12.748, 11.053, 4.787 and 4.750 and P was $<0.005>0.001$, <0.001 , <0.001 , $<0.010>0.005$ and $<0.010>0.005$ respectively. Again using PMN from the same subject, the chemostatic stimulus of blastospores was greater than that shown by mycelium.

Variation in PMN from different subjects with respect to Candida induced chemotaxis

Having found that a standard suspension of C. albicans blastospores (1/100 dilution of O.D. 0.30) produced a mean migration of approximately 100 PMN, variation in the PMN from different healthy subjects and patients with diabetes were investigated. The standard blastospore suspensions used gave viable counts of 7×10^4 and the results are shown in Tables 20 and 21.

The mean numbers of PMN from different normal subjects' blood varies in their migration from 71.2 to 144.5 (mean = 99.6 S.D. = 22.6) and those from the 2 diabetics fell within this range. A larger number of blood samples from patients with

Table 19. Comparative numbers of PMN migrating towards mycelium grown in medium B (Chattaway *et al.*, 1968) in the absence of plasma, and blastospores grown in Sabouraud Broth respectively.

INOCULUM		NUMBER OF PMN MIGRATED					
MATERIAL	WEIGHT OF MATERIAL/ML.	NUMBERS COUNTED AS MIGRATED IN 5 FIELDS OF THE MICROSCOPE					MEAN MIGRATION
		1	2	3	4	5	
MYCELIUM	1 mg	31	32	33	34	38	33.6
	100 µg	19	23	24	24	27	23.4
	10 µg	15	16	17	19	19	17.2
	1 µg	8	9	10	11	16	10.8
	0.1 µg	3	4	5	6	7	5.0
BLASTOSPORES	1 mg	43	46	49	52	58	49.8
	100 µg	35	35	41	41	42	38.8
	10 µg	26	28	29	30	32	29.0
	1 µg	19	20	20	21	25	21.0
	0.1 µg	11	15	16	18	22	16.4
CONTROL Blastospores O.D. 0.30 diluted $\frac{1}{100}$		84	90	92	102	110	95.6

Table 20. Numbers of PMN from different blood samples which migrated towards blastospores of C. albicans strain 207/53A.

PATIENT	NUMBER OF PMN MIGRATED					MEAN MIGRATION
	NUMBERS COUNTED AS MIGRATED IN 5 FIELDS OF THE MICROSCOPE					
	1	2	3	4	5	
<u>(1) PMN FROM NORMAL SUBJECTS</u>						
1	42	76	85	95	108	89.1
	68	94	105	112	115	
	59	73	88	107	110	
2	76	79	82	85	98	94.6
	78	99	105	114	126	
	82	93	95	100	107	
3	99	105	110	122	137	112.3
	98	107	115	118	126	
	92	104	112	115	125	
<u>(2) PMN FROM BLOOD OF DIABETIC PATIENT</u>						
4	79	85	112	127	151	97.5
	68	75	85	132	150	
	65	76	78	86	93	

Table 21. Numbers of PMN from different blood samples which migrated towards blastospores of C. albicans strain 792/53B.

PATIENT	NUMBER OF PMN MIGRATED					
	NUMBERS COUNTED AS MIGRATED IN 5 FIELDS OF THE MICROSCOPE					MEAN MIGRATION
	1	2	3	4	5	
<u>(1) PMN FROM NORMAL SUBJECTS</u>						
5	51	56	65	72	78	71.2
	68	73	74	79	85	
	65	68	75	77	82	
6	52	55	73	82	115	71.5
	73	76	78	85	89	
	42	55	56	61	80	
7	92	99	103	105	135	112.6
	90	112	115	126	127	
	105	109	110	125	136	
8	105	112	166	173	177	144.5
	167	176	178	184	203	
	96	103	105	110	112	
<u>(2) PMN FROM BLOOD OF DIABETIC PATIENT</u>						
9	97	105	108	121	142	102.9
	89	96	102	115	120	
	82	84	85	97	101	

diabetes would need to be examined to obtain a significant result and this might not be meaningful unless related to the clinical state of the patients.

C. albicans mycelium was prepared in the plasma of time-expired blood, incubated for 3 days at 37° without shaking. The growth was washed twice in water.

Variations in the numbers of PMN from 4 normal subjects and 2 diabetic patients which migrated towards standard mycelial suspensions are shown in Tables 22 and 23.

Smaller numbers of PMN migrated towards mycelium of C. albicans than towards a suspension of blastospores of the same Optical Density (Tables 20, 21, 22, 23). The difference was statistically significant in every case, for serotype A, t was 23.258, 26.610, 11.233 and 16.313, and for serotype B, t was 14.058 and 7.899. In every case $P < 0.001$.

More PMN from the blood of patients with Diabetes mellitus migrated towards mycelium than PMN from normal subjects. This difference was statistically significant, for serotype A $t = 2.947$ and $P < 0.010 > 0.005$ and for serotype B $t = 2.723$ and $P < 0.025 > 0.010$.

Nuclear division in C. albicans and the numbers of PMN migrating

To determine whether the process of active budding or hyphal growth affected the chemotaxis of PMN, a mycostatic agent,

Table 22. Comparison of numbers of PMN from 4 different persons migrating towards C. albicans strain 207/53A mycelium grown in plasma.

INOCULUM	NUMBER OF PMN MIGRATED					
	NUMBERS COUNTED AS MIGRATED IN 5 FIELDS OF THE MICROSCOPE					MEAN MIGRATION
	1	2	3	4	5	
(1) <u>PMN FROM NORMAL PERSONS</u>						
MYCELIUM	22	22	25	31	34	24.8
	30	33	38	44	52	
	15	17	19	21	26	
	14	15	19	20	23	
	14	15	22	23	25	
BLASTOSPORES	78	86	92	96	104	91.2
MYCELIUM	27	28	33	41	42	30.0
	22	25	28	29	32	
	19	26	27	35	36	
BLASTOSPORES	95	106	108	110	115	106.8
MYCELIUM	36	48	49	52	57	43.3
	20	33	39	44	55	
	27	38	46	49	55	
	25	43	48	50	61	
	29	38	39	45	56	
BLASTOSPORES	78	79	82	82	114	87.0
(2) <u>PMN FROM PATIENTS WITH DIABETES</u>						
MYCELIUM	35	46	53	67	72	53.7
	34	37	43	48	55	
	42	44	51	56	67	
	39	46	53	56	89	
	44	58	66	69	72	
BLASTOSPORES	85	96	102	105	112	100.0

Table 23. Comparison of numbers of PMN from 2 different persons migrating towards C. albicans strain 792/53B mycelium grown in plasma.

INOCULUM	NUMBER OF PMN MIGRATED					MEAN MIGRATION
	NUMBERS COUNTED AS MIGRATED IN 5 FIELDS OF THE MICROSCOPE					
	1	2	3	4	5	
(1) <u>PMN FROM NORMAL PERSONS</u>						
MYCELIUM	56	61	61	62	70	62.8
	52	64	72	76	85	
	51	54	64	74	78	
	35	43	46	52	75	
	55	64	68	71	82	
BLASTOSPORES	92	104	112	116	124	109.6
(2) <u>PMN FROM A PATIENT WITH DIABETES</u>						
MYCELIUM	54	56	71	75	82	73.6
	81	88	94	98	130	
	40	46	55	67	76	
	51	55	60	73	82	
	63	79	82	84	98	
BLASTOSPORES	98	105	107	110	112	106.4

5-fluorocytosine, was used to arrest nucleic acid synthesis.

A 1/100 dilution of an Optical Density 0.30 suspension of synchronous C. albicans serotype A was prepared. 5 μ g of 5-fluorocytosine was added to 10 ml. of the synchronous blastospore suspension 2 hours before the yeasts were placed in the Boyden Chamber. A similar 10 ml. untreated suspension was used as a control. 5 μ g of 5-fluorocytosine was added to 10 ml. Ringer-Locke solution and inoculated into a Boyden Chamber to investigate whether the compound itself had any effect on the PMN. The result is shown in Table 24.

At the end of the experimental three hour period, the blastospore cultures were examined under the microscope. Growth of hyphae was observed only in the culture which did not contain 5-fluorocytosine.

The numbers of PMN migrating towards a synchronous blastospore suspension in which nuclear division was inhibited did not differ statistically from a synchronous suspension in which normal nuclear division occurred, $t = 1.578$ and $P < 0.400 > 0.300$.

Furthermore, 5-fluorocytosine had no effect on the migration of PMN, the numbers which migrated towards Ringer-Locke solution containing 5-fluorocytosine being similar to those migrating to Ringer-Locke solution alone.

Table 24. Numbers of PMN migrated towards C. albicans blastospores in which cell division was inhibited with 5-fluorocytosine.

INOCULUM	NUMBER OF PMN MIGRATED					
	NUMBERS COUNTED AS MIGRATED IN 5 FIELDS OF THE MICROSCOPE					MEAN MIGRATION
	1	2	3	4	5	
BLASTOSPORES + 0.5 µg/ml. 5-FLUOROCYTOSINE	61	96	108	111	112	113.5
	98	123	124	131	142	
	99	115	118	126	137	
	96	105	108	128	131	
	92	104	109	126	138	
CONTROL BLASTOSPORES	88	96	109	118	122	106.6
5-FLUOROCYTOSINE IN RINGER-LOCKE SOLUTION	4	6	7	11	19	9.4

Complement, *C. albicans* and the migration of PMN

To investigate whether complement was essential for the positive chemotaxis of PMN towards *C. albicans*, experiments were undertaken in which the plasma was heated for 30 minutes at 56° to inactivate the complement.

From a comparison of the results of the previous experiments with those shown in Tables 25 and 26, it is obvious that complement plays an essential role in the chemotaxis of PMN towards *C. albicans*.

Comparative effects of *C. albicans* cell wall (polysaccharide) and cell sap (protein) antigens on the chemotactic stimulation of PMN

Cell wall and cell sap enriched antigens were prepared as described (p.107-8). The data given in Table 27 shows clearly that greater numbers of PMN migrated towards the cell wall enriched antigen, and these results were statistically significant with antigen concentrations of 5 mg., 500 µg., 50 µg., 5 µg., 0.5 µg. per ml. and t was 16.254, 26.976, 17.734, 9.079 and 7.016, and P was 0.001, 0.001, 0.001, 0.001 and 0.005 0.001.

To evaluate the role of protein, a preparation of *C. albicans* in which the protein had been denatured by heat was used as a *Candida* polysaccharide antigen (Table 28).

Table 25. Numbers of PMN migrated towards blastospores of C. albicans strain 207/53A following inactivation of complement.

PATIENT	NUMBER OF PMN MIGRATED					MEAN MIGRATION
	NUMBERS COUNTED AS MIGRATED IN 5 FIELDS OF THE MICROSCOPE					
	1	2	3	4	5	
<u>(1) PMN FROM NORMAL SUBJECTS</u>						
1	0	0	0	2	4	1.3
	0	0	0	1	3	
	0	0	2	2	6	
2	1	1	2	4	5	1.4
	0	1	1	1	2	
	0	0	0	1	2	
3	3	6	7	8	12	3.3
	0	0	1	2	5	
	0	0	0	2	3	
<u>(2) PMN FROM A PATIENT WITH DIABETES</u>						
4	2	3	4	6	8	2.7
	0	0	1	3	4	
	0	1	2	2	5	

Table 26. Numbers of PMN migrated towards blastospores of C. albicans strain 792/53B following inactivation of complement.

PATIENT	NUMBER OF PMN MIGRATED					MEAN MIGRATION
	NUMBERS COUNTED AS MIGRATED IN 5 FIELDS OF THE MICROSCOPE					
	1	2	3	4	5	
<u>(1) PMN FROM NORMAL SUBJECTS</u>						
5	0	0	0	2	5	1.8
	0	1	2	4	5	
	0	0	1	2	5	
6	0	0	2	2	3	3.6
	3	7	8	8	9	
	1	2	2	3	4	
7	2	3	5	6	12	5.0
	0	2	2	5	7	
	3	3	6	8	11	
<u>(2) PMN FROM PATIENTS WITH DIABETES</u>						
8	0	0	0	2	3	0.8
	0	0	0	0	5	
	0	0	0	0	2	
9	0	0	1	2	3	1.2
	0	1	2	3	5	
	0	0	0	0	1	

Table 27. Numbers of PMN migrated towards cell wall and cell sap antigens of C. albicans.

INOCULUM		NUMBER OF PMN MIGRATED					
ANTIGEN	WEIGHT OF ANTIGEN PER ML.	NUMBERS COUNTED AS MIGRATED IN 5 FIELDS OF THE MICROSCOPE					MEAN MIGRATION
		1	2	3	4	5	
CELL WALL ANTIGEN	5 mg	111	135	138	146	152	136.4
	500 µg	62	68	75	78	82	73.0
	50 µg	43	45	49	53	59	49.8
	5 µg	18	19	22	24	24	21.4
	0.5 µg	12	14	17	18	22	16.6
	0.05 µg	4	5	8	11	11	7.8
CELL SAP ANTIGEN	5 mg	55	56	60	68	72	62.2
	500 µg	19	23	27	28	29	25.2
	50 µg	12	13	15	16	17	14.6
	5 µg	12	12	12	13	14	12.6
	0.5 µg	8	8	10	11	12	9.8
	0.05 µg	4	5	6	7	9	6.2
CONTROL		84	93	95	98	102	100.4
$\frac{1}{100}$ DILUTION OF O.D. = 0.30 BLASTOSPORES		96	105	108	111	112	

Table 28. Numbers of PMN migrating towards polysaccharide (cell wall) antigen.

ANTIGEN	NUMBER OF PMN MIGRATED					
	NUMBERS COUNTED AS MIGRATED IN 5 FIELDS OF THE MICROSCOPE					MEAN MIGRATION
	1	2	3	4	5	
POLYSACCHARIDE ANTIGEN	93	106	112	121	140	113.9
	95	105	118	120	129	
PROTEIN AND POLYSACCHARIDE ANTIGEN	119	122	125	127	136	112.2
	82	98	99	105	109	
CONTROL $\frac{1}{100}$ DILUTION O.D. 0.30 VIABLE BLASTOSPORES	113	122	128	131	135	124.6
	115	117	122	124	139	

The polysaccharide antigen consisted of blastospores which were heated for 30 minutes, cooled, reheated and then dialysed. The protein and polysaccharide antigen comprised a blastospore suspension which was simply dialysed to remove small molecules.

It may be seen that there was no statistically significant difference in the numbers of PMN which migrated towards the polysaccharide and protein and the polysaccharide antigens, t being 0.252 and $P > 0.500$.

It must therefore be concluded that the chemotaxis of PMN is induced by the polysaccharide complex. This investigation was then repeated with heat-killed blastospores and mycelium which were subjected to tryptic digestion (see p. 108). The results (Table 29) show that trypsin-heated blastospores stimulated a greater taxis of PMN than trypsin-treated mycelium, but the trypsin-treated antigens were less stimulatory than the control suspension of viable blastospores. Moreover, when the results shown in Tables 16 and 17 were compared with those of the present experiment (Table 29) the reduction in the numbers of PMN migrating to the trypsin-treated antigen were significantly reduced, t being 8.641 and 13.930 and $P < 0.001$ respectively for blastospore and mycelial antigens. The apparent reduction in chemotactic stimulus presented by the trypsin-treated preparations may be due to an ineffective removal of the trypsin during washing and the enzyme affecting

Table 29. Numbers of PMN migrated towards heat-killed blastospores and mycelium treated with trypsin.

INOCULUM		NUMBER OF PMN MIGRATED					
ANTIGEN	DECIMAL DILUTION	NUMBERS COUNTED AS MIGRATED IN 5 FIELDS OF THE MICROSCOPE					MEAN MIGRATION
		1	2	3	4	5	
TRYPSIN-TREATED BLASTOSPORES	0	87	95	105	107	112	101
	$\frac{1}{10}$	85	89	94	97	105	94
	$\frac{1}{100}$	38	41	44	49	73	49
TRYPSIN-TREATED MYCELIUM	0	38	40	41	44	52	43
	$\frac{1}{10}$	19	25	29	32	39	28.8
	$\frac{1}{100}$	14	14	17	19	29	18.6
CONTROL $\frac{1}{100}$ DILUTION O.D. = 0.30 BLASTOSPORES		110	115	116	122	125	117.6

the complementary system upon which the PMN migration depends.

Mannan antigen and the migration of PMN

The antigen was prepared as described on p. 108. The results are shown in Table 30. It may be seen that 1 µg/ml. of the mannan preparation has approximately the same chemotactic stimulus as a 1/100 dilution of the Optical Density 0.30 *Candida* blastospores, i.e. 7×10^4 viable cells per ml.

The numbers of PMN migrating towards equal weights of freeze-dried *Escherichia coli* and *C. albicans* are given in Table 31.

Nearly twice as many PMN migrated towards *E. coli* as towards an equal weight of *C. albicans*. For the concentrations of 10 µg/ml. and 1 µg/ml. and PMN from the first person t was 12.053 and 14.381 and $P = 0.001$. For concentrations of 10 µg., 1 µg. and 0.1 µg. with PMN from the second person t was 9.073, 22.920 and 3.674 and $P < 0.001$, < 0.001 and < 0.025 , > 0.010 respectively.

DISCUSSION

C. albicans is rapidly phagocytosed by human PMN (Wang and Schwarz, 1959) and the movement of PMN towards *C. albicans* was observed microscopically (p. 83).

In infected tissue, *C. albicans* frequently persists in the

Table 30. Numbers of PMN migrating towards a mannan (cell wall) antigen.

PATIENT	WEIGHT OF ANTIGEN PER ML.	NUMBER OF PMN MIGRATED					
		NUMBERS COUNTED AS MIGRATED IN 5 FIELDS OF THE MICROSCOPE					MEAN MIGRATION
		1	2	3	4	5	
1	0.01 μ g	14	15	15	17	18	21.1
		21	22	24	27	28	
		19	19	22	27	28	
	0.1 μ g	44	52	53	53	60	53.7
		44	46	48	54	55	
		53	58	60	62	64	
	1.0 μ g	86	97	98	103	104	104.9
		98	98	107	109	112	
		96	112	115	118	120	
2	0.01 μ g	19	21	23	25	25	23.8
		19	23	25	25	26	
		23	24	24	27	28	
	0.1 μ g	53	54	55	57	62	50.3
		46	47	53	53	57	
		41	41	42	44	52	
	1.0 μ g	100	106	116	118	122	110.7
		96	102	102	108	118	
		106	108	114	120	124	
1	CONTROL O.D. = 0.30 BLASTOSPORES DILUTED $\frac{1}{100}$	80	94	97	102	103	95.2
2		103	106	109	110	122	110.0

Table 31. Numbers of PMN migrating towards equal weights of freeze-dried E. coli and C. albicans.

PATIENT	MICROBE	WEIGHT OF ANTIGEN PER ML.	NUMBER OF PMN MIGRATED					MEAN MIGRATION
			NUMBERS COUNTED AS MIGRATED IN 5 FIELDS OF THE MICROSCOPE					
			1	2	3	4	5	
1	<u>E. COLI</u>	10 µg	109	120	125	132	141	123.1
			115	122	125	128	134	
			110	112	119	120	132	
	<u>E. COLI</u>	1 µg	64	67	68	72	75	67.7
			53	59	64	72	83	
	<u>C. ALBICANS</u>	10 µg	69	72	73	74	82	79.6
74			76	79	83	88		
76			82	85	89	92		
<u>C. ALBICANS</u>		1 µg	29	31	32	32	32	31.0
	28		29	30	32	35		
2	<u>E. COLI</u>	10 µg	105	112	118	120	124	115.8
		1 µg	42	48	52	53	54	49.5
			49	52	55	56	61	
			41	43	44	45	48	
		0.1 µg	6	9	10	10	15	10.0
	<u>C. ALBICANS</u>	10 µg	66	68	70	72	78	70.8
		1 µg	24	27	28	30	32	27.9
			26	32	33	34	35	
			20	22	23	25	27	
0.1 µg		3	4	4	5	7	4.6	

hyphal form and only a mild local inflammation occurs (Richart and Dammin, 1960). It is noteworthy therefore that, in the experiments described, significantly fewer PMN migrated towards a mycelial suspension than towards a blastospore suspension with the same Optical Density. Hyphae deeply embedded in tissue may exert little chemotactic stimulation and escape phagocytosis by PMN.

Here the fact that the membranes used had a mean pore size of 3 μ must be emphasized since this may be a greater barrier to penetration than occurs in vivo.

Blastospores, because of their form and smaller size, are better adapted for dissemination in the blood than hyphae and because they induce a greater stimulation of PMN are more likely to be phagocytosed.

Hasenclever and Mitchell (1961) described two serotypes of C. albicans of which serotype A possesses at least one more antigen than serotype B. No difference in PMN chemotaxis towards the two serotypes was detected in either blastospores or mycelium.

About 50% of the population carry C. albicans as a commensal in the normal body flora (Davies and Leese, personal communication). More PMN migrate chemotactically towards C. albicans than towards T. glabrata which is much less frequently found in the indigenous flora. The variation in the

numbers of PMN migrating towards standard suspensions of C. albicans may indicate immunological memory of previous exposures to this microbe in some persons and lack of previous exposure in others. Diabetics are particularly prone to super-infection with Candida (Winner, 1966) and their PMN migrated in significantly greater numbers towards C. albicans hyphae than did normal PMN.

Complement was shown to be essential for PMN chemotaxis towards bacteria (Müller-Eberhard, 1968). Heating the plasma to 56° for $\frac{1}{2}$ hour prevented PMN migration towards C. albicans demonstrating a similar role for complement in PMN chemotaxis towards C. albicans.

It seemed possible that the poorer PMN migration towards mycelium compared with blastospores might be due to the fact that most of the mycelium used was prepared by growing it in plasma and antigen or antibody from the plasma, bound to the cell surface, prevented the formation of chemotaxis-inducing complexes in this system. T. glabrata blastospores grown in plasma did not induce less PMN chemotaxis than if grown in broth and C. albicans mycelium grown in the absence of plasma still attracted less PMN migration than did blastospores.

In a medium containing 12.5% plasma C. albicans cells begin to grow as hyphae (Table 12). Actively growing cells would seem to pose a greater threat to the immune defence mechanisms

than non-growing cells. To see if PMN migration was more highly stimulated during hyphal growth, a population of blastospores in which nuclear division was inhibited by 5-fluorocytosine was prepared. There was no difference in PMN migration towards the treated and normally growing cells.

Polysaccharides and proteins have been shown to exert chemotactic stimulation of PMN (Harris, 1954). The *Candida* cell wall is mainly composed of mannan and glucan (Bartnicki-Garcia, 1968). Cell wall mannan-enriched antigens from *C. albicans* were highly stimulatory for PMN, whereas cell-sap antigens by comparison had little effect. The destruction of *Candida* cell protein by boiling did not reduce positive chemotaxis. It must be concluded therefore that it is the cell wall polysaccharide and especially the enriched antigens which are important. These form the complexes which induce the chemotactic stimulation of PMN. These studies demonstrate the chemotaxis of PMN towards *C. albicans* in vitro. It must be emphasized that in vivo there is a constant supply of PMN and complement. The large numbers of *C. albicans* required to produce PMN migration in satisfactory numbers is probably related to the difficulty the PMN (mean diameter 15 μ) encounter in penetrating the pores of the rigid membrane in the Boyden Chamber. In vivo it is probable that the soft tissues present less physical resistance to migration.

It is concluded that the chemotaxis of PMN towards C. albicans is an important part of the normal immune defences. Non-specific antibody in the plasma probably forms a complex with Candida cellular surface antigens, and, in the presence of complement, a substance is formed which stimulates the positive chemotaxis of PMN towards the complex.

Two complement-derived chemotactic factors which appear to be involved have been described. A high molecular weight factor (Ward, Cochrane and Müller-Eberhard, 1966) constitutes a trimolecular complex of C'5, C'6 and C'7:-

where "c" = chemotactic activity.

The associated form (C'5, 6, 7)c activates a proesterase in the polymorphonuclear leucocyte membrane and this enzyme may be a chemotactic receptor in PMN (Ward and Becker, 1967).

Secondly, a low molecular weight factor has been described. This is a split product of C'3 (Ward, 1967).

The PMN are thus enabled to phagocytose and subsequently kill the invading Candida (Sections 6 and 7).

SUMMARY

The chemotactic migration of dextran-sedimented PMN through

the pores of a Millipore membrane towards microbial suspensions was studied in a modified Boyden Chamber.

Fewer PMN migrated towards suspensions of C. albicans mycelium than towards blastospores, but there was no difference between the two serotypes. Inter-patient variation was observed in PMN migration and, although only a few diabetics were studied, their PMN showed greater migration towards mycelium than did normal PMN. Inactivation of complement prevented PMN migration.

More PMN migrated towards C. albicans than towards Torulopsis glabrata. PMN migration towards T. glabrata was unaffected by whether the cells were grown in plasma or broth. C. albicans mycelium grown in Chattaway's Medium B produced less migration than blastospores, a similar result to plasma-grown mycelium. PMN migration towards C. albicans blastospores treated with 5-fluorocytosine to arrest nuclear division did not differ from that towards untreated blastospores.

Destruction of Candida cell protein by heat did not reduce the positive chemotaxis of PMN. C. albicans cell sap only weakly stimulated PMN migration, but cell wall preparations and purified mannan preparations were highly stimulatory for PMN migration.

E. coli caused the migration of more PMN than equivalent weights of C. albicans.

S E C T I O N 9

THE CANDIDACIDAL ACTIVITY OF CATIONIC PROTEINS

FROM THE LYSOSOMES OF PMN

INTRODUCTION

The degranulation which accompanies the bactericidal activity of PMN was first described by Kanthak and Hardy (1894). Spitznagel and Chi (1963) gave the first indication that the anti-microbial substances of the PMN may be polycationic in nature. Zeya and Spitznagel (1966a) in a study of the electrophoretic resolution of the antibacterial activity of PMN lysosomes demonstrated that this activity was associated with cationic protein components. They also showed (Zeya and Spitznagel, 1966b) that this fraction was lethal to C. albicans.

In this study, the lysosomal cationic protein fraction was prepared from the PMN of normal volunteers and its candidacidal activity at a range of pH values measured in vitro.

METHODS

Isolation of leucocytes from venous blood

The method used was that of Chodirker, Bock and Vaughan (1968).

25 ml. of blood was collected by antecubital venepuncture

without anticoagulant. The blood was immediately ejected into 475 ml. of 0.85% saline containing 0.1% albumin cooled to 3°. This mixture was centrifuged at 800 g. for $\frac{1}{2}$ hour and the supernatant was removed and discarded.

The erythrocytes were lysed by adding 100 ml. chilled hypotonic 0.2% saline for 20 seconds. Immediately, 100 ml. of chilled hypertonic 1.61% saline was introduced to restore the isotonicity. The mixture was centrifuged at 400 g. for 10 mins. The osmotic lysis procedure was then repeated once and centrifuged at 110 g. for 5 minutes.

The remaining leucocytes were then washed twice in cold saline.

Isolation of granules from leucocyte homogenates

The cell suspension was diluted ten-fold in 0.2 M sucrose containing 3.6 mg/ml. heparin and centrifuged at 800 g. for 15 minutes to remove lymphocytes, erythrocyte fragments, etc. The supernatant was then centrifuged at 25,000 g. for 10 minutes. This yielded a dirty white pellet of leucocyte granules. These granules were washed in 0.34 M sucrose and resuspended in 0.2 ml. of 0.25 M sucrose.

Preparation of the ethyl alcohol fraction

The method used was that of Zeya and Spitznagel (1966b).

The granule suspension was extracted with 1.8 ml. of 0.2 N sulphuric acid at 4° for $\frac{1}{2}$ hour. The mixture was centrifuged at 10,000 g. for 10 minutes. The clear supernatant was removed to an ice-chilled glass centrifuge tube and the temperature adjusted to -4° . Absolute ethanol at -4° was slowly added with constant gentle stirring to give a final concentration of 20% V./V.

The mixture was allowed to stand for 3-4 hours at -4°C . The precipitate was then collected by centrifugation at 250 g. for 15 minutes. The precipitate was immediately redissolved in ice-cold distilled water to a volume of 5 ml. and dialysed for 16 hours against two changes of distilled water. The dialysate was freeze-dried and stored in a stoppered bottle at 3° .

Preparation of microbes to test the activity of lysosomal cationic proteins

C. albicans strain 207/53A was grown overnight in shaken Sabouraud Broth. A synchronous culture was then prepared by the linear density gradient technique.

E. coli was grown overnight in shaken Glucose Nutrient Broth and the cells washed twice.

The microbial cells were resuspended in phosphate-citrate buffer to a desired Optical Density in the Hilger "Spekker" absorptiometer and decimal dilutions prepared in buffer.

Preparation of the lysosomal cationic protein (LCP) fraction

The freeze-dried material was dissolved in phosphate-citrate buffers of pH 4, 5, 5.6 and 7, to give concentrations of 10 mg., 1 mg., 100 µg., 10 µg., 1 µg. and 0.1 µg. per ml. at each pH.

The experimental system

0.1 ml. inoculum of a decimal dilution of microbe was inoculated into 0.9 ml. lots of LCP in buffer in a Wasserman rack. The suspensions were incubated for 5 hr. at 37° and the rack shaken by hand at intervals.

Following incubation, 0.1 ml. samples were withdrawn from each tube and spread over the surface of 4 inch diameter plates containing Sabouraud dextrose agar.

RESULTS

The results of experiments with cationic proteins are given in Tables 32-35.

DISCUSSION

Zeya and Spitznagel (1966b) showed that PMN granules contain strongly cationic proteins which, because of their unique physico-chemical properties, were lethal to C. albicans and may

Table 32. Survival of C. albicans strain 207/53A and E. coli in LCP at pH 4.0

INOCULUM							WEIGHT OF LCP PER ML. LOT	SURVIVAL			
MICROBE	PLATE COUNTS							PLATE COUNTS			% SURVIVAL
	1	2	3	4	5	MEAN		1	2	MEAN	
<u>C. ALBICANS</u>	20	20	22	24	26	22.4	9 mg	0	0	0	0
							900 µg	0	0	0	0
							90 µg	1	3	2.0	89.3
							9 µg	3	5	4.0	100.0
							0.9 µg	4	8	6.0	100.0
							0.09 µg	2	7	5.0	100.0
<u>E. COLI</u>	58	60	68	71	73	66.0	9 mg	0	0	0	0
							900 µg	0	0	0	0
							90 µg	0	1	0.5	75.8
							9 µg	0	1	0.5	75.8
							0.9 µg	0	1	0.5	75.8
							0.09 µg	1	3	2.0	100.0

Table 33. Survival of C. albicans strain 207/53A and E. coli in LCP at pH 5.0

INOCULUM							WEIGHT OF LCP PER ML. LOT	SURVIVAL			
MICROBE	PLATE COUNTS							PLATE COUNTS			% SURVIVAL
	1	2	3	4	5	MEAN		1	2	MEAN	
<u>C. ALBICANS</u>	27	35	45	50	63	44.0	9 mg.	0	0	0	0
							900 µg	1	2	1.5	100.0
							90 µg	3	4	3.5	100.0
							9 µg	7	9	8.0	100.0
							0.9 µg	3	5	4.0	100.0
							0.09 µg	2	11	6.5	100.0
<u>E. COLI</u>	18	21	28	28	28	24.6	9 mg	0	0	0	0
							900 µg	0	0	0	0
							90 µg	0	1	0.5	100.0
							9 µg	2	2	2.0	100.0
							0.9 µg	0	3	1.5	100.0
							0.09 µg	1	4	2.5	100.0

Table 34. Survival of C. albicans strain 207/53A and E. coli in LCP at pH 5.6

INOCULUM							WEIGHT OF LCP PER ML. LOT	SURVIVAL			
MICROBE	PLATE COUNTS							PLATE COUNTS			% SURVIVAL
	1	2	3	4	5	MEAN		1	2	MEAN	
<u>C. ALBICANS</u>	35	37	43	44	45	40.8	9 mg	0	0	0	0
							900 µg	0	0	0	0
							90 µg	1	3	2.0	100.0
							9 µg	2	3	2.5	100.0
							0.9 µg	6	9	7.5	100.0
							0.09 µg	8	12	10.0	100.0
<u>E. COLI</u>	168	170	183	185	192	179.6	9 mg	0	0	0	0
							900 µg	0	0	0	0
							90 µg	0	1	0.5	2.8
							9 µg	6	17	11.5	64.0
							0.9 µg	11	13	12.0	66.8
							0.09 µg	28	35	31.5	100.0
<u>C. ALBICANS</u>	260	280	290	300	320	290.0	9 mg	7	9	8.0	27.6
							900 µg	11	11	11.0	37.9
							90 µg	9	16	12.5	43.1
							9 µg	12	17	14.5	50.0
							0.9 µg	21	27	24.0	82.8
							0.09 µg	30	33	31.5	100.0
<u>E. COLI</u>	390	410	420	440	460	424.0	9 mg	0	0	0	0
							900 µg	4	7	5.5	13.0
							90 µg	35	38	36.5	86.1
							9 µg	28	32	30.0	70.8
							0.9 µg	35	37	36.0	84.9
							0.09 µg	43	47	45.0	100.0

Table 35. Survival of C. albicans strain 207/53A and E. coli in LCP at pH 7.0

INOCULUM							WEIGHT OF LCP PER ML. LOT	SURVIVAL			
MICROBE	PLATE COUNTS							PLATE COUNTS			% SURVIVAL
	1	2	3	4	5	MEAN		1	2	MEAN	
<u>C. ALBICANS</u>	64	75	78	80	82	75.8	9 mg	0	2	1.0	13.2
							900 µg	2	4	3.0	39.5
							90 µg	1	5	3.0	39.5
							9 µg	3	4	3.5	46.1
							0.9 µg	1	4	2.5	32.9
							0.09 µg	9	10	9.5	100.0
<u>E. COLI</u>	13	16	18	19	24	18.0	9 mg	0	0	0	0
							900 µg	0	0	0	0
							90 µg	0	4	2.0	100.0
							9 µg	3	5	4.0	100.0
							0.9 µg	4	6	5.0	100.0
							0.09 µg	3	4	3.5	100.0

thus constitute an important biochemical determinant in host defence.

Using the technique of Zeya and Spitznagel, five preparations of LCP were investigated. The activity of different preparations showed variability in candidacidal activity.

Zeya and Spitznagel used the technique of Hirsch (1958) for the assay of LCP. 1 ml. lots of dilutions of LCP were contained in cups in a plastic tray, 50-200 microbial cells added and the whole incubated for 2 hours at 38°. Molten agar was added to each cup and, following incubation, the number of colonies per cup visually estimated.

A more precise assessment of the lethal effects of LCP on C. albicans and E. coli by a plate count method was used in this series of experiments.

The LCP preparations investigated in this study were not as potent as those described by Zeya and Spitznagel who obtained 50% inhibition of growth of 150-200 cells of C. albicans and E. coli with concentrations of LCP of 0.07 and 0.15 µg. per ml. respectively.

In the present study, 9 mg. per ml. of LCP produced complete killing of C. albicans except at pH 7.0 and with the higher inoculum at pH 5.6. A concentration of 900 µg. per ml. gave even more variable results but 100% killing was

observed in some preparations. Concentrations of less than 900 µg. per ml. resulted in little or no killing of *Candida*.

E. coli was completely killed by 900 µg. per ml. LCP except for the higher inoculum at pH 5.6. Less than 900 µg. per ml. gave little or no killing.

In contrast to the results of Zeya and Spitznagel, in this study *E. coli* was slightly more sensitive to LCP than *C. albicans*. The pH of the buffer over the range pH 4.0-7.0 appeared to make little difference to the candidacidal activity of LCP.

The lysosomal enzyme myeloperoxidase has also been implicated in the candidacidal activities of PMN and purified human myeloperoxidase in the presence of hydrogen peroxide and potassium iodide was rapidly lethal to *C. albicans in vitro* (Lehrer, 1969).

A patient with disseminated candidosis was demonstrated to lack myeloperoxidase in the PMN, though other lysosomal enzymes were normal (Lehrer and Cline, 1969b). The patient's PMN failed to kill *C. albicans in vitro* and it was concluded that myeloperoxidase was necessary for the candidacidal activity of human PMN.

The candidacidal powers of the myeloperoxidase in vitro was not investigated in this study because of the complexities of its purification (Schultz and Schmukler, 1964).

SUMMARY

Lysosomal cationic protein from the PMN of healthy volunteers was lethal to C. albicans in vitro in phosphate-citrate buffer in the range pH 4-7 at higher concentrations than those quoted by Zeya and Spitznagel (1966b). The activity of each LCP preparation varied considerably. In contrast to the results of Zeya and Spitznagel, in this study E. coli was more sensitive to LCP than C. albicans. LCP in the PMN at suitable concentration may constitute a candidacidal mechanism in vivo.

SECTION 10

GENERAL DISCUSSION

C. albicans is present in the normal microbiota of about half the population (Davies and Leese, personal communication). Although superficial Candida infections are common, systemic candidosis is rare and only occurs in those debilitated by other diseases (Winner and Hurley, 1964). It follows, therefore that the normal subject has immunity to infection with this organism.

Conflicting opinions had been reported on the role of serum and phagocytosis in the eradication of C. albicans (see Sections 5 and 7).

The researches described were undertaken to investigate this controversy.

C. albicans is a dimorphic organism. The budding blastospore is the propagule of rapid asexual multiplication and dissemination. The hyphal form is able to penetrate host cells (Stanley and Hurley, 1967), resist phagocytosis by certain cells (Louria and Brayton, 1964; Stanley and Hurley, 1969) and is considered by many authors to be the invasive form in infection (Hill and Gebhardt, 1956; Young, 1958;

Blyth, 1959). Although the change from blastospore production to hyphal growth in serum effects a reduction in numbers, the hyphae are less well adapted to dissemination in the blood but the hyphal elements are able to penetrate the tissues.

Broth cultures of C. albicans are a heterogeneous population of single and budding blastospores and hyphae. The use of the linear sucrose density gradient to select a homogeneous population of single synchronous blastospores gave accurately known inocula and facilitated experimentation with reproducible results.

Although in vitro studies of the bactericidal power of blood have been reported (Miles and Misra, 1938), no comparable studies on the candidacidal activity of blood appeared to have been made. In this study, inocula of synchronous blastospores of between 10-100 cells and 100-1,000 cells showed a mean kill of 80% and 70% respectively. Thus 1 ml. lots of blood possess considerable candidacidal power in vitro. In vivo the PMN pool is constantly replenished so that the fungicidal efficacy is very much greater.

There have been several reports of human serum being lethal to *Candida* (Roth et al., 1959; Roth and Goldstein, 1961;

Louria and Brayton, 1964). In these studies, inoculation of synchronous blastospores into serum showed 100% tubulation and a linear growth of the hyphae during an 8½ hour period. It is evident that, in serum, C. albicans is not killed but grows in the hyphal form. These hyphae clump together as colony-forming units when cultured and this results in a drop in viable count which appears to have been mistaken for killing.

Normal blood contains 5,000-9,000 leucocytes per cubic mm., of which 56% are PMN. Microscopically, PMN preparations were observed to engulf Candida cells. Only rarely was the yeast seen to bud or produce a hypha within the leucocyte, an observation in contrast with other reports (Louria and Brayton, 1964; Stanley and Hurley, 1969). Under the interference microscope PMN were observed to phagocytose and kill Candida cells as evidenced by colour change and disintegration of the blastospores. Long hyphae, to which many PMN were attracted, appeared to be killed following engulfment, possibly by extracellular enzymes.

The present investigation confirms the observations of Chilgren, Oh, Hong and Quie (1969) and Lehrer and Cline (1969a) that C. albicans is killed by PMN in serum. Very little killing was found when washed PMN were resuspended in Ringer-Locke solution or TC199 fluid. Anticomplementary treatment of

the serum reduced the numbers of *Candida* killed by PMN. It may be concluded, therefore, that serum complement is necessary for optimum phagocytosis of *Candida* by PMN.

The chemotaxis of PMN in the Boyden Chamber (Boyden, 1962) was significantly greater towards blastospores than towards mycelium, but all activity was lost if the complement system was inactivated. PMN from different subjects showed variations in their chemotaxis towards *C. albicans* and PMN from diabetic patients were especially motile.

Cell wall and mannan-enriched preparations from *C. albicans* were highly stimulatory for PMN chemotaxis. However, nearly twice as many PMN migrated towards *E. coli* compared with an equal freeze-dried weight of *C. albicans*.

It must be concluded therefore that whilst serum itself is not fungicidal, the complement system is essential for the chemotaxis of PMN towards *Candida*, and in this manner has a vital role in its destruction.

Zeya and Spitznagel (1966a) reported that the antimicrobial activity of PMN lysosomes was associated with the cationic protein components. In the present study, it was found that this fraction did possess anti-*Candida* activity but considerable variation was present in different preparations.

In conclusion, this study has demonstrated the killing

of C. albicans blastospores and hyphae by whole blood.

The candidacidal activity of blood stems from the phagocytic activities of the PMN whose chemotaxis is mediated by serum complement.

S E C T I O N 11

APPENDIX

Description of *Candida albicans*

Cited Lodder and Kreger-van Rij (1952):

Growth in malt extract: After 3 days at 25°C cells are usually round or short-oval, seldom elongate, often highly refractive. According to the size of the cells three groups can be distinguished, although transitions may occur. The first group shows the smallest cells, measuring (2-5) x (3-7,5)μ. The second group, to which the greater part of the strains belong, shows cells from (3-6,5) x (3,5-12,5)μ. The largest cells, belonging to the third group, measure (3-8,5) x (5-14)μ. A sediment is formed.

After one month at 17°C a sediment and a thin ring.

Streak culture on malt agar: After one month at 17°C the colour of the streak culture is cream to yellowish-cream, at the bottom of the tube with a somewhat reddish tinge. The colour is usually deeper in the middle and at the bottom of the tube; rarely does the cream colour change into a greenish

tone. The streak culture is soft, glistening, rather flat; it is rarely tough, dull and raised. Either the surface is quite smooth or it shows small pimples at the bottom of the tube, in the middle or on the whole surface. In most strains the margin is smooth and under the streak strands of hyphae grow into the agar; hyphae rarely develop at the surface surrounding the margin. Besides this appearance very rarely strains occur with a tough, raised, almost dull streak culture of a yellow colour with a reddish tinge and a surface which is folded or wrinkled (R-modification). In the ordinary soft cultures spots of the tough consistence may be found.

Slide cultures: Generally there is a rich development of pseudomycelium; this may be formed not on the surface but in the medium. Besides pseudomycelium also true mycelium is produced. The development of ball-like clusters of blastospores on the pseudomycelium (type "Mycotorula") is very characteristic of this species. Blastoconidia, too, are formed. They may be elongate and one or more blastospores can arise on them forming typical "blastospore clusters on stems".

Very characteristic too, although not always present, are the large round chlamydo spores which are formed usually terminally, but also intercalary, pleurally or in clusters. The thin-walled protochlamydo spores on which the

chlamydospores may arise are round, elongate or somewhat bottle-shaped to pear-shaped.

It can also occur that in addition to this so characteristic aspect the blastospores are arranged in another way, viz. according to the types "Blastodendrion", "Candida", "Mycotoruloides". Moreover, bare strands of hyphae may occur.

Very seldom strains are found which do not show at all clusters of blastospores, but which develop a coarse (pseudo) mycelium with hyphae of simple or branched chains of long cells which often adhere to each other. The production of blastospores is much reduced. Chlamydospores are rarely formed. The streak culture is tough and folded. This type clearly represents the R-modification. It may also be found besides the ordinary type in the same strain.

Fermentation:

Glucose +

Maltose +

Galactose + (often weak)

Lactose -

Saccharose - (seldom a few
gas bubbles may develop)

Sugar assimilation:

Glucose +	Maltose +
Galactose +	Lactose +
Saccharose +	

Assimilation of potassium nitrate: Absent.

Ethanol as sole source of carbon: No growth.

Growth in litmus milk: A film is formed. The milk turns blue or is decolourised except the upper part of the tube which turns blue. The milk is usually not coagulated.

Splitting of arbutin: Absent or very rarely a slight brown colouring.

Three strains of C. albicans were used in this investigation.

Strains Hasenclever 207/53A and 792/53B, which were serotypes A and B respectively, were kindly provided by Dr. I. Murray of the London School of Hygiene and Tropical Medicine.

Strain WF 668A from a collection of cultures isolated from patients at St. Mary's Hospital.

PREPARATION OF MEDIA USED

Sabouraud Broth

Glucose	40 g.
Mycological Peptone	10 g.
Distilled water	1 l.

Wickerham Fluid

6.7 g. Difco Yeast Nitrogen Base and 5.0 g. glucose were dissolved in 100 ml. distilled water and Seitz-filtered. This stock solution was diluted ten-times with sterile distilled water for use.

Sabouraud Agar

15 g. of Davis agar was added to each litre of Sabouraud Broth.

Ringer-Locke Solution

NaCl	9.00 g.
KCl	0.24 g.
CaCl ₂	0.42 g.
Glucose	1.00 g.
NaHCO ₃	0.20 g.
Distilled water	1 l.

Buffered TC 199

TC 199 (Glaxo Laboratories Ltd.) stock solution was diluted ten-fold and buffered with 4.4% sterile sodium bicarbonate solution.

Dextran solution

Dextraven 110 (Fisons Pharmaceuticals Ltd.), a sterile solution of 6% w/v. dextrans of average Molecular Weight 110,000 in 0.9% sodium chloride, was used.

APPARATUS

Absorptiometer

Hilger and Watts "Spekker" absorptiometer type CS-330 fitted with 0.5 ml. cells.

Rotary shaker

A Matburn Mixer rotary shaker, 50 r.p.m., was used in a 37° incubator.

SECTION 12

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