بسم الله الرحمن الرحيم

Development, Standardisation and Application of Radiometric Assays for Measuring Interaction of Phagocytes, Serum Substances and Fungal Organisms.

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ABSTRACT

Objective radiometric methods for measuring independently opsonisation, phagocytosis and intracellular killing of Candida albicans and Saccharomyces cerevisiae have been developed and standardised. Opsonisation and phagocytosis were measured as inhibition of ^{3}H-uridine uptake into the microorganisms caused by phagocytosis. Intracellular killing of C.albicans was measured by ^{51}Cr release assay. The methods are easy to perform, require a small amount of blood and are therefore suitable for clinical application. Using these methods, the following original observations were made:

(1) Using human sera neither antibody nor complement were required for opsonisation of C.albicans but the opsonisation of S.cerevisiae required the activation of the alternative complement pathway.

(2) Intracellular killing of C.albicans was found to be dependent on the presence of C3. Thus in the absence of C3, C.albicans was normally ingested but not killed. It was therefore concluded that C3 participates directly in the intracellular processes leading to phagocytic killing of C.albicans.

(3) Using S.cerevisiae, it was possible to measure selectively the opsonising capacity of the alternative pathway. (The alternative pathway opsonising capacity (APOC) assay).

(4) Preliminary clinical application of the APOC assay suggested that a large proportion (30-40%) of patients with recurrent infection and suspected immune deficiency, without other detectable immunological abnormality, have decreased opsonising capacity of the alternative complement pathway.

ABBREVIATIONS

B-lymphocytes	Bone marrow derived lymphocytes
C2D	C2 deficient
C3D	C3 deficient
C5D	C5 deficient
C6D	C6 deficient
CGD	Chronic granulomatous disease
CPM	Counts per minute
DNase	Deoxyribonuclease
EDTA	Ethylene diamine tetraacetic acid
Hepes	N-2- Hydroxyethypiperazine-N'-2-ethanesulphonic acid
Ig	Immunoglobulin
KAF	Conglutinogen-activating factor
LPS	Lipopolysaccharide
MIF	Migration inhibitory factor
MNP	Mononuclear phagocytes
N.D.	Not done
NBT	Nitroblue tetrazolium
PBS	Phosphate buffered saline
PMN	Polymorphonuclear leukocytes
PPO	Diphenyl oxazole
R3	Zymosan treated serum
rpm	Revolutions per minute
C.albicans	Candida albicans
S.cerevisiae	Saccharomyces cerevisiae
T-lymphocytes	Thymus derived lymphocytes

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Chapter 1

General Introduction

GENERAL INTRODUCTION

I. Definition of terms used to describe various components of phagocytic activity.

II. Defence mechanisms against infection.

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IX. Objectives of this study.

I. Definition of terms used to describe various components of phagocytic activity.

Phagocytes: Phagocytes are cells which exhibit amoeboid behaviour, including spontaneous changes of form, the ability to migrate by extending pseudopodiae and engulf foreign material (e.g. microorganisms). The two major categories of phagocytic cells are polymorphonuclear phagocytes (PMN) and mononuclear phagocytes (MNP). PMN are cells which are predominantly found in the peripheral blood while MNP are primarily tissue

associated cells of which two types may be distinguished : free macrophages mainly present in blood, serous cavities and the lung ; and fixed macrophages found mainly in the liver (Kupffer cells), spleen, lymph nodes and connective tissue.

Opsonin: Opsonin is a substance which renders microorganisms more susceptible to engulfment by phagocytes.

Procidin: Procidin is a substance which increases the susceptibility of phagocytosed microorganisms to the action of intracellular killing or digestion processes. A detailed description is given in Chapter 5.

Phagocytosis: Phagocytosis is defined as the internalization of particulate material, including microorganisms; it may or may not lead to intracellular killing (Mudd et al, 1934).

Intracellular killing: Intracellular killing is defined as death of phagocytosed microorganism(s).

Phagocytic activity: Phagocytic activity is a collective term describing all events associated with the interaction of serum, microorganisms and phagocytes, including the generation of chemotactic agents, chemotactic migration of phagocytic cells, opsonisation, phagocytosis, killing and digestion.

II. Defence mechanisms against infection.

At birth , man is immediately surrounded by an immense number of microorganisms, but is normally protected from infection by mechanisms which prevent invasion and which, if infection occurs, identify and eliminate the invading organisms. Immunity mechanisms are both non-specific and specific.

(a) Non-specific immunity.

Surface barriers and their secretions are the first line of non-specific defence against infection. Many other factors are also involved in preventing infection. These include coughing, sneezing, the lavaging action of tears, the cleansing action of the urethra by the flow of urine etc. Lysozyme (Fleming, 1922), can lyse certain types of Gram positive microorganisms such as Micrococcus lysodeikticus. Lysozyme is present in tears, nasal secretions and saliva. Man is therefore protected against microorganisms that are lysed by lysozyme. It is probable that complement too plays a role in non-specific defence. For example, certain bacteria can activate complement directly through the alternative pathway and may thereby become lysed in the absence of antibody (Bjornson & Bjornson, 1977). Furthermore complement coated bacteria are more susceptible to the action of lysozyme (Muschel et al., 1959). Once microorganisms have established an infection, various elimination mechanisms become involved, of which phagocytic activity, which will be discussed in greater detail below, is of critical importance.

(b) Specific immunity.

Specific immunity is established as the result of either recovery from an infection or vaccination. Two distinct but functionally integrated mechanism of specific immunity operate; humoral and cellular immunity. Humoral immunity is dependent on antibody production by plasma cells, which are derived from B lymphocytes, and it can be transfered to animals by means of serum. Cellular immunity is mediated by sensitised T lymphocytes and can usually only be transfered using viable cells (Landsteiner & Chase, 1942). Antibodies can bind to microorganisms and some of the latter are susceptible to lysis following complement fixation (Buchner, 1889). However the major role of antibody in defence is to enhance phagocytosis directly by opsonisation (Wright & Douglas, 1903), or

indirectly through complement activation (Meakins, 1909). Sensitised T lymphocytes alone may be able to destroy abnormal cells by direct contact (Perlmann & Holm, 1969) , but they also produce substances, lymphokines, (David & David, 1972), such as macrophage inhibitory factor,(MIF) (Bloom & Bennett, 1966; David, 1966), chemotactic factor (Ward et al., 1969), etc. These substances not only recruit and activate other non-sensitised lymphocytes but they are also thought to attract MNP and enhance their function. Although specific substances may directly neutralise microorganisms (Wheelock, 1965), their ability to activate and enhance non-specific mechanisms involving complement and phagocytes is more important for resistance to infections.

III. Complement.

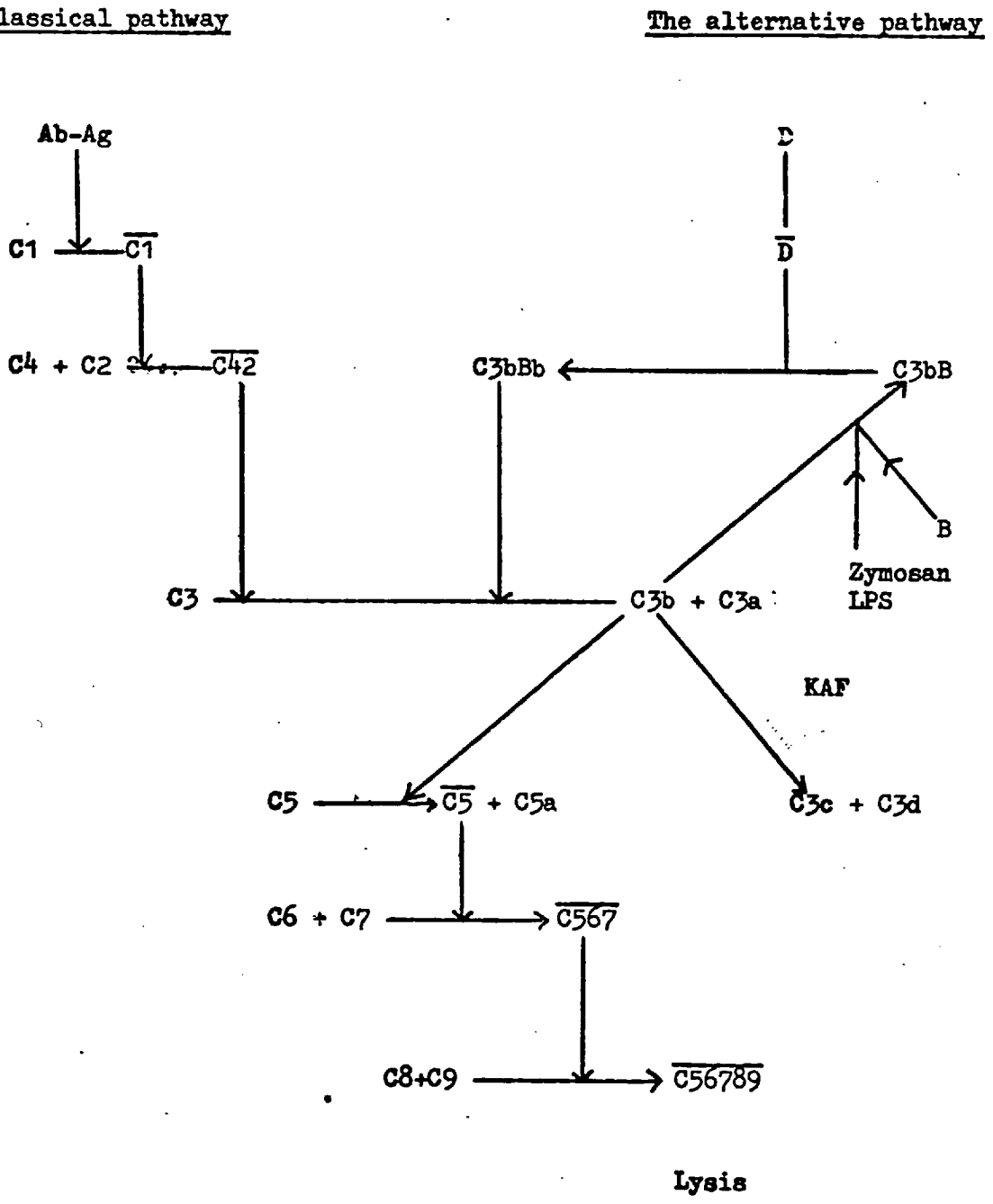
The major biological functions of complement are:

(i) lysis of bacteria in the presence or absence of antibody (Pillemer et al., 1954; Buchner, 1889), and

(ii) generation of biologically active substances, such as opsonins, chemotactic substances, anaphylatoxins, etc. (Lachmann,1975). The reaction sequence of the complement system is shown in Fig 1. Two major activation pathways are recognised, the classical pathway and the alternative pathway (Lachmann, 1975). The classical pathway is activated by antigen-antibody complexes (IgG_1, IgG_3, and IgM). The alternative pathway is activated for instance, by yeast cell wall (zymosan), or lipopolysaccharide, some bacteria such as Escherichia coli and Staphylococcus aureus (Bjornson & Bjornson, 1977), in the absence of antibody. For defence, C3 is of central importance. It is the component present in highest concentration and is activated to C3b by both pathways. The activation of C3 has a positive feed-back effect in the alternative pathway and is also

necessary for initiating the membrane lysing capacity of C5 - C9. Patients who lack C3 have severe immunity deficiency while selective absence of other complement components is usually not associated with greatly increased susceptibility to infection (Lachmann, 1975).

Fig. 1 The reaction sequences of the complement system.



IV. Various aspects of phagocytic activity.

Phagocytic activity includes chemotaxis, opsonisation, phagocytosis, intracellular killing and digestive processes. It requires not only the phagocytes' intrinsic ability to move, ingest, kill and digest, but also the generation of chemotactic and opsonising factors which are provided by the body fluids (etc.). Phagocytic elimination therefore involves the interaction of phagocytes, serum substances and microorganisms.

(a) Chemotaxis.

Chemotaxis of leukocytes is defined as their movement towards increasing concentration of a chemotactic substance (Keller et al., 1977). It can be measured by in vivo or in vitro methods. In vivo methods have been applied only to animals but not to human beings, with one exception (Rebuck & Crowley, 1955). In vitro assays can be used not only to assess the ability of cells to move, but also the capability of serum and other body fluids to generate chemotactic substances. The method currently most widely used was described by Boyden (Boyden, 1962), who introduced chambers which consist of two compartments separated by a millipore membrane. Cells are added to one compartment and chemotactic substances to the other. After incubation at 37°C for given periods, the millipore membrane is removed and stained by a suitable method and examined microscopically. The results are either expressed as the distance over which the cells migrated or the number of cells which have migrated through the membrane. The membrane pore size is dependent on the cells tested, usually 3 µm for PMN and 8 µm for MNP.

(b) Phagocytosis.

The process of phagocytosis can be separated into two stages : first attachment of the particles to the phagocytes and secondly internaliza-

tion of the particles. During the early stage of engulfment, pseudopodiae of the phagocytic cell surrounds the particle, and eventually they meet and fuse to form phagocytic vacuoles. Phagocytosis is facilitated by opsonins. Specific antibodies, complement factors and perhaps some unknown serum substances have opsonic activity (Stossel, 1975). Opsonisation may be defined as the interaction of foreign materials (e.g. microorganisms) with opsonins. The mechanisms of opsonisation at the molecular and cellular levels are not yet fully understood.

(c) Intracellular killing and digestion.

Intracellular killing processes can be divided into several stages:

(i) establishment of contact between lysosomes and phagosomes;

(ii) fusion of the lysosomal membrane with the phagosomes;

(iii) discharge of contents of the lysosomes into phagosomes;

(iv) oxygen-independent and oxygen-dependent bacteriocidal system can act on microorganisms. The former comprises acidity, lysozyme, cationic proteins (phagocytins) and lactoferrin, the latter includes the production of hydrogen peroxide and superoxide by oxidases (Klebanoff, 1975);

(v) finally, disintegration of microorganisms by digestive enzymes such as acid phosphatase, ribonuclease, deoxyribonuclease, β-glucoronidase and cathepsin (Stossel, 1975).

Methods for measuring intracellular killing will be discussed in later chapters.

V. Humoral substances which influence the function of phagocytes.

(a) Chemotactic substances.

Chemotactic substances are derived either from the interaction between humoral substances and microorganisms, or directly from the microorganisms themselves (Wilkinson, 1974). Some complement components become chemotac-

tic if the activation of complement occurs either by the classical or alternative pathway. These include C5a and a related C5a-derived peptide (Shin et al., 1968), C3a and a related C3-derived peptide (Ward, 1967), and the $\overline{C567}$ complex (Ward et al., 1965). IgG molecules treated with proteases have also been found to be chemotactic (Yoshinaga et al., 1970). Peptides derived from Candida albicans were found to be chemotactic, (Cutler, 1977) and many denatured proteins such as albumin, casein and peptone have also been shown to possess chemotactic activity (Keller & Sorkin, 1965). Furthermore supernatants taken from stimulated lymphocytes contained chemotactic substances (Ward et al., 1969).

(b) Opsonins.

The term "opsonin" was introduced by Wright and Douglas some seventy years ago (Wright & Douglas, 1903), but the substances involved have not yet been fully characterised chemically and their mechanism of action is still not completely understood. Immunoglobulin (natural antibody), specific antibody and complement enhance phagocytosis. The function of an opsonin is to coat particulate matter (e.g.microorganisms), thus enabling recognition to occur via the Fc and C3b receptors present on the phagocyte membrane (Henson, 1969).

VI. Summary of phagocytic activity.

Phagocytic activity is a complex mechanism which operates not only as a non-specific first line of defence but also in the final stage of elimination of invading organisms by processes of specific immunity. It comprises chemotaxis, phagocytosis , intracellular killing and digestion and it requires or is enhanced by humoral substances.

VII. General considerations concerning methodology.

Phagocytic elimination of microorganisms can be studied *in vivo* or *in vitro*. The former approach is not suitable for analysing the contribution of the individual components of phagocytic function such as the effects of different humoral substances or the relative importance of polymorphonuclear and mononuclear cells; nor can it help to distinguish between ingestion and intracellular killing of microorganisms. *In vivo* studies are therefore largely limited to the measure of the clearance of intravenously injected colloids, red blood cells or bacteria which are mainly phagocytosed by the fixed macrophages of the liver and spleen (Rowley, 1962).

In vitro studies, on the other hand, have distinct analytical advantages, because it is possible to measure chemotaxis, phagocytosis and intracellular killing independently. Phagocytosis and intracellular killing have been measured by methods involving both microscopic examination (Metchnikoff, 1905), and biochemical analysis (Karnovsky, 1962) such as carbon dioxide production (Stähelin et al., 1957), nitroblue tetrazolium reduction (Baehner & Nathan, 1968), oxygen consumption (Baldridge &Cerarrd, 1933) as well as microbiological assays such as colony counting (Cohn & Morse, 1959). The difficulties of microscopic examination include the problems of distinguishing between engulfment and attachment as well as changes in cell morphology. Furthermore, counting under the microscope is inevitably subjective. The biochemical methods are based on the assumption that certain biochemical changes reflect phagocytosis in a direct manner, but this may not always be correct (Cohn & Morse, 1960). Colony counting measures the decrease of viable (i.e. colony forming) microorganisms following incubation of phagocytes, micro-

organisms and serum. The advantage of the colony counting technique is that it is possible to measure both phagocytosis and intracellular killing independently and simultaneously. However , this approach is time consuming and variable clumping of the microorganisms (Chilgren et al., 1968) is a major problem. The use of inert particles such as carbon and quartz (Fenn. 1920-1921), or paraffin oil particles containing oil red O (Stossel, 1973), makes it possible to measure phagocytosis quantitatively and more objectively, but these particles clearly cannot be employed for measuring intracellular killing. Ideally assays for measuring opsonisation, phagocytosis and intracellular killing should satisfy the following criteria:

(i) the method of mixing and the number of particles and microorganisms must be as constant as possible (Fenn, 1920-1921);

(ii) opsonisation, engulfment and intracellular digestion should be determined in relation to a defined type of particle ;

(iii) the assay must be objective and easy to perform;

(iv) the assay must discriminate between cellular and humoral components of phagocytic activity;

(v) if the assay is to be applied in clinical investigation, it should require as little blood as possible;

(vi) the results should preferably be available within 24 hours.

If all the above criteria are fulfilled, the use of microorganisms is preferable to that of inert particles. Microorganisms to be used should be as follows:

(i) pathogenic without being virulent and hence dangerous to handle;

(ii) not multiply during the assay period;

(iii) easy to check for number and viability;

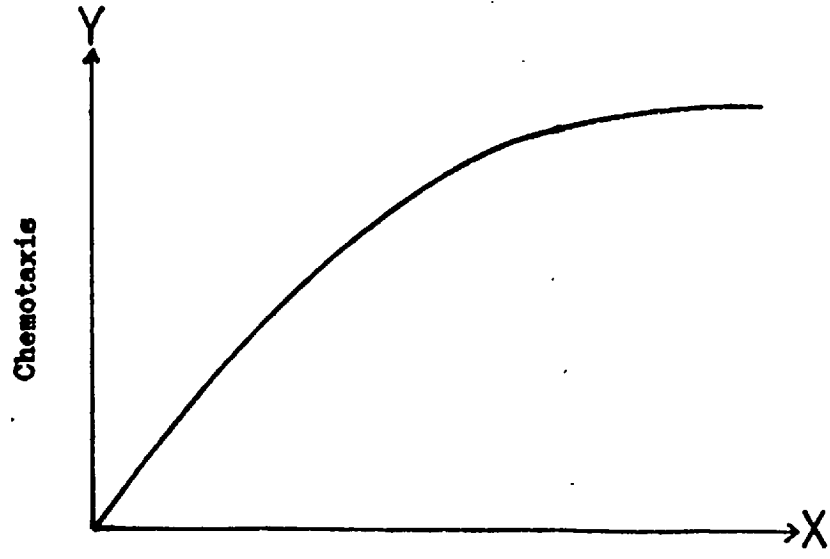
(iv) stable during storage.

VIII. Expression of phagocytic activity.

Phagocytic activity in the body is made up of several parameters, of which three are measurable at the moment; chemotaxis, phagocytosis and killing. An attempt is made to construct a model to express these activities.

Chemotaxis should be considered as a function of two independent activities, migration of cells on the one hand and generation of chemotactic factors on the other. It can therefore be expressed in a two dimensional diagram (Diagram I), in which the X-axis represents the production of chemotactic substances, and the Y-axis directional leukocyte migration. The production of chemotactic substances can result e.g. from the interaction of serum and microorganisms.

DIAGRAM I CHEMOTAXIS.

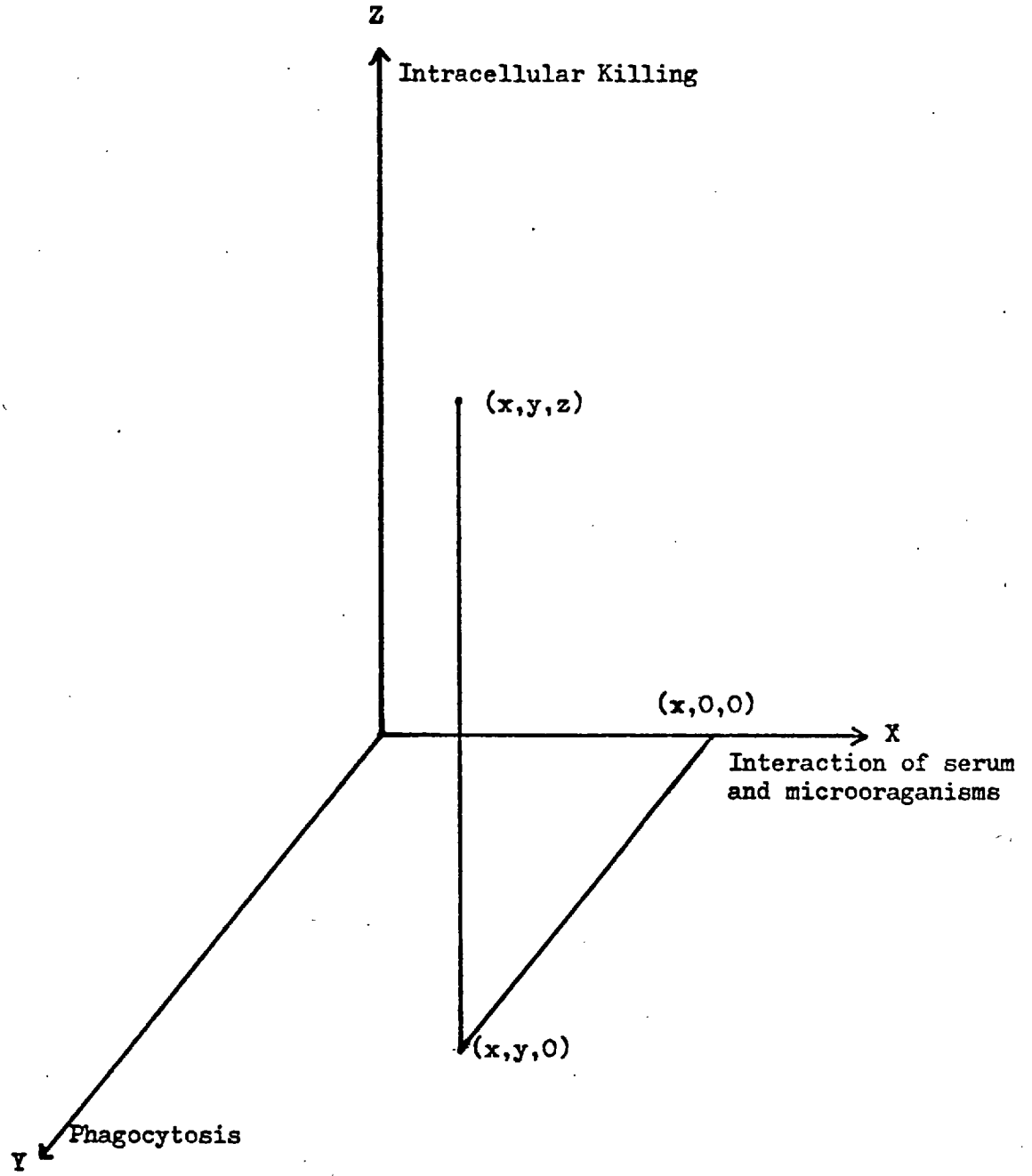


Phagocytosis and intracellular killing can be considered as one entity because intracellular killing cannot occur if phagocytosis does not take place. Phagocytosis requires opsonins, and intracellular killing may also require humoral substances (Yamamura & Valdimarsson, 1977). It is possible to express phagocytosis and intracellular killing in a three dimensional diagram (Diagram II). The X-axis represents interaction between serum and microorganisms, including the generation of opsonins and chemotactic substances. The Y-axis represents phagocytosis, and the Z-axis intracellular killing. To express intracellular killing geometrically the series of events leading to killing has to be analysed sequentially to determine first the function of X, then of Y and finally Z. According to Diagram II, the interaction of serum substances and microorganisms (expressed as x,0,0) should be measured in the absence of phagocytic cells. Phagocytosis is expressed as x,y,0 and intracellular killing x,y,z.

Thus phagocytosis may be calculated as the function of x and y and intracellular killing as the function x,y,z. However, because in practice opsonisation (x) is measured as phagocytosis (x,y,0), y must be kept constant . Phagocytosis (y) is measured under conditions where x is constant. Furthermore x and y must be kept constant when intracellular killing (z) is measured.

(See overleaf for Diagram II).

Diagram II Expression of phagocytosis and intracellular killing.



IX. Objectives of this study.

The main objectives of this study were:

(i) to develop and standardise radiometric assay systems for measuring opsonisation, phagocytosis and intracellular killing of fungal organisms; and,

(ii) to apply these assays clinically and for analysing the various mechanisms of phagocytic activity.

Chapter 2

Materials and Methods

MATERIALS AND METHODS

Medium

RPMI 1640 buffered with Hepes was used throughout this study and is referred to as "medium" in the text.

Mononuclear phagocytes

Mononuclear phagocytes (MNP) were obtained by differential centrifugation using a Ficoll-Hypaque density gradient (density=1.075 g/ml) (Bøyum, 1964). Heparinised (20-40 units heparin/ml) or defibrinated blood was carefully layered on the gradient and centrifuged at 400g for 30min at 20°C. Mononuclear leukocytes were collected from the interphase between plasma (or serum) and the gradient, washed at least three times with medium and then adjusted to the appropriate cell concentration. The suspensions usually consisted of 70-80% lymphocytes and 20-30% monocytes, and it was contaminated by less than 2% PMN; the yield of mononuclear cells ranged between 20-40%.

Polymorphonuclear leukocytes

Polymorphonuclear leukocytes (PMN) were obtained from either heparinised whole blood or from the bottom layer after centrifugation on Ficoll-Hypaque.

(1) One volume of heparinised blood was mixed with half a volume of Dextran 110 (6% w/v in 0.9% NaCl) and allowed to stand at 37°C for 30 minutes. The leukocyte-rich plasma was collected and centrifuged and the leukocytes were washed at least five times with medium and adjusted to the appropriate concentration. The suspension contained 50-60% PMN, 30-40% lymphocytes and 5-10% monocytes, the yield of PMN usually being around 50%.

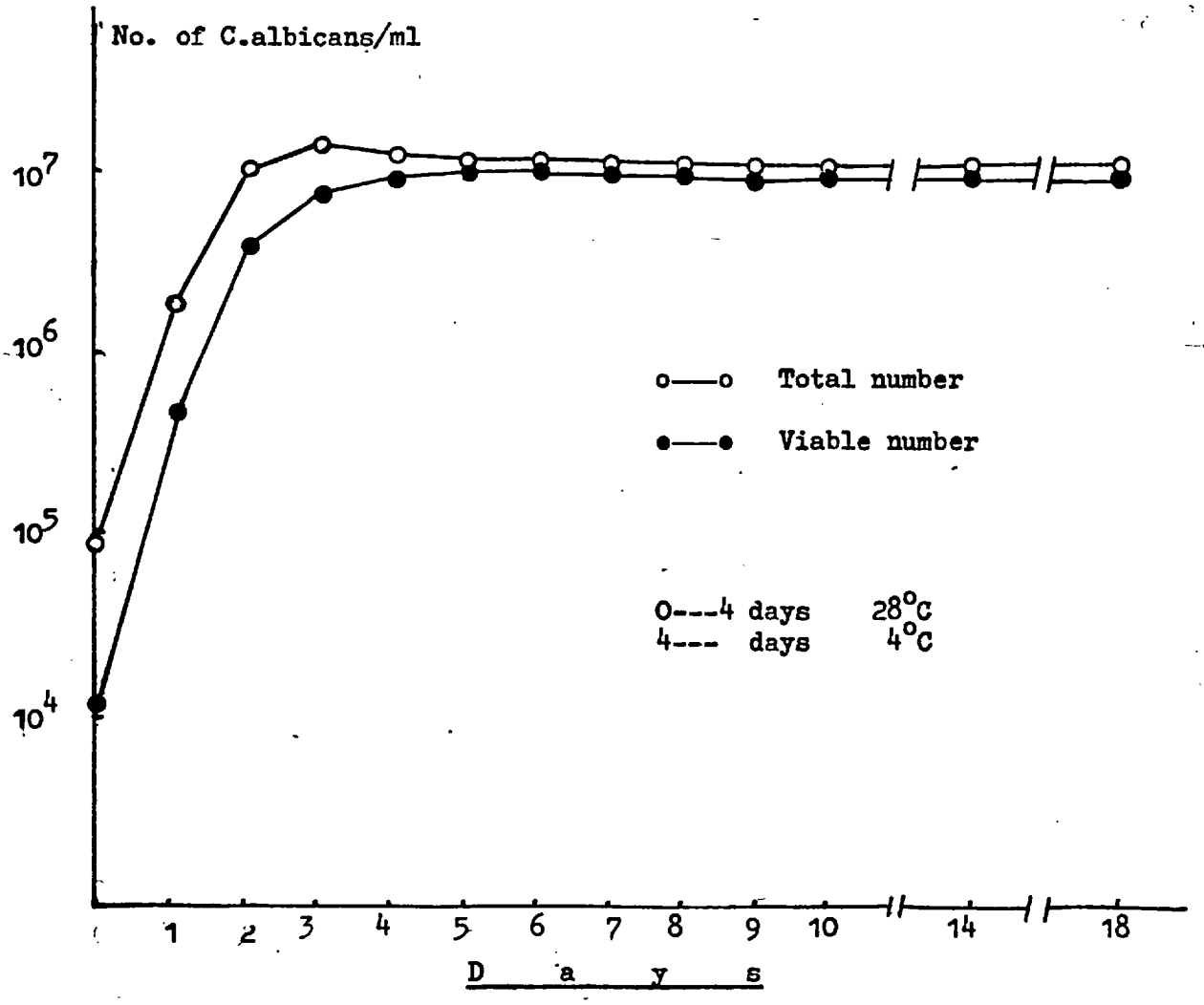
(2) The pellet obtained after Ficoll-Hypaque centrifugation was diluted twice with medium to which 1/3 volume of Dextran 110 was added. The mixture was allowed to stand for 15-30 min at 37°C before the leukocyte-

rich upper layer was collected and centrifuged. The leukocytes were washed at least three times with medium, counted and adjusted to the appropriate concentration. The cell suspension usually contained 80-95% PMN, 5-20% lymphocytes and 1% monocytes. The yield of PMN was 60-70% i.e. better than that obtained by the first method. There were no functional differences in the PMN isolated by the two methods.

Candida albicans

Candida albicans strain UC 820 was maintained on 2% agar slopes containing 1% peptone and 2% glucose. Subcultures were made in 1% peptone and 2% glucose broth for 3-4 days at 28°C and kept at 4°C up to 2 weeks without loss in number or in viability (98%). The broth cultures gave a homogeneous yeast form of C.albicans.

Fig.2 Candida albicans growth curve



The growth curve of C.albicans is shown in Fig. 2 . After 3 days of culture in broth , there was no increase in number nor loss of viability, the culture having reached a steady state. It was therefore decided that more than three days-old C.albicans should be used as the test organism.

Saccharomyces cerevisiae

Saccharomyces cerevisiae, strain L, was isolated from CWE Brewer's yeast, which is specially marketed for rapid fermentation . The yeast was maintained on 2% agar slopes containing 1% peptone and 2% glucose. Subcultures were made in medium for 3 days at 28°C and these could be kept at 4°C for up to two weeks without loss in number or viability (the latter being greater than 98%). The growth curve was similar to that of C.albicans.' The strain was kindly characterised by Mrs Barbara Kirsop of the Brewing Industry Research Foundation, Redhill, Surrey. It is now maintained at the National Collection of Yeast Cultures and has been given the following number NCYC 873.

Table 1 shows that when S.cerevisiae and C.albicans were incubated in medium containing 2.5% human serum, there was no increase in the number of organisms up to three hours and no loss of viability. It could therefore be concluded that during the time required for the various assays the number of viable organisms would remain constant. S.cerevisiae and C.albicans were counted in a haemocytometer chamber, every cell was counted even if cells were sticking together forming one unit.

TABLE 1 The number of organisms of C.albicans and S.cerevisiae cultured for 3 hours in medium containing 2.5% serum.

Time (hours)	C.albicans ($x10^6$/ml)	S.cerevisiae ($x10^6$/ml)
0	2.5± 0.2	2.5± 0.2
1	2.6± 0.2	2.4± 0.2
2	2.4± 0.1	2.4± 0.1
3	2.5± 0.1	2.5± 0.1

Few mycelial forms of C.albicans were observed after 3 hours of incubation and there was no change in number and viability which remained more than 98%.

Labelling C.albicans with ^{51}Cr

C.albicans which had been kept in culture for at least 3days, was centrifuged for 10min. 100 µCi-200 µCi (0.1ml-0.2ml of 1mCi/ml Sodium chromate solution) was gently added onto the pellet of C.albicans and carefully mixed with the cells with a pasteur pipette, without spreading the cells too widely over the container. The mixture was allowed to stand at 28°C for 60-120 min with occasional mixing. The C.albicans was then washed at least three times with phosphate buffered saline or saline, and resuspended in cold medium. The number of organisms was adjusted to 25 x 10^6/ml. This suspension was kept at 4°C until used. The labelled organisms could be kept up to 3-4 weeks, provided that the original ^{51}Cr incorporation was high enough. The release of ^{51}Cr from the organisms was minimal during storage at 4°C, any decrease in radioactivity being mainly due to the physical half life of ^{51}Cr, which is 27.8 days.

Sera

All sera were kept at -190°C until used. Sera deficient in C2, C3, C5 and C6 were kindly supplied by Dr P.J.Lachmann, Cambridge, UK.

R3 reagents

R3 reagents were made according to the method of Lachmann, Hobart and Aston,(1973). Human serum was incubated with zymosan (Sigma) 1.25mg/ml, for 45 minutes at 37°C under constant rotation (30rpm). The zymosan was removed by centrifugation and the serum sterilised by filtration through a 0.22 μm millipore membrane. The reagents retained normal levels of C1, C4, and C2 but native C3 was approximately 40% of the original level, as determined by two dimensional immunoelectrophoresis (Clarke & Freeman, 1968). There was no haemolytic or alternative pathway activity.

Heat-inactivation

Sera were incubated at 56°C for 30 minutes for whole complement pathway inactivation and at 50°C for 15 minutes for factor B inactivation.

MATERIALS AND EQUIPMENT

The following items were used.

Agar	Oxoid
Dextran 110 (6%w/v in 0.9% NaCl)	Fisons
DNase DN 100 (Deoxyribonuclease)	Sigma Chemical Co.
Ficoll	Pharmacia
Glucose	British Drug House (BDH)
Heparin (1,000IU/ml)preservative free	Paines & Byrne Ltd.
Hepes	Hopkins & Williams Co.
Hypaque	Winthrop Laboratory
Lymphoprep (density 1.077g/ml)	Nyegaard & Co. A/S,Oslo,Norway
Peptone	Difco
PPO (Diphenyloxazol)	BDH
RPMI 1640	Flow Laboratory
Sodium chromate $Na_2{}^{51}Cr_2O_7$	Radiochemical Centre
Sodium deoxycholate	BDH
Toluene (ANALAR)	BDH
^{3}H-Uridine	Radiochemical Centre
Zymosan	Sigma Chemical Co.
β-Counter	Packard Tri-carb Scintillation spectrometer Model B 2450
γ-Counter	Packard
Hamilton syringe with automatic dispenser	Hamilton
LP3 Plastic tubes 7 x 65 mm	Luckam
Microtitre plate	Cooke
Millipore filter	Millipore
Multiple Cell Harvester	Skatron
Rotator (30rpm)	Jencons
Scintillation vials	Packard

Chapter 3

Phagocytosis of Candida albicans measured as Inhibition of Uridine Incorporation.

INTRODUCTION

Drawbacks of existing methods

Phagocytosis has been measured by using ; (1) microscopic assessment, (2) the decrease in the number of colony forming cells, (3) metabolic indicators e.g. the rate of glucose oxidation to CO_2 or the rate of nitroblue tetrazolium (NBT) reduction, or (4) colourimetric or radiometric assays using inert particles. (Wright & Douglas, 1903; Metchnikoff, 1905; Fenn, 1920-1921; Baldridge & Cerarrd, 1933; Cohn & Morse, 1959; Karnovsky, 1962; Baehner & Nathan, 1968; Stossel, 1973).

Microscopic estimation is time consuming and liable to subjective errors. The use of metabolic indicators is based on the assumption that such events reflect phagocytosis. However, certain particles can stimulate cellular metabolism without the occurence of phagocytosis (Cohn & Morse, 1960). Moreover, in chronic granulomatous disease phagocytosis does not result in increased glucose oxidation or NBT reduction (Good et al., 1968). Variable clumping of microorganisms is a major source of error in colony counting methods, which are also cumbersome and involve complex microbiological techniques (Chilgren et al., 1968).

Techniques in which inert materials such as carbon, quartz (Fenn, 1920-1921), coloured paraffin oil (Stossel, 1973) are used, are quantitative and objective. However, large numbers of cells are required and inert particles cannot be used for measuring subsequent intracellular killing. The advantage of using the same particles for assessing both phagocytosis and killing has been discussed in Chapter 1.

Ideally, opsonisation should be measured by using phagocytes with constant and defined ingestive properties, under conditions in which the amount of available opsonins limits the number of particles that can be engulfed. Conversely the intrinsic ability of phagocytes to ingest should be measured under conditions in which opsonins do not limit the phagocytosis.

Ideal assay conditions

In theory it should be possible to measure opsonisation without using phagocytes because opsonisation represents the interaction between opsonins and microorganisms. However, in practice, phagocytosis must be used as an end point for opsonin activity until the opsonising substances can be measured chemically. Objective and quantitative assays for phagocytosis are therefore required for accurate measurement of opsonin activity under conditions in which the efficiency of phagocytes is constant. Conversely, it is not possible to measure accurately the intrinsic efficiency of phagocytes to engulf unless conditions of optimal and constant opsonin levels have been established. Thus, the ingestive capacity of phagocytes and the opsonic capacity of serum must be measured independently.

Assuming that each phagocyte can ingest N optimally opsonised particles, the number engulfed by each cell is dependent on the number of particles available as long as that number is less than N. However, the amount engulfed will not increase if the particle number exceeds N. An assay for measuring intrinsic efficiency of phagocytes to ingest must therefore employ more than N optimally opsonised particles per phagocyte.

To summarise, the intrinsic efficiency of phagocytes to engulf and the opsonising capacity of serum must be measured independently. The condition of measuring the intrinsic efficiency of phagocytes to ingest must include (1) phagocytes, (2) excess number of microorganisms and (3) a sufficient amount of serum. It will be demonstrated that the radiometric assay to be described here fulfills the above criteria.

METHOD

The preparation of PMN and C.albicans has already been described in Chapter 2.

Can phagocytosed C.albicans incorporate ^{3}H-uridine from the culture medium?

To answer this question, PMN leukocytes from patients with chronic granulomatous disease (CGD) were used. These are known to have a normal capacity to engulf but are not capable of killing or preventing germination of engulfed C.albicans. It was found that PMN from three such patients, inhibited uridine incorporation by C.albicans to the same extent as PMN from healthy control subjects (Table 2). It was therefore concluded that engulfed live C.albicans cannot incorporate ^{3}H-uridine from the culture medium.

TABLE 2 Effect of PMN leukocytes from CGD patients on ^{3}H-uridine incorporation by C.albicans.

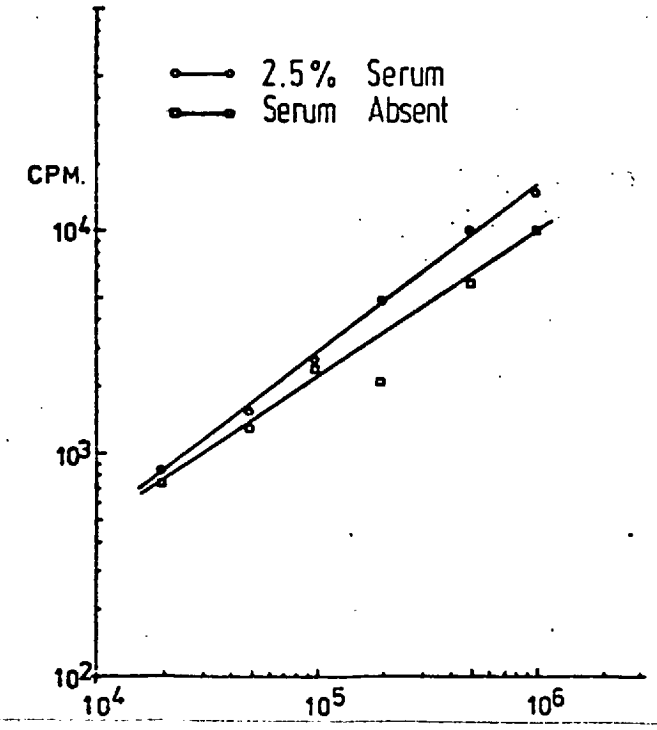
	Phagocytosis (% Inhibition) *		
	Experiment I		Experiment II
CGD (a)	44 ± 4	CGD (c)	65 ± 5
CGD (b)	45 ± 5	Father of c	73 ± 7
Mother of a & b	44 ± 4	Mother of c	75 ± 8
Normal control	48 ± 5	Normal control	73 ± 6
Normal range	33 - 71	Normal range	33 - 71

* The results are expressed as per cent inhibition of ^{3}H-uridine incorporation.

Incorporation of ^{3}H-uridine into C.albicans.

As shown in Table 1, the number of C.albicans does not increase during three hours of incubation. It was also found that the incorporation of ^{3}H-uridine into C.albicans was directly related to the number of cells, i.e. there was a direct linear correlation between the incorporation of ^{3}H-uridine and the number of C.albicans (Fig. 3). It is therefore possible to determine the number of C.albicans by measuring the incorporation of ^{3}H-uridine. Furthermore, if incorporation of ^{3}H-uridine by phagocytes and ingested C.albicans is negligible, it should be possible to determine the number of non-ingested C.albicans by comparing the uridine incorporation of a fixed number of C.albicans in the presence and absence of phagocytic cells during a given time of incubation.

Fig. 3 Incorporation of ^{3}H-uridine into C.albicans.

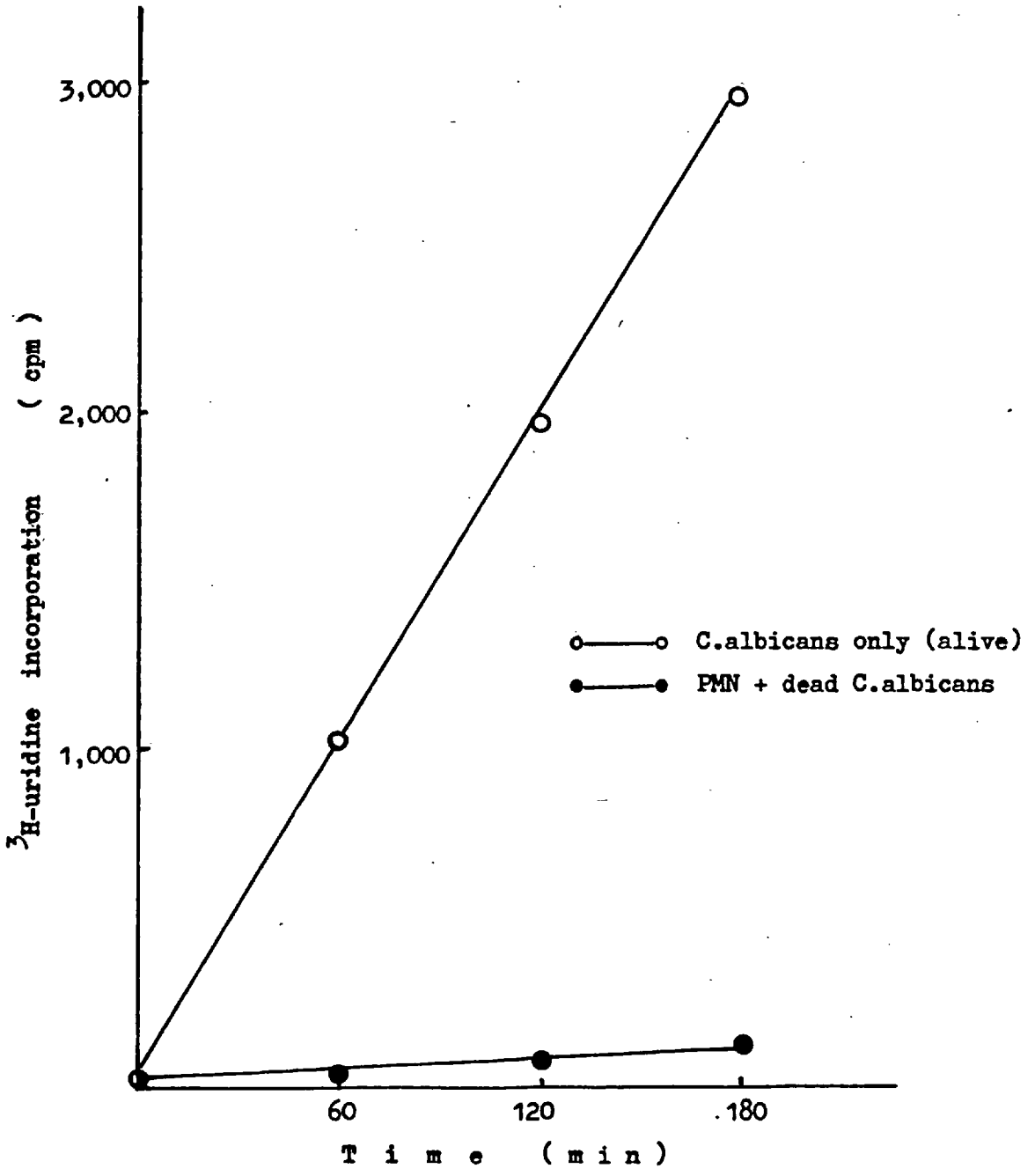


Incorporation of ^{3}H-uridine by varying numbers of *C. albicans* incubated at 37°C for 1 h. □——□, Serum free medium. ○——○, 2.5% serum present.

Is ^{3}H-uridine incorporation into phagocytes negligible ?

Experiments were carried out using heat-killed C.albicans and phagocytes. As shown in Fig. 4, incorporation of ^{3}H-uridine by PMN was negligible during a culture period of one hour.

Fig. 4 Incorporation of ^{3}H-uridine into phagocytes.



Standard assay conditions.

Unless otherwise stated assays were performed as follows. A mixture of 5×10^5 C.albicans and 5×10^4 PMN in 0.5ml of medium containing 2.5% serum, was incubated for 30 minutes at 37°C under constant rotation (30 rpm). 0.1ml aliquots of the mixture were then transferred in duplicates to microtitre wells to which 0.1 µCi of ^{3}H-uridine had been added. The microtitre plate was incubated for a further 60 minutes at 37°C. The cells were then harvested by an automatic cell harvester onto glass fibre discs which were dried and placed in scintillation bottles to which 5 ml of scintillation fluid (4g PPO/l toluene) was added. The radioactivity was counted in a Packard Tri-carb scintillation spectrometer, model B 2450. As a control, 5×10^5 C.albicans were incubated in 0.5ml of medium containing 2.5% serum. These cultures were treated and their uridine incorporation measured as described above. Controls gave the uridine incorporation by the C.albicans in the absence of phagocytes.

Presentation of data

As discussed above, inhibition of ^{3}H-uridine reflects the degree of phagocytosis. The results are presented as per cent inhibition of uridine incorporation using the following equation A:

$$I = 100 \left(1 - \frac{\text{cpm (C.albicans + PMN)}}{\text{cpm (C.albicans only)}}\right)$$

Phagocytosis can also be expressed as a phagocytic index, i.e. the average number of organisms ingested by each phagocyte using the following equation B:

$$P = \frac{I \times R}{100}$$

I = per cent inhibition of ^{3}H-uridine incorporation

R = the ratio of C.albicans/PMN

P = phagocytic index

RESULTS

The following assay conditions were investigated:

(i) the most suitable number of C.albicans,

(ii) the optimal serum concentration,

(iii) a suitable ratio of C.albicans and PMN,

Once standard condition had been determined, the following was also studied:

(a) the effect of pH,

(b) serum (or plasma) requirements,

(c) the normal range for human PMN.

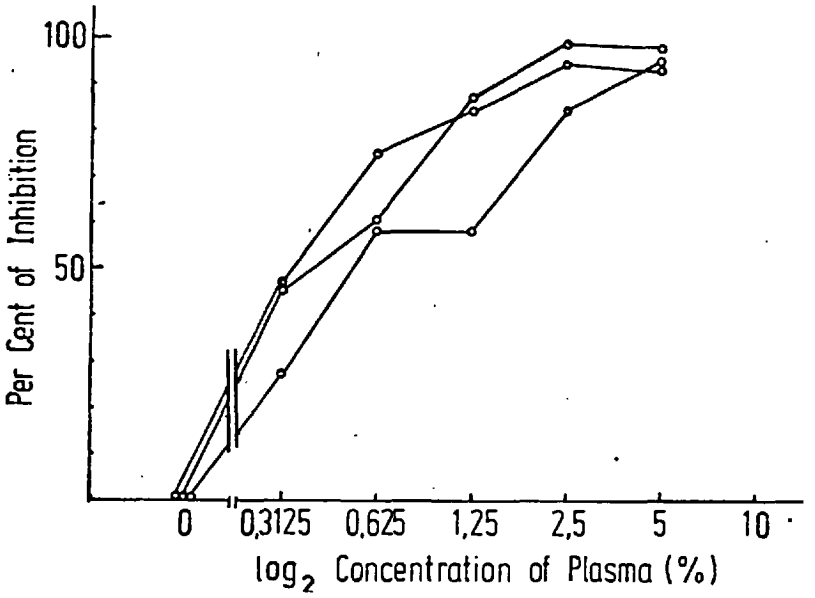
(i) Selection of the most suitable number of C.albicans.

As shown in Fig. 3, the smallest number of C.albicans which was detected by incorporation of ^{3}H-uridine was 2 x 10^{4} per well. The number used had therefore to be greater than this. Incorporation by PMN was less than 100 cpm (Fig. 4), which is regarded as negligible provided that incorporation of ^{3}H-uridine by C.albicans is more than 2000cpm. This amount was incorporated by 1 x 10^{5} of C.albicans which was therefore the number used per well. Aliquots of 0.1ml from 0.5ml culture (5 x 10^{5} C.albicans) contains this number of C.albicans.

(ii) Determination of optimal serum concentration.

Phagocytosis was measured using 5 x 10^{5} C.albicans and 1 x 10^{5} PMN with various serum concentrations from 0 to 5 %. As shown in Fig. 5 phagocytosis (per cent inhibition), was dependent on the concentration of serum up to 2.5%, beyond which it reached a plateau. Thus, 2.5% serum provided optimal opsonisation for 5 x 10^{5} C.albicans. It should be noted that if the number of C.albicans increases the concentration of serum may also have to be increased in order to achieve optimal opsonisation.

Fig.5 Effect of varying serum concentration on phagocytosis.



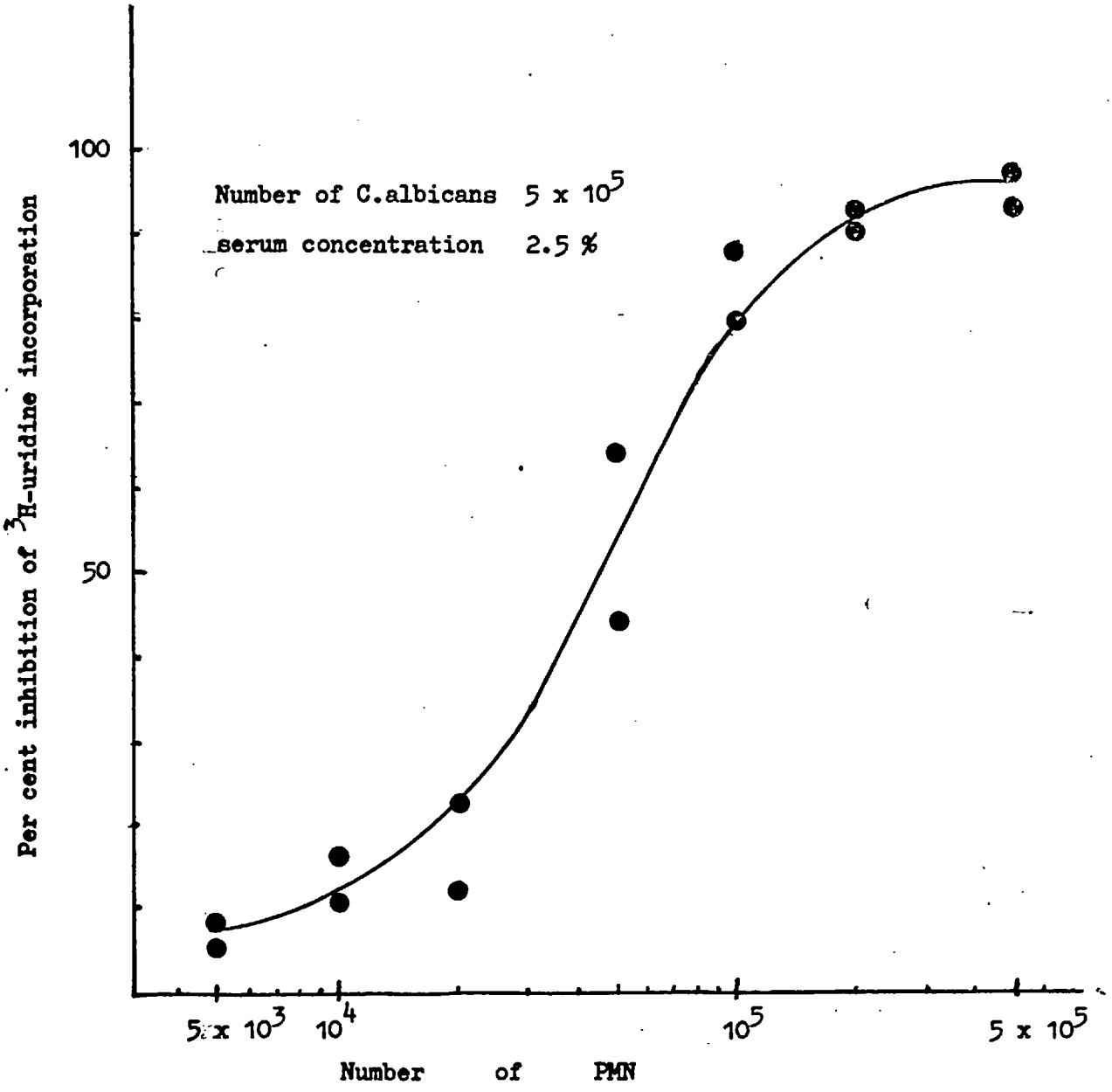
Effects of different plasma concentrations on the incorporation of ^{3}H-uridine by *C. albicans* in 3 different experiments. The PMN/*C. albicans* ratio was 1/5.

(iii) Effects of varying the ratio between C.albicans and PMN.

Using 5×10^5 C.albicans and 2.5% serum, the number of PMN was varied from 5×10^3 to 5×10^5. The results are shown in Fig. 6. There was a linear increase of phagocytosis from 2.5×10^4 to 1×10^5 PMN. It was therefore decided to use 5×10^4 PMN in the standard assay together with 5×10^5 C.albicans and 2.5% serum.

Thus, standard conditions for measuring phagocytosis were chosen so that 5×10^5 C.albicans , 5×10^4 PMN and 2.5% serum were used.

Fig. 6 Effect on phagocytosis of varying numbers of PMN.



(a) The effect of pH.

Although minimal changes in pH (± 0.1) were observed during the assay, it was nevertheless necessary to study the effect of varying pH because pH changes alter the rate of uridine incorporation into C.albicans. Fig. 7 shows that the incorporation decreased in a linear fashion with increasing pH.

Fig. 7 Effect of pH on ^{3}H-uridine incorporation in C.albicans.

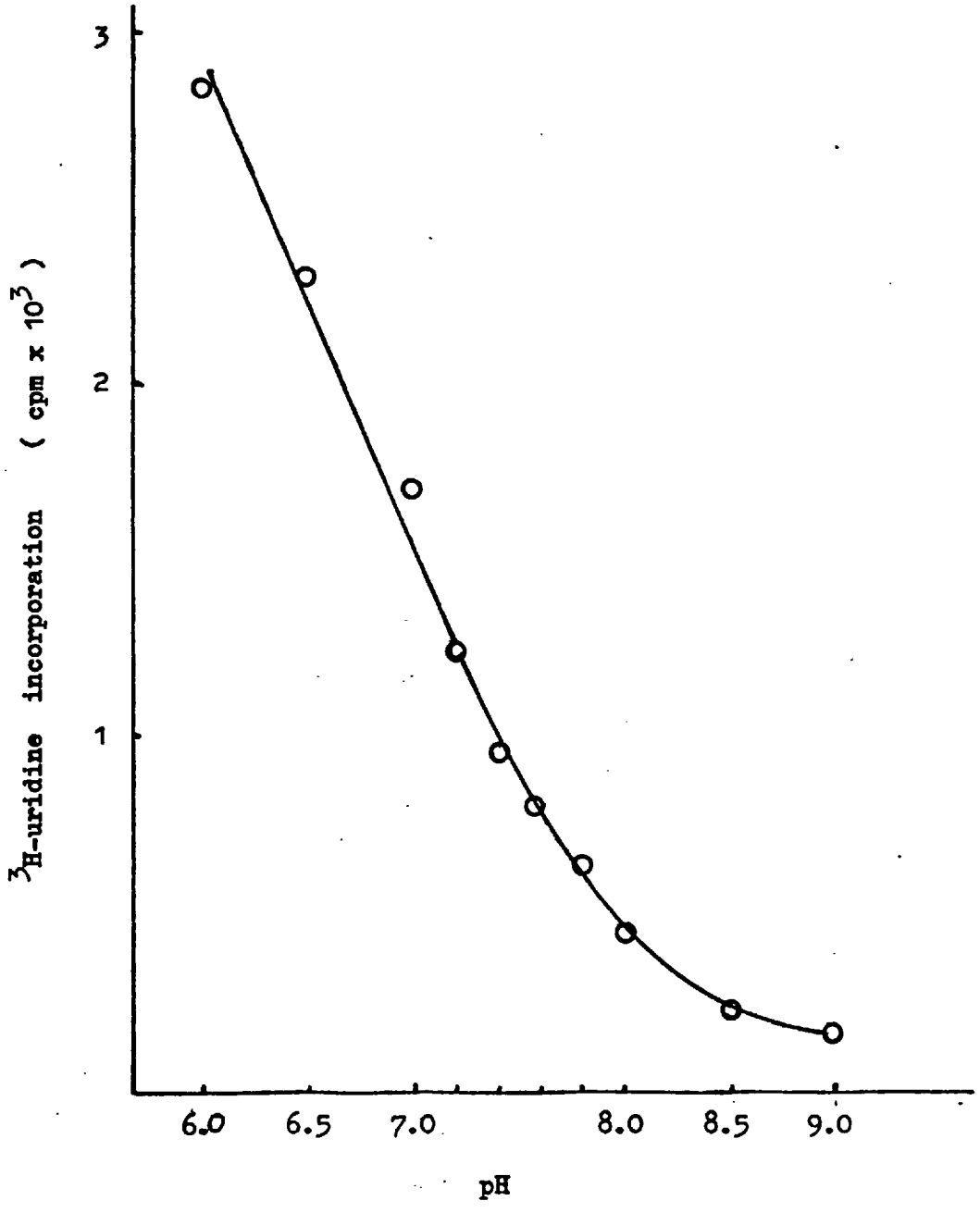
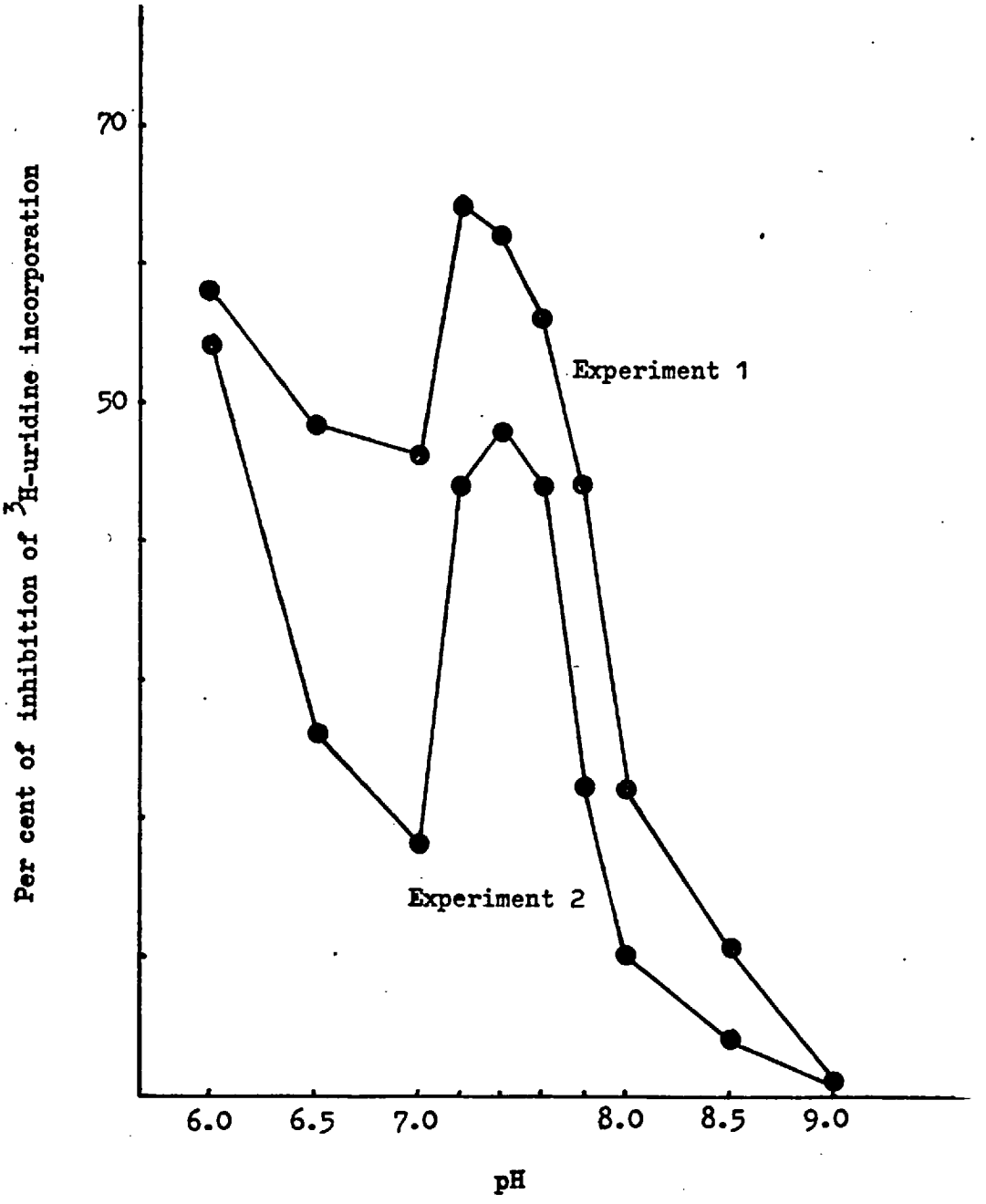


Fig.8 Effect of pH on phagocytosis of C.albicans.



Apart from varying the pH, standard assay conditions were used. The results are shown in Fig. 8. At pH 7.0, phagocytosis was somewhat less than at pH between 7.2 and 7.6. It is therefore important to maintain the pH around 7.4 throughout the assay period. This may be difficult if a large number of C.albicans is used. For this reason, bicarbonate buffer should not be used because of its susceptibility to pH changes.

(b) Serum requirements

EDTA treated, heat-inactivated (56°C, 30min), zymosan treated, complement deficient, agammaglobulinaemic and heparinised sera were investigated. Table 3 summarises the results.

TABLE 3 Serum (plasma) requirements.

Type of sera*	Phagocytosis (%)	
	Experiment I	Experiment II
Heated (56°C,30min)	46	56
Zymosan treated	51	46
C2 deficient	49	62
C3 deficient	65	62
C6 deficient	41	45
Agammaglobulinaemic (50 mg/l Igs)	48	43
EDTA treated	2	1
Heparin-plasma	50	61
No serum	3	2
Normal range	33 - 75	33 - 75

*2.5% serum or plasma used throughout.

All the sera as well as the heparinised plasma possessed normal opsonising capacity for C.albicans, while no engulfment occured in EDTA trea-

ted serum. Two points should be stressed here: first, all sera tested possessed opsonic activity to C.albicans; second, heat-inactivated serum had only marginally less opsonic activity than the intact serum when compared within an experiment. The experimental error within an experiment was ± 5%, and between experiments ± 10%.

(c) Normal range

Using standard assay conditions, the normal range was determined using PMN obtained from 20 normal healthy individuals (Table 4).

Table 4 The normal range

	Ratio C.albicans : PMN	
	10 : 1	5 : 1
Mean (Medium)	50	92
Range (± 2SD)	33 - 75	74 - 99

Thus, when a 10:1 ratio was used, a Poisson's distribution was observed while a 5:1 ratio gave a logarithmic distribution. It is possible to calculate the phagocytic index according to equation 8, which is five C.albicans per phagocyte.

DISCUSSION

The ingestive capacity of phagocytes and the opsonising capacity of serum can be objectively measured by this radiometric method. Phagocytosis is determined by calculating the number of non-ingested C.albicans. This type of approach was first adopted by Fenn(1920-1921), using carbon and quartz. More recently a number of workers have adopted this principle, for example by using the Coulter counter (Harvey et al., 1978,personal communication), or microscopic counting (Leijh et al., 1977).

My radiometric assay has 5% error within an experiment and 10% error between experiments. This error may be due to various factors including variability in both leukocytes and C.albicans. Neither complement nor immunoglobulin appears to be required for effective opsonisation of C.albicans (Table 3).

No attempts were made in this study to identify the nature of this opsonin, that will have to be done in future experiments by its isolation and purification.

My method is particularly suitable for clinical investigations because it only requires 1×10^5 PMN per test. It can therefore easily be applied to study young children as well as patients with severe neutropenia. It can also be applied for measuring opsonisation of other microorganisms , provided that conditions discussed earlier in the chapter are satisfied. A radiometric method for measuring opsonisation of Saccharomyces cerevisiae will be discussed in a later chapter.

Chapter 4

Intracellular Killing of Candida albicans measured by ^{51}Chromium Release Assay.

INTRODUCTION

Intracellular killing is defined as the death of microorganisms due to intracellular processes.This may or may not lead to digestion of the microorganisms, but so far it has not been possible to measure intracellular killing and digestion independently. Thus it is not possible in practice to dissociate the two processes.

In previous methods killing has been assessed either by counting colony forming units (Cohn & Morse, 1959), or measuring the release of radioisotopes such as ^{32}P from microorganisms labelled with radioactive precursors (Miya & Marcus, 1961). The former method involves time consuming microbiological techniques and clumping of the microorganisms can be a major drawback (Chilgren et al., 1968). For the latter method, the microorganisms must be cultured in the presence of the radioisotope for a lenghthy period usually for more than 16 hours.

^{51}Cr release assays for measuring the lysis of ^{51}Cr labelled mammalian target cells have been widely used (Perlmann & Holm, 1969). The ^{51}Cr is incubated with the target cells at a suitable concentration, is readily taken up by the cells and binds to their proteins. When the cells are killed a large proportion of the chromium is liberated and cannot be reutilised by the surviving cells. A chromium release assay could therefore be applied to measure intracellular killing of C.albicans.

Kinetic consideration.

As mentioned in the general introduction, intracellular killing cannot occur without phagocytosis. Most microorganisms, including C.albicans must be opsonised before they can be ingested. In theory, therefore, intracellular killing could be defined as the rate of killing under defined conditions for opsonisation and phagocytosis (x, y are constant, see Diagram II). If opsonisation is not optimal, then intracellu-

lar killing will be limited by the rate of phagocytosis and not by the killing capacity of the phagocytes. If the conditions for a killing assay are to be standardised one must, therefore, know the optimal level of opsonisation.

Killing efficiency can be defined as the average number of organisms killed by each phagocyte during a given period in an assay. Efficiency may or may not depend on the number of organisms ingested per phagocyte. At a low ratio between organisms and phagocytes the killing is limited by the number of available organisms, but the killing mechanisms may be overwhelmed if too many organisms are ingested. The ratio at which the killing efficiency is greatest must therefore be defined for different microorganisms in relation to each type of phagocyte. This ratio is defined as the optimal killing ratio.

Experimental design.

Initially, the following questions were studied in order to ascertain whether intracellular killing of C.albicans could be measured by a ^{51}Cr release assay.

(1) Is ^{51}Cr released as a result of intracellular killing ?

(2) If release occurs, does it correlate with a conventional intracellular killing assay ?

METHODS

The preparation of C.albicans and PMN suspensions has already been described in Chapter 2.

As shown in Table 5, C.albicans (5×10^5), incorporate a sufficient amount of ^{51}Cr. It was therefore decided to use this number in the standard assay. The ratio between C.albicans and PMN could be determined through theoretical considerations. On average, each phagocyte can engulf up to five C.albicans (Chapter 3), therefore if the killing efficiency is independent of the number of ingested C.albicans the ratio should be five. However if the killing efficiency decreased when phagocytes ingested too many organisms a ratio of less than five should be used.

TABLE 5 ^{51}Cr incorporation into C.albicans

Age of C.albicans (days)	^{51}Cr incorporation/ 5×10^5 C.albicans (cpm)
3	21 576
4	15 600
5	6 652
6	12 364
7	13 696
8	5 601
10	8 096
14	5 857
18	4 812

Standard assay conditions.

Unless otherwise stated, standard experimental conditions were as follows: 5×10^5 C.albicans and 5×10^5 PMN suspended in 0.5ml of medium containing 2.5% serum. The mixture was incubated for 60 minutes at 37°C under constant rotation (30 rpm) and 20 µg DNase and 1.2mg sodium deoxycholate then added. The mixture was incubated for a further 10 minutes at 37°C under constant rotation and then centrifuged at 1 000 g for 10 minutes. Aliquots of supernatant were pipetted into plastic tubes to count the radioactivity.

Presentation of data.

The results were expressed by the following equation C;

$$^{51}\text{Cr release} = \frac{X - S}{T - S} \times 100$$

X = the radioactivity released in the presence of PMN,

S = the spontaneous release after 60min in medium containing 2.5% serum,

T = Total releasable chromium.

The total releasable chromium (T) was determined by incubating C.albicans with PMN for 60 minutes, followed by sodium deoxycholate, DNase treatment and boiling for 30 minutes. This resulted in the release of approximately 60% of total chromium label used (Table 6).

TABLE 6 ^{51}Cr released from C.albicans after heat treatment.

Number of C.albicans	C.albicans (cpm)	^{51}Cr released (cpm)	^{51}Cr released / Total ^{51}Cr (%)
1×10^6	24 825	14 998	60
5×10^5	12 312	7 452	61
2.5×10^5	6 090	3 582	59
1×10^5	2 518	1 587	63
5×10^4	1 293	724	56

Methylene blue dye exclusion test.

The method described by Lehrer & Cline (1969), was modified as follows: 5×10^5 C.albicans (^{51}Cr labelled) and 5×10^5 PMN were mixed in 0.5ml of medium containing 2.5% serum and incubated for 60 minutes at 37°C under constant rotation (30 rpm). The mixture was then incubated for a further 10 minutes at 37°C with DNase (20 µg) and sodium deoxycholate (1.2mg) and aliquots of the supernatant removed for counting the radioactivity. 1ml of 0.01% methylene blue solution was then added to each tube and after centrifugation the pellet was examined under the microscope. Dead C.albicans stain blue but live C.albicans do not take up the stain. At least three hundred C.albicans were counted and the percentage of dead organisms was scored. If correction is made for clumping, this method correlates well with the plating method and stained C.albicans do not form colonies (Lehrer & Cline, 1969). The dye exclusion method originally described has two technical problems; (1) formation of mycelia and (2) extensive clumping after the sodium deoxycholate treatment. In the modified assay described here, C.albicans did not form mycelia and the clumps were successfully dispersed by the enzymatic action of DNase. Thus counting of stained and unstained organisms was made much easier because a homogeneous suspension of C.albicans in the yeast form was obtained.

RESULTS

Standardisation of the method.

Standardisation of the method was achieved by investigating the following wing parameters:

(i) the effect of DNase and sodium deoxycholate,

(ii) the concentration of serum,

(iii) the C.albicans/ PMN ratio,

(iv) the time course,

(v) the effect of pH.

(i) The effect of DNase and sodium deoxycholate.

The effect of DNase is to split clumps of polymerised DNA and thereby allowing C.albicans to be released from them. Table 7, shows that there was no significant release of ^{51}Cr unless DNase was used. The table also shows that DNase and sodium deoxycholate did not induce ^{51}Cr release from PMN. This was confirmed by microscopic examination using the methylene blue exclusion test.

TABLE 7 Conditions for ^{51}Cr release from C.albicans*.

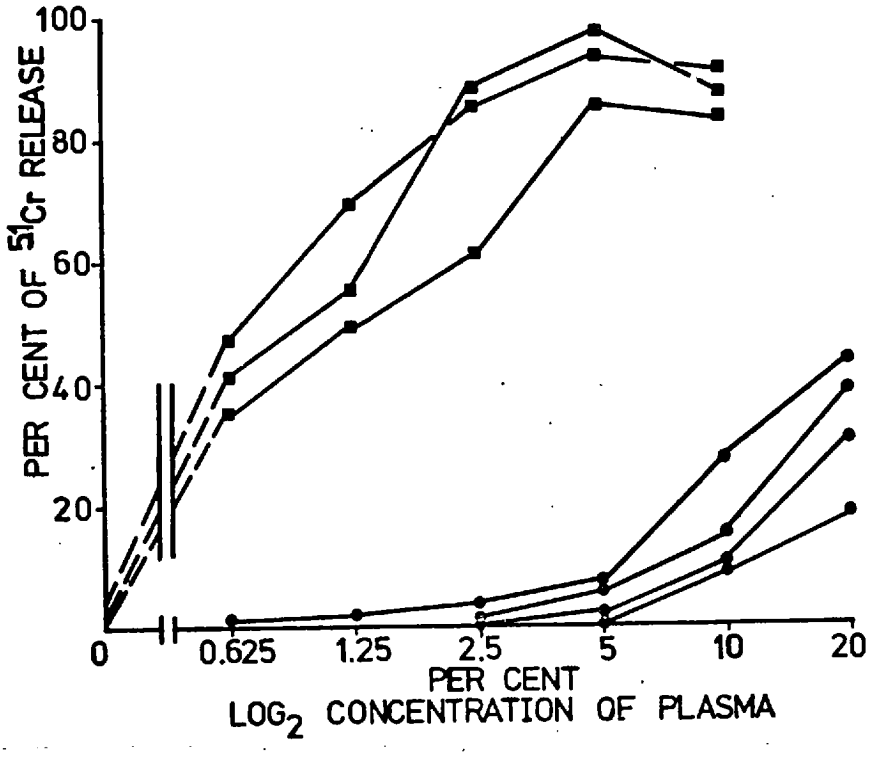
	Treated with: Sodium deoxycholate	DNase	Cpm in supernatant
C.albicans only	-	-	371
C.albicans only	+	-	389
C.albicans only	+	+	401
C.albicans + PMN	-	-	378
C.albicans + PMN	+	-	382
C.albicans + PMN	+	+	631

*Plasma at 2.5% used throughout.

(ii) The concentration of serum.

Intracellular killing of C.albicans was measured by ^{51}Cr release in the presence of serum concentrations ranging from 0 - 10%. The results are shown in Fig. 9. A linear increase of intracellular killing of C.albicans was observed up to a serum concentration of 2.5% and a plateau was usually reached at concentrations of 2.5 to 5%.

Fig. 9 Effect of varying serum concentrations on intracellular killing.



Effects of plasma concentration on the chromium release. ●——●: plasma only; ■——■: PMN and plasma.

(iii) The effect of varying C.albicans/ PMN ratio.

As discussed in the introduction of this chapter, intracellular killing efficiency of PMN leukocytes may or may not depend on the number of C.albicans engulfed by each PMN. If it is independent, the C.albicans/ PMN ratio will not matter in relation to intracellular killing , so long as the rate of ingestion is not a limiting factor. If it is dependent, the ratio is an important variable. As it has already been shown that the maximal average number of C.albicans engulfed by a phagocyte is five, the C.albicans/ PMN ratio was varied from 0.5 to 10 by using a fixed number of C.albicans (5×10^5) and the number of PMN ranged between 5×10^4 to 1×10^6 per tube. Fig. 10 shows that killing efficiency was almost constant when ratios between 1 and 10 were used. As can be seen in Fig. 11, when the ratio was decreased from 10 to 1, there was a linear increase in the percentage of ^{51}Cr released. The ratio 1 was selected for the standard assay because this ratio gave the optimal combination of killing efficiency and chromium release.

Fig. 10 Effect of varying the C.albicans/PMN ratio.

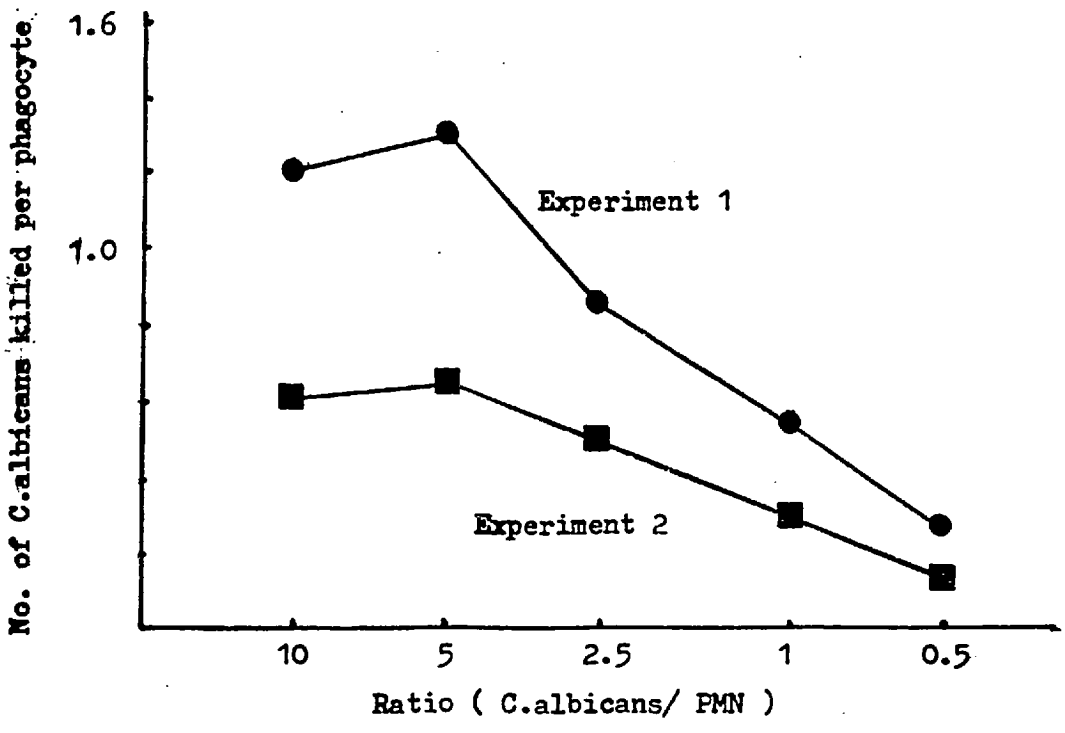
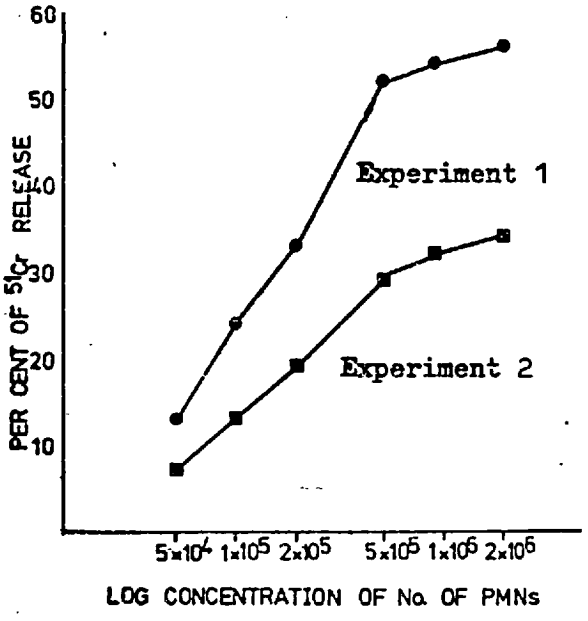


Fig. 11 Effect of varying C.albicans/PMN ratio.

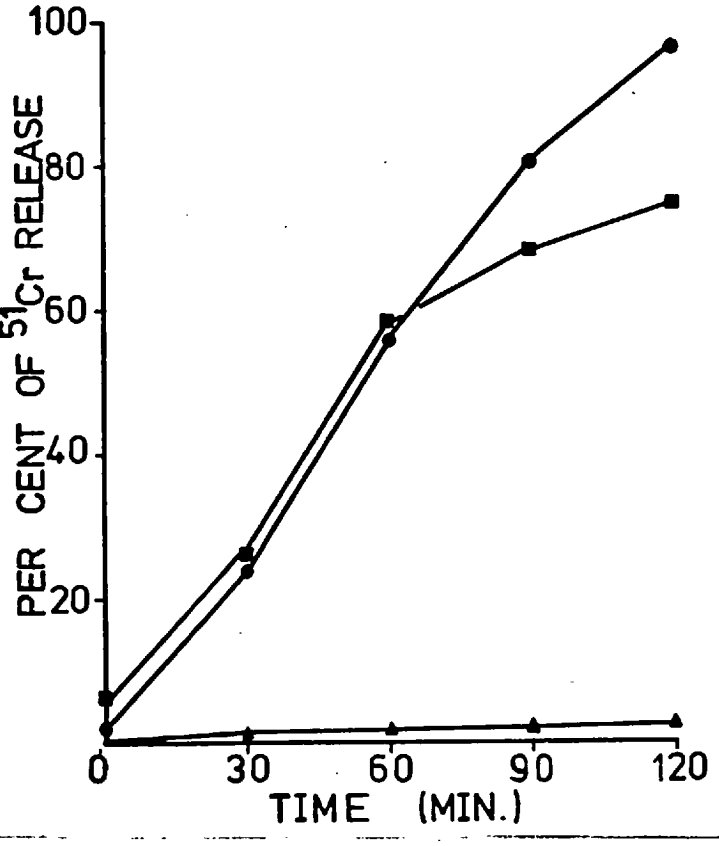


Chromium release curves from 2 experiments in which increasing numbers of PMNs were incubated with 5×10^5 *C. albicans*.

(iv) Time course of intracellular killing.

The time course of intracellular killing was studied using standard assay conditions, ^{51}Cr release being determined at 0, 30, 60, 90 and 120 minutes. The results are shown in Fig. 12. The release was related in linear fashion to the time of incubation up to 60 minutes, when it reached a plateau. It was therefore concluded that 60 minutes is a suitable incubation time.

Fig 12 Time course measuring intracellular killing as ^{51}Cr release.



Time course of chromium release. ▲——▲: 2.5% plasma only; ●——● and ■——■: PMN and 2.5% plasma.

(v) The effect of pH.

Although significant pH changes did not occur during this assay under standard conditions, the effect of varying pH was studied because it was known that spontaneous ^{51}Cr release increases as the pH decreases (Fig. 13). The pH was varied from 6.0 to 9.0 by using either 0.1M NaOH or 0.1M HCl. Fig. 14 shows the results.

Fig.13

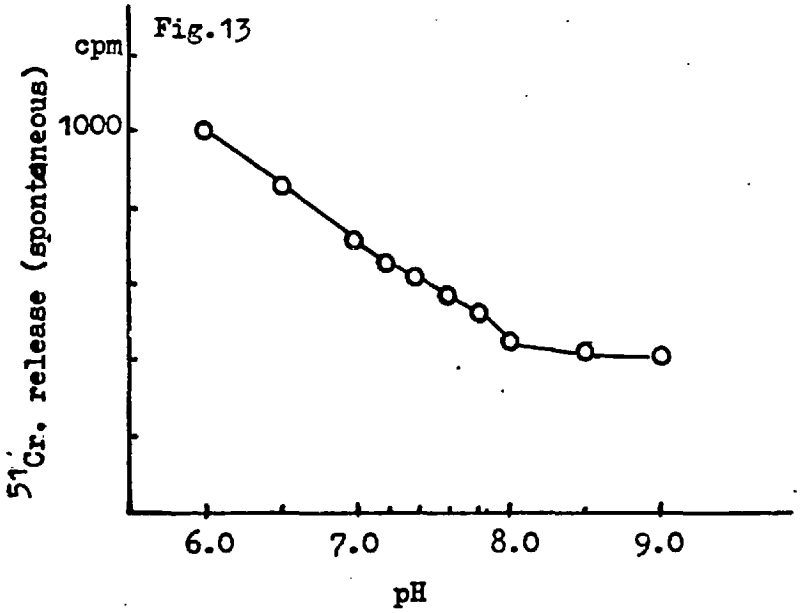
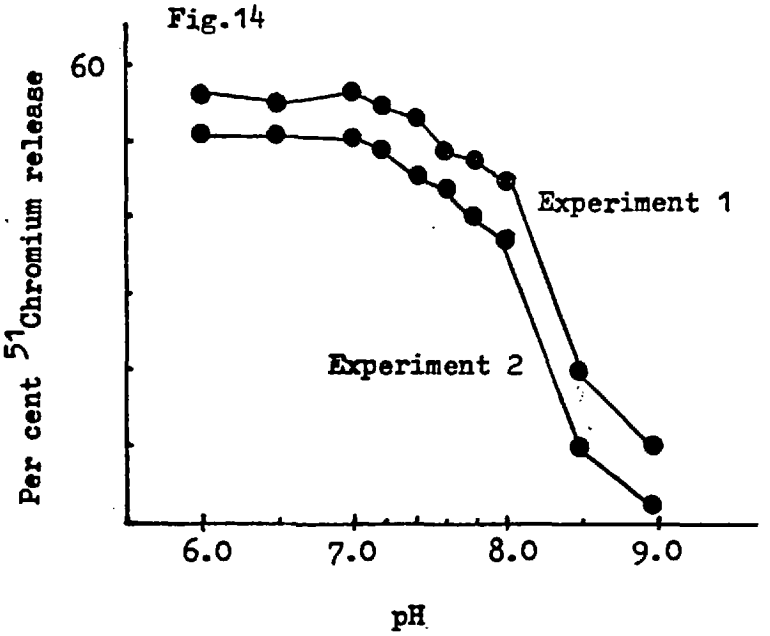


Fig.14



There was no significant difference in ^{51}Cr release when the pH was between pH 6.0-7.8, but ^{51}Cr release ceased above pH 8.0. Therefore the assay can be performed between pH 6.0 and 7.8.

Comparison of the ^{51}Cr release technique with the methylene blue test.

The chromium release technique was compared with the methylene blue dye exclusion test, because the methylene blue test is relatively easy to perform and has been shown to correlate well with the colony forming assay (Lehrer & Cline, 1969). Table 8 shows that the ^{51}Cr release correlated well with the methylene blue exclusion test. PMN from patients with chronic granulomatous disease (CGD) were also tested. Phagocytes from these patients are normal in their ability to ingest but are unable to kill C.albicans, and their mothers have the same abnormality, affecting 50% of their phagocytes. As expected, PMN from CGD patients failed to release ^{51}Cr and PMN from mothers of CGD patients liberated approximately 50% less radioactivity than PMN from control subjects. Further experiments were carried out, in which the two assays were compared using varying C.albicans to PMN ratios. Fig. 15 shows that there is good correlation between the methylene blue exclusion test and the ^{51}Cr release assay ($r=0.99$). It can therefore be concluded that ^{51}Cr release reliably measures the killing of C.albicans.

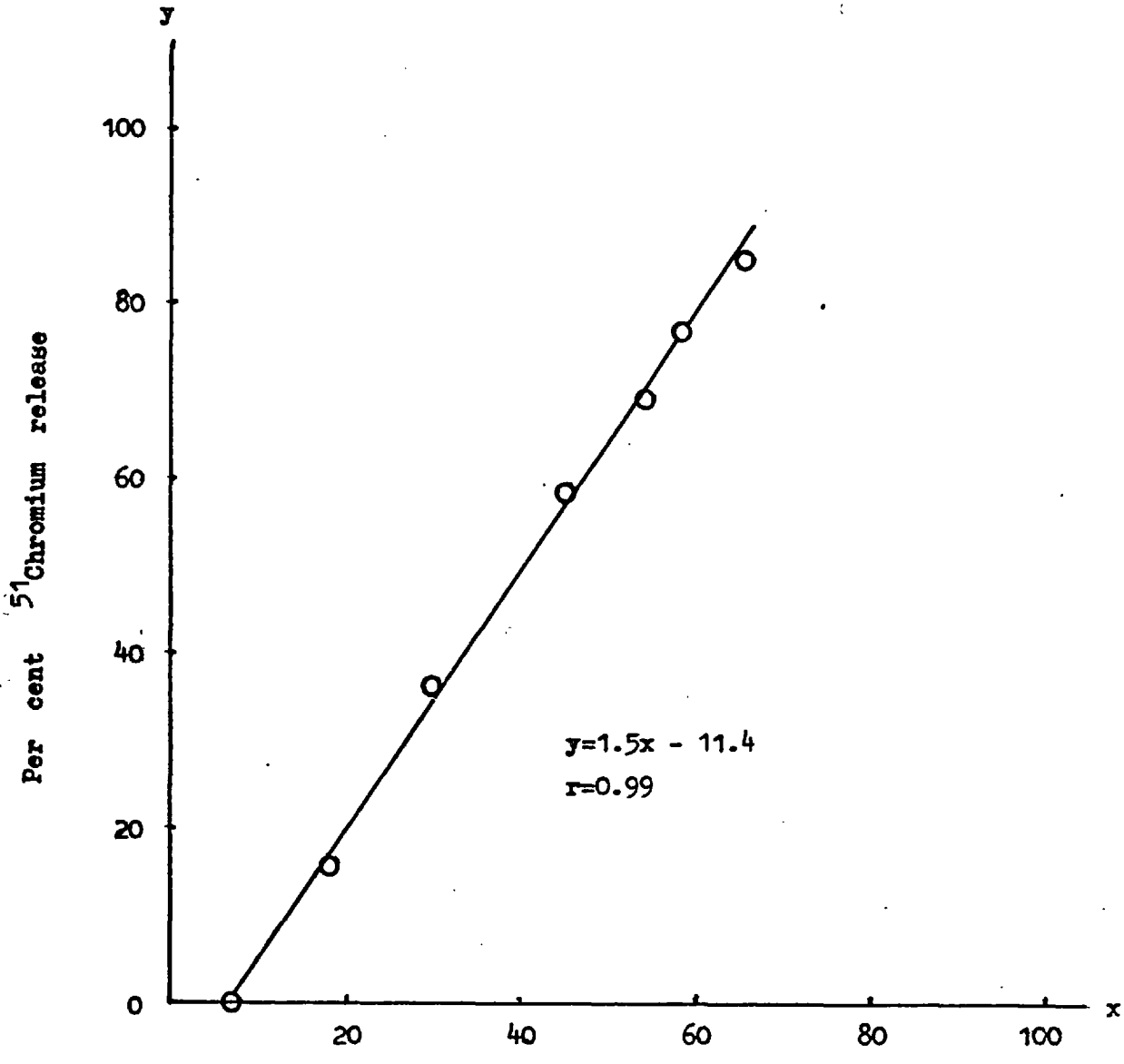
TABLE 8

Comparison between the methylene blue exclusion test and ^{51}Cr release.

Subjects tested	Methylene blue test (% *C. albicans* killed)	^{51}Cr release (%)
1	39	66
2	42	64
3	32	62
4	42	58
5	35	60
6	30	42
7	44	52
8	34	48
9	44	68
10	45	60
11 *	7	1
12 *	0	2
13 **	46	15

* Patients with chronic granulomatous disease (CGD).
** Mother of 2 boys with CGD.

Fig.15 Comparison between the methylene blue exclusion test and and ^{51}Cr release assay.



The effect of EDTA and heat-inactivated serum.

No phagocytosis occurs if 0.01M EDTA is present in the system (see Table 4). Consequently, no intracellular killing of C.albicans was observed under such conditions. On the other hand, as shown in Table 3, C.albicans was effectively engulfed in the presence of heat-inactivated serum. However, in spite of this normal or near normal ingestion no killing was observed when C.albicans was opsonised in heat-inactivated serum (Table 9). The reason why killing does not occur under these conditions will be the subject of the next chapter.

TABLE 9 The effect of EDTA and heat-inactivated serum.*

	^{51}Cr release (%)		
	Exp.1	Exp. 2	Exp. 3
Untreated heparin plasma	61	76	67
Untreated serum	60	69	61
Serum heated 56°C for 30min	1	2	ND
EDTA 0.01M	2	3	ND
No serum or plasma	2	1	ND

*2.5% serum or plasma used in all experiments.

Reproducibility

This method had an experimental error of 5% within an experiment and 10% between experiments.

Normal range.

PMN from 20 healthy individuals were tested in the presence of 2.5% AB serum. A range of 35 - 71% chromium release was observed, with a

median of 45%.

Using standard phagocytes, sera obtained from 20 healthy individuals were tested for activity of procidin. A range of 30 - 55% chromium release was observed, with a median of 43%.

DISCUSSION

Phagocytic killing of C.albicans can be quantitatively measured by an objective and simple ^{51}Cr release assay. Only 5 x 10^5 PMN are required for this assay and it should be possible to reduce the number further by using ^{51}Cr of higher specific activity, thus making the organisms more radioactive. This possibility should be considered if one wants to test infants or patients with neutropaenia. In that event, the mixing must be particularly efficient and the loss of material due to spreading onto the plastic wall, etc., must be minimised.

The method has been applied for assessing the intracellular killing function of blood monocytes and also for analysing the phagocytic function of peritoneal and spleen macrophages of mice (Cooper et al., 1978; Sljivic, personal communication).

The observation that heat-inactivated serum can opsonise but fails to support intracellular killing of C.albicans suggests that a heat labile humoral substance participates in the intracellular killing processes (Li & Mudd, 1965). The participation of heat labile substances in chemotaxis and opsonisation is well known but it has not been conclusively demonstrated before in relation to intracellular killing. The term "procidin" is suggested for this new activity. Thus phagocytes require heat labile humoral substances for expressing all components of phagocytic activity, namely chemotaxis, phagocytosis and intracellular killing. The nature of "procidin" in relation to C.albicans will be discussed in the following chapter.

Chapter 5

Participation of C3 in Intracellular Killing of Candida albicans.

INTRODUCTION

The importance of humoral substances which assist phagocytic activity have already been discussed. They are present in, or are generated as the result of the interaction of microorganisms with body fluids. In their absence phagocytic activity is minimal. Intrinsic phagocytic activity can be considered as defective, only if phagocytes are impaired in the presence of optimal amounts of the humoral substance. Li and Mudd, (1965), have suggested that a heat-labile serum factor is required for intracellular killing of Staphylococcus aureus. However this evidence is not conclusive and no attempt was made to identify the substance involved, because suitable techniques were not available. The radiometric assays described in the previous chapters are suitable for analysing the requirement of serum substances for intracellular killing of C.albicans. A complement component is an obvious candidate for this activity.

METHODS

Phagocytosis was tested as described in Chapter 3. Intracellular killing was measured as described in Chapter 4. The analysis was made in relation to C.albicans throughout. C2 deficient serum was used for analysing the role of the early components of the classical complement pathway; it had a serum factor B level which was 50% of the mean normal level. C6 deficient serum and zymosan treated serum were used for analysing a possible role for the late complement pathway. Serum heated at 50°C for 15 min, was used for analysing the role of the alternative complement pathway (Lachmann et al., 1973). Agammaglobulinaemic serum, containing less than 0.05g/l of immunoglobulin was used for analysing the role of antibody. C3 deficient serum was used for analysing the role of C3. The above complement deficient sera were kindly supplied by Dr PJ Lachmann.

RESULTS

Table 10 summarises the results of the effect of various complement or antibody deficient sera on phagocytosis of Candida albicans.

TABLE 10 Effect of various complement and antibody deficient sera on phagocytosis of C.albicans.

Type of serum used	Percentage serum concentration	Phagocytosis (% inhibition of uridine incorporation)	
		Expt. 1	Expt. 2
Normal, untreated	2.5	51	40
Normal, untreated	5.0	56	45
Heated 56°C for 30 min	2.5	56	46
Heated 56°C for 30 min	5.0	N.D.	N.D.
C2-deficient	2.5	49	62
C2-deficient	5.0	N.D.	N.D.
C6-deficient	2.5	41	50
C6-deficient	5.0	43	37
Zymosan-treated	2.5	46	51
Zymosan-treated	5.0	N.D.	N.D.
C3-deficient	2.5	65	62
C3-deficient	5.0	N.D.	N.D.
C3-deficient + C3	2.5	N.D.	N.D.
Agammaglobulinaemic	2.5	48	51
Agammaglobulinaemic(heated)*	2.5	42	44
Normal ranges	2.5	33–75	

* 56°C, 30 min

None of these sera were found to be deficient in their ability to opsonise C.albicans.The same was true too for over hundred sera taken from patients with suspected immunodeficiency, including patients with chronic candidiasis, who were referred to Dr H.Valdimarsson.

This is an important consideration in analysing the effects of complement deficient sera on intracellular killing. Thus, if intracellular killing is found to be absent or defective, it cannot be due to an absence of phagocytosis but must indicate the failure of the intracellular killing processes.

The effect of complement deficient sera on intracellular killing of C.albicans.

(i) C2 deficient serum.

The results are shown in Fig. 16. Minimal intracellular killing was noted at 2.5% serum concentration but a normal level of killing was observed above 5.0% concentration. This may be due to inadequate activation of the alternative pathway because of the low factor B level in the C2 deficient serum used. Alternatively, a higher serum concentration is required for direct activation of C3 via the alternative pathway than is required for its classical activation by $\overline{C42}$ convertase, two conclusions can be drawn from this observation: (i) activation of C1-C4 was not required in intracellular killing and; (ii) the alternative pathway can be activated directly by C.albicans.

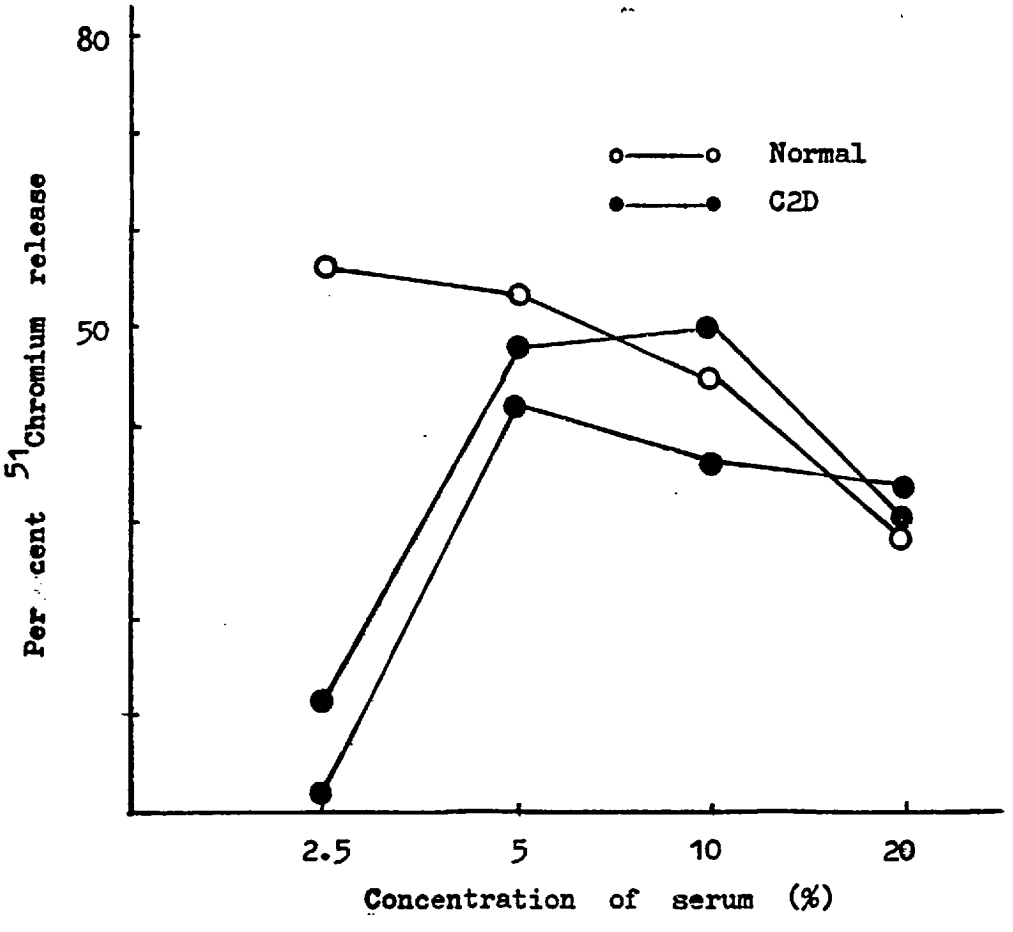


Fig. 16 Effect of C2 deficient serum on intracellular killing of C.albicans.

(ii) C6 deficient serum.

The results , shown in Fig. 17, suggest that the late complement components were not significantly involved in the intracellular killing of C.albicans.

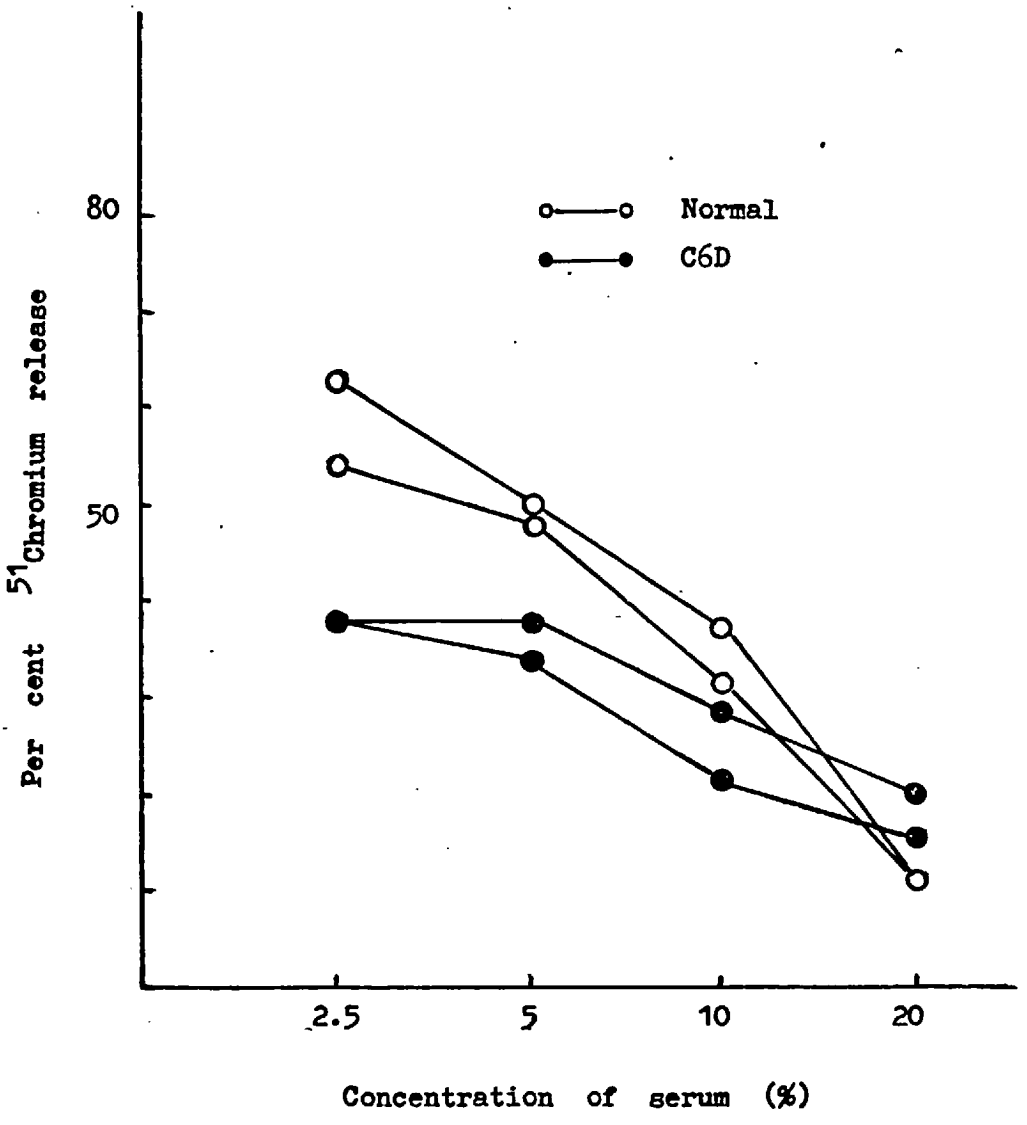
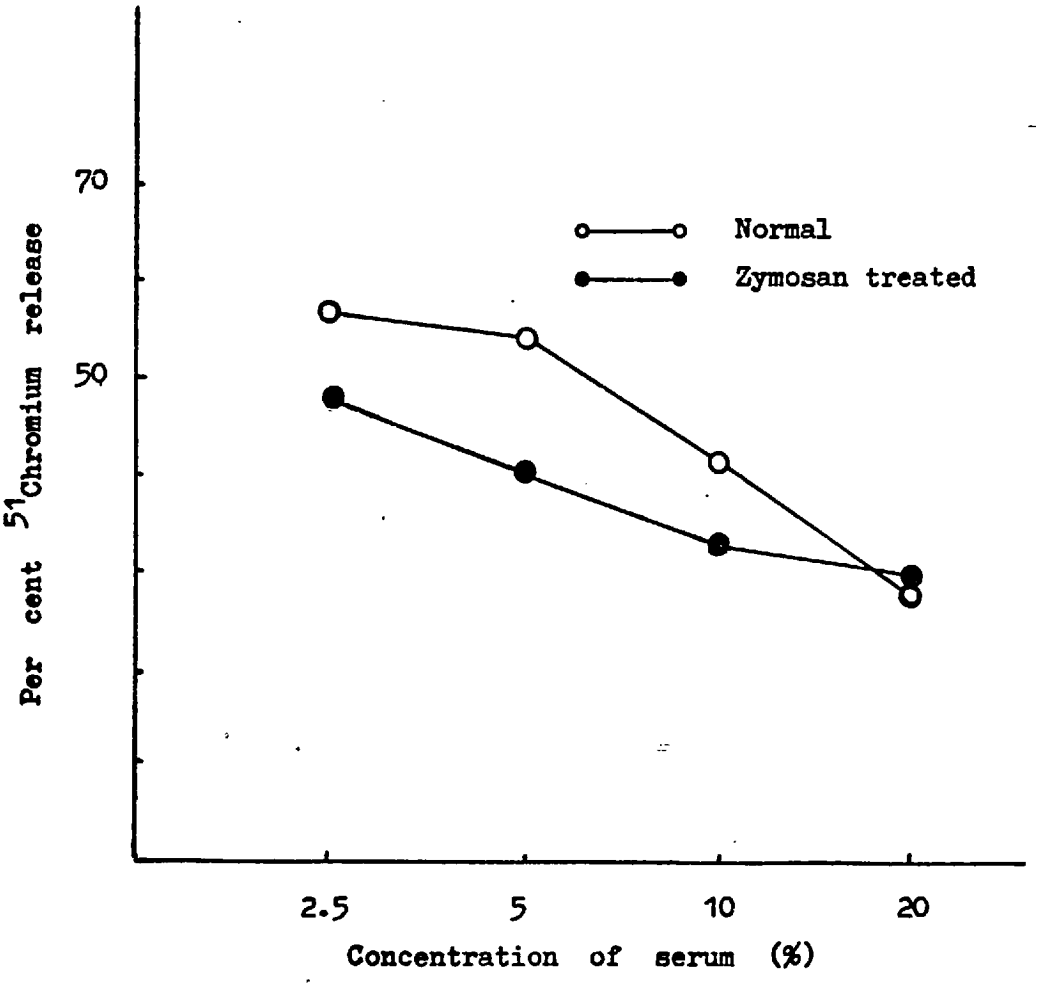


Fig. 17 Effect of C6 deficient serum on intracellular killing of C.albicans.

(iii) Zymosan-treated normal serum.

Zymosan-treated serum supported intracellular killing to the same degree as normal serum (Fig. 18). This serum contained normal levels of C1, C4 and C2 and approximately 40% of unconverted C3, but its alternative pathway was depleted. It therefore seemed likely that C3 might play an essential role in intracellular killing of C.albicans.

Fig. 18 Effect of zymosan-treated normal serum on intracellular killing of C.albicans.



(iv) C3 deficient serum.

No intracellular killing of C.albicans was observed in the presence of 2.5% and 5.0% C3 deficient serum. However, killing was restored when purified C3 was added to the system to a concentration of 25mg/ml. Purified C3 alone did not support killing, indicating that C3 activation is required in order to assist intracellular killing. These results are summarised in Table 11. It was concluded that C3 participates in the intracellular processes leading to killing of C.albicans.

TABLE 11 The effect of C3 deficient serum and purified C3.

Serum used	(conc.%)	Intracellular killing % ^{51}Cr release	Phagocytosis % Inhibition
C3D	2.5	0 , 0	65 , 62
	5.0	0 , 0	ND
C3D + C3	2.5	- 25	ND
C3	2.5	- 0	0
Control	2.5	44 ,35	65 , 60

DISCUSSION

It has been demonstrated that C3 participates directly in intracellular killing of C.albicans. The term "procidin" is proposed for humoral substances which render microorganisms susceptible to the action of intracellular killing. In the case of C.albicans, it has been established that C3 has "procidin" activity. The "procidin" may not be required for phagocytic killing of all microorganisms, some will undoubtedly be automatically killed following phagocytosis.

The analysis of "procidin" activity in relation to other organisms can only be achieved by developing assays for measuring independently phagocytosis and intracellular killing of defined strains of these organisms.

The mechanism whereby C3 participates in the intracellular killing of C.albicans was not investigated further in this study. However, the following possibilities have been considered:

(i) activation of the phagocytes through binding of C3 products to their plasma membrane,

(ii) stimulation of lysosome adherence to phagosomes,

(iii) stimulation of the fusion process between the phagosome-lysosome membranes,

(iv) activation of lysosomal enzyme involved in the candidacidal process,

(v) induction of lysosomal enzyme release as demonstrated by Schorlemmer & Allison (1976) in a different system,

(vi) C.albicans coated by C3 may be more susceptible to the action of lysozyme (Muschel et al., 1959; Brumfitt & Glynn, 1961), phagocytin (Hirsh, 1956) or other lysosomal compounds, (De Duve & Wattian, 1966).

Analysis of some of these possibilities may have to await the developement of techniques whereby individual steps of the intracellular killing process can be measured independently.

Chapter 6

A Radiometric Opsonin Assay:

Selective measurement of opsonising capacity of the alternative pathway of complement.

INTRODUCTION

Over 70 years ago, Wright and Douglas observed that fresh serum promoted the ingestion of microorganisms by phagocytes and coined the term "opsonin" for substances in the body fluid which render particles more susceptible to the action of phagocytosis. Two major categories of opsonins exist; thermostable and thermolabile. Antibodies (e.g. IgG) possess thermostable opsonic activity and complement components are largely responsible for the thermolabile opsonic activity, activated C3 in particular (Stossel, 1975). Since C3 is the common convergence point for both the classical and the alternative complement pathways, opsonisation by C3 occurs when either pathway is activated .

In addition to activated C3 and antibody, some other serum proteins have been proposed as candidates for opsonic activity. Thus α- and β-globulins have been reported to enhance ingestion of starch particles (Tullis & Surgenor, 1956), and C-reactive protein has also been shown to have opsonic activity (Williams & Quie, 1968).

Recently sera from cord blood have shown to be deficient in complement dependent opsonising capacity (Miller, 1969) and a large proportion of allergic patients has also been shown to have such opsonin deficiency (Soothill & Harvey, 1977). It is also found in 5-7% of the healthy normal population (Soothill & Harvey, 1976).

Theoretical considerations.

Opsonisation is a process involving interaction between opsonins and microorganisms, and can therefore occur in the absence of phagocytes. Consequently it should be possible to measure opsonisation in the absence of phagocytes. Thus if a standardised purified opsonin for

microorganisms would be available, this could be labelled with a radioactive substances such as ^{125}I, and used for opsonin measurement in a competitive binding assay. This would give a value for (x) measured independently of (y) in diagram II.

Practical considerations.

Since it is not possible to measure opsonisation by the above method, it must be measured as phagocytosis under conditions where particle ingestion is limited in a linear fashion by the amount of available opsonins. Standard phagocytes, should be used if available. Alternatively, phagocytes obtained from a healthy individual can be used in each particular experiment as done in this study.

It has been shown that one particular serum may have varying opsonising capacity for different strains of a single microorganism (Ward, 1930; Ward & Enders 1933). Therefore the type and the number of the microorganisms used must be strictly defined. The objective of this study was to establish an assay, which would measure selectively the opsonising capacity of the alternative pathway of complement. S.cerevisiae was chosen because it is capable of activating this pathway directly (Pillemer et al., 1954) and normal human sera do not contain opsonising antibodies to this organisms.

The method devised is a modification of the technique described for phagocytosis of C.albicans (see Chapter 3). Its development and standardisation will be discussed as well as its application for measuring the opsonising activity of the alternative complement pathway.

METHODS

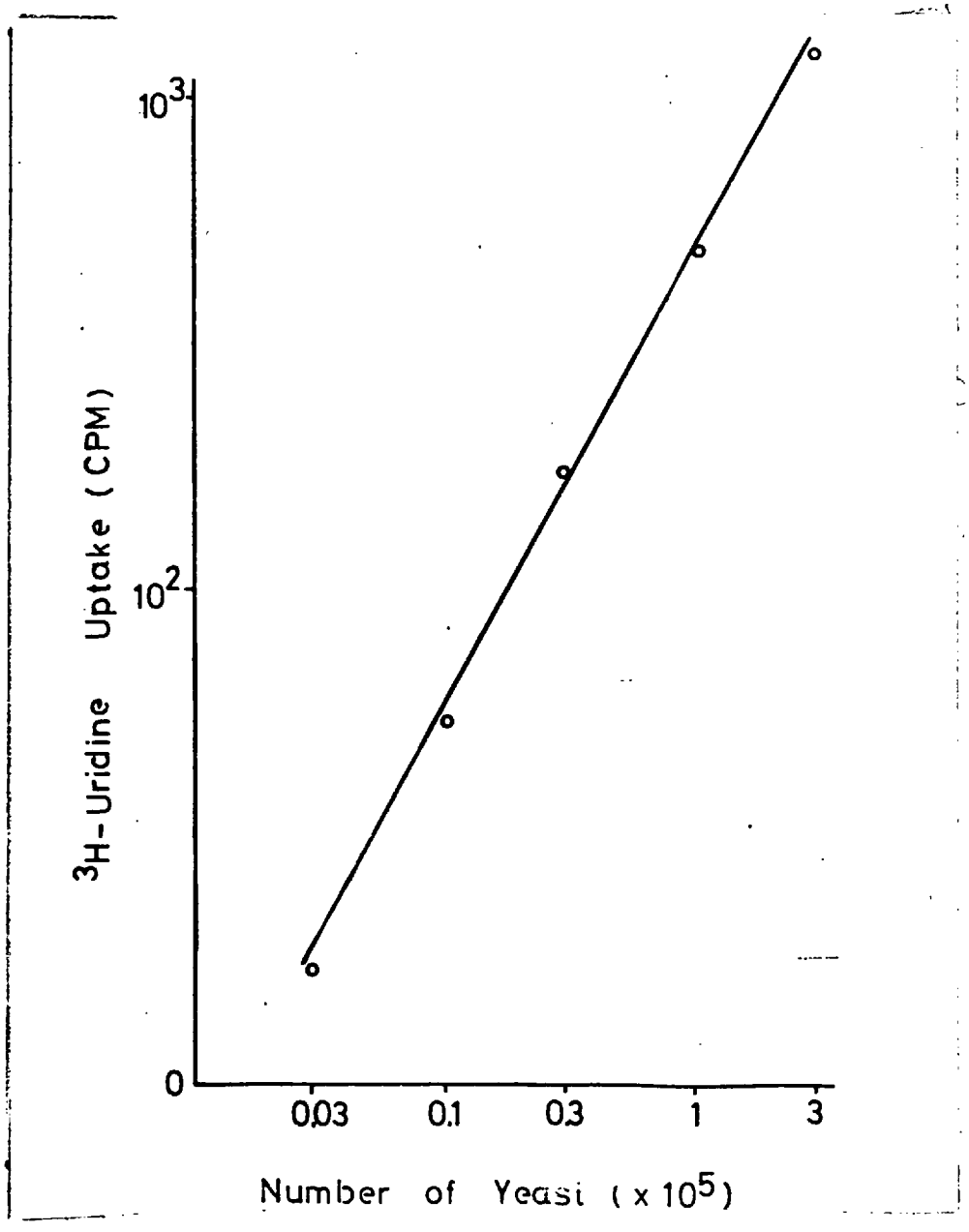
The preparation of S.cerevisiae, PMN and serum has already been described in Chapter 2.

The relationship between the number of S.cerevisiae and the incorporation of ^{3}H-uridine.

As shown in Table 1,* S.cerevisiae does not replicate during an incubation period of 3 hours in the presence of 2.5% serum. Fig. 19 shows that the incorporation of ^{3}H-uridine is directly related in a linear fashion to the number of S.cerevisiae. Since ingested C.albicans did not incorporate ^{3}H-uridine from the culture medium, (Yamamura et al., 1977), it was assumed that the same would apply to S.cerevisiae. Inhibition of ^{3}H-uridine incorporation was therefore used to measure phagocytosis and opsonisation of S.cerevisiae.

* (p.18)

Fig. 19 Relationship between number of S.cerevisiae and ^{3}H-uridine uptake



Standard assay procedure.

Unless otherwise stated the opsonin assays were performed as follows: A mixture containing 5×10^5 *S.cerevisiae* with 5×10^4 or 10×10^4 PMN were incubated at 37°C for 30 minutes under constant rotation of 30 rpm in 0.5ml of medium containing 2.5% or 1.25% serum. Duplicate 0.2ml aliquots were then transferred to microtitre wells, to which 0.2 µCi of ^{3}H-uridine had previously been added and the microtitre plate incubated for a further 60 minutes at 37°C. The cells were then collected onto glass fibre filter discs with a Skatron harvester, the dried discs placed in scintillation vials, counted by a Packard Tri-carb liquid scintillation spectrometre model B 2450.

Presentation of data.

Opsonin activity has traditionally been expressed as Opsonin Index, which is the average number of particles ingested per phagocyte at a predetermined serum concentration. In the assay described here, the results can be expressed as per cent inhibition of uridine incorporation caused by phagocytosis (Equation D) or as Opsonin Index (Equation E).

Equation D:

$$I = \left(1 - \frac{\text{CPM PMN present}}{\text{CPM PMN absent}}\right) \times 100$$

Equation E:

$$\text{Opsonin Index} = \frac{I \times R}{100}$$

In these equations I is the percent inhibition and R is the *S.cerevisiae*/PMN ratio.

RESULTS

Standardisation of assay conditions.

Assay conditions for opsonin activity must be adjusted so that the number of particles ingested is limited in a linear fashion by the opsonin concentration and not by the number of particles available for phagocytes. The following parameters were investigated:

(i) the concentration of serum,

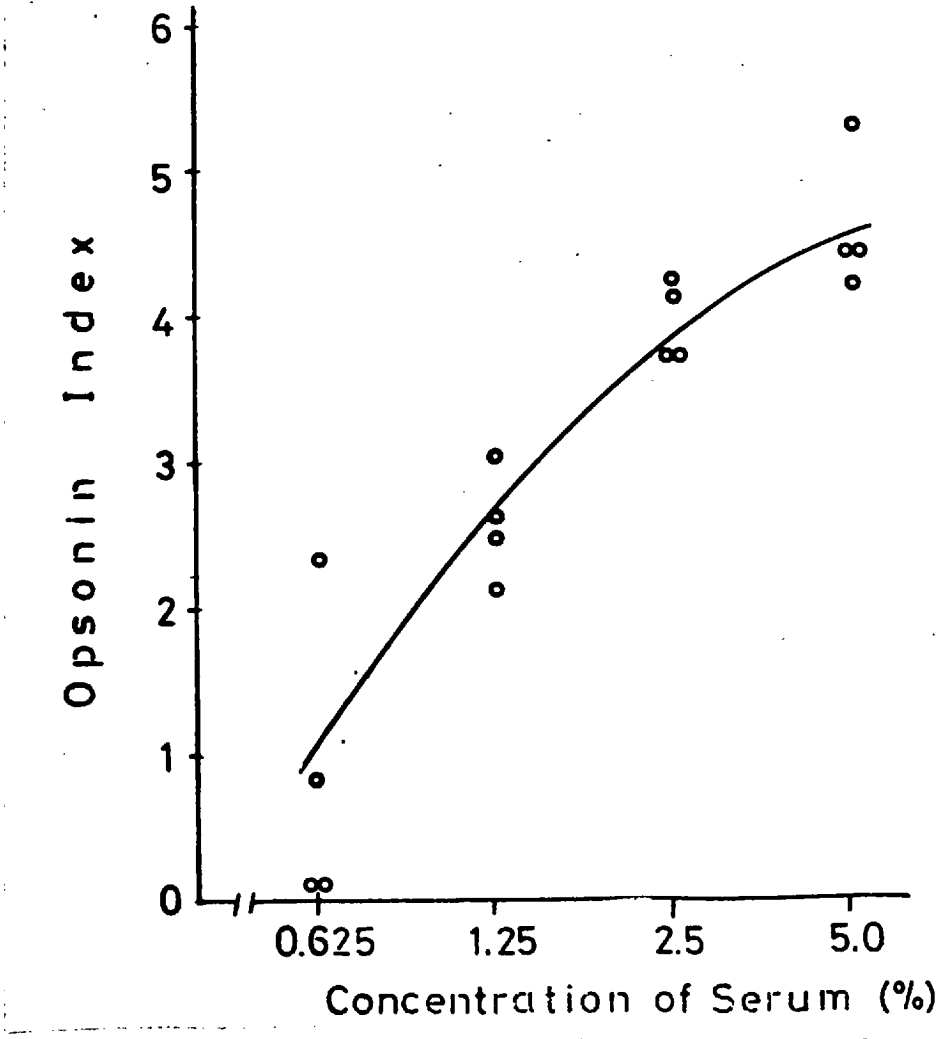
(ii) S.cerevisiae / PMN ratio,

(iii) the combination of (1) and (2).

(i) The concentration of serum.

Phagocytosis was measured in serum ranging in concentration from 0.625% to 5% and as shown in Fig. 20 a plateau was reached at 2.5%. As the opsonin index varied greatly in 0.625% serum, a concentration of 1.25% or 2.5% were chosen for the standard assay.

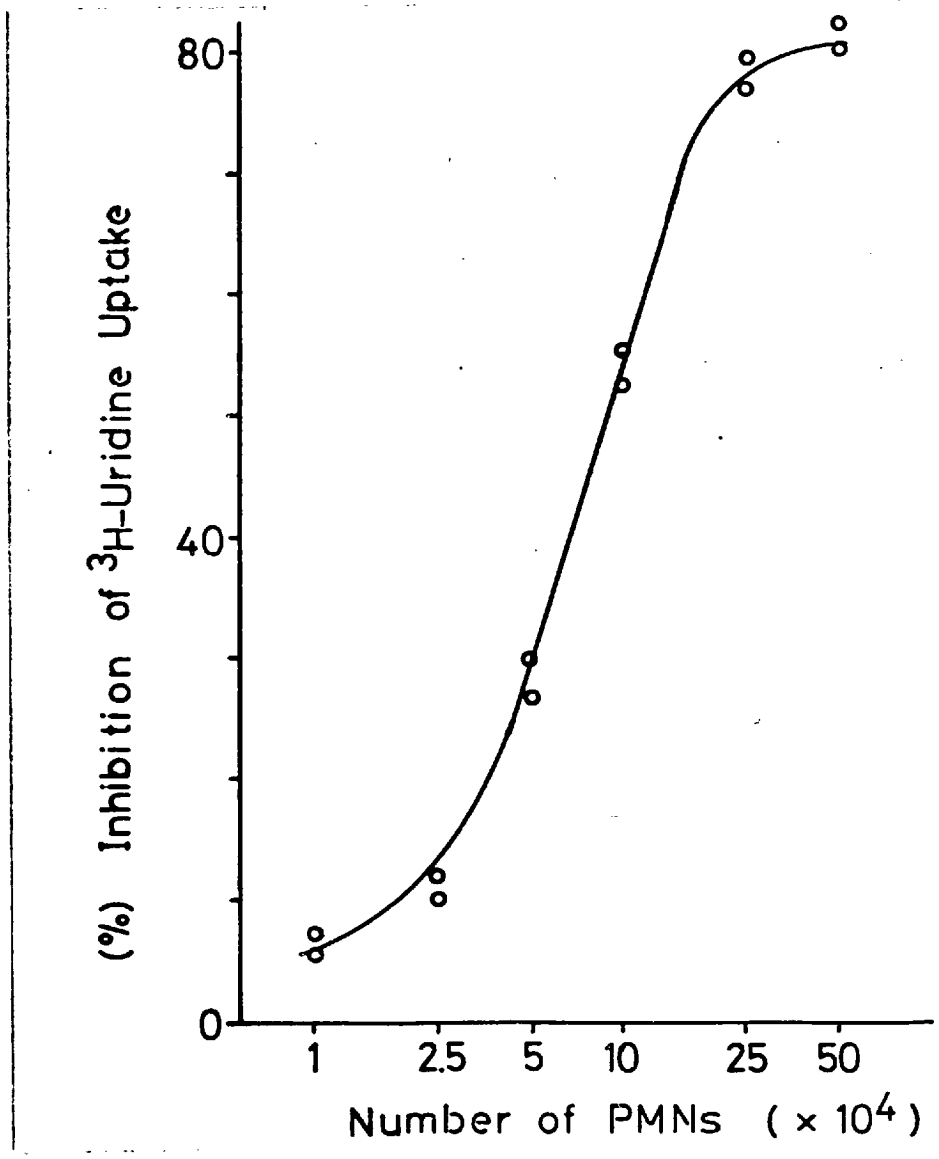
Fig. 20 Effect of serum concentration on opsonising capacity.



(ii) S.cerevisiae / PMN ratio.

A fixed number of S.cerevisiae was incubated with varying numbers of PMN in the presence of 1.25% and 2.5% serum. It was found that phagocytosis was dependent on the availability of yeast when there were less than five organisms per PMN leukocyte but remained constant when the yeast/ PMN ratio exceeded 5:1 (Fig. 21)

Fig. 21 S.cerevisiae / PMN ratio, using constant number of yeast ($5x10^5$).



(iii) The combination of condition (i) and (ii).

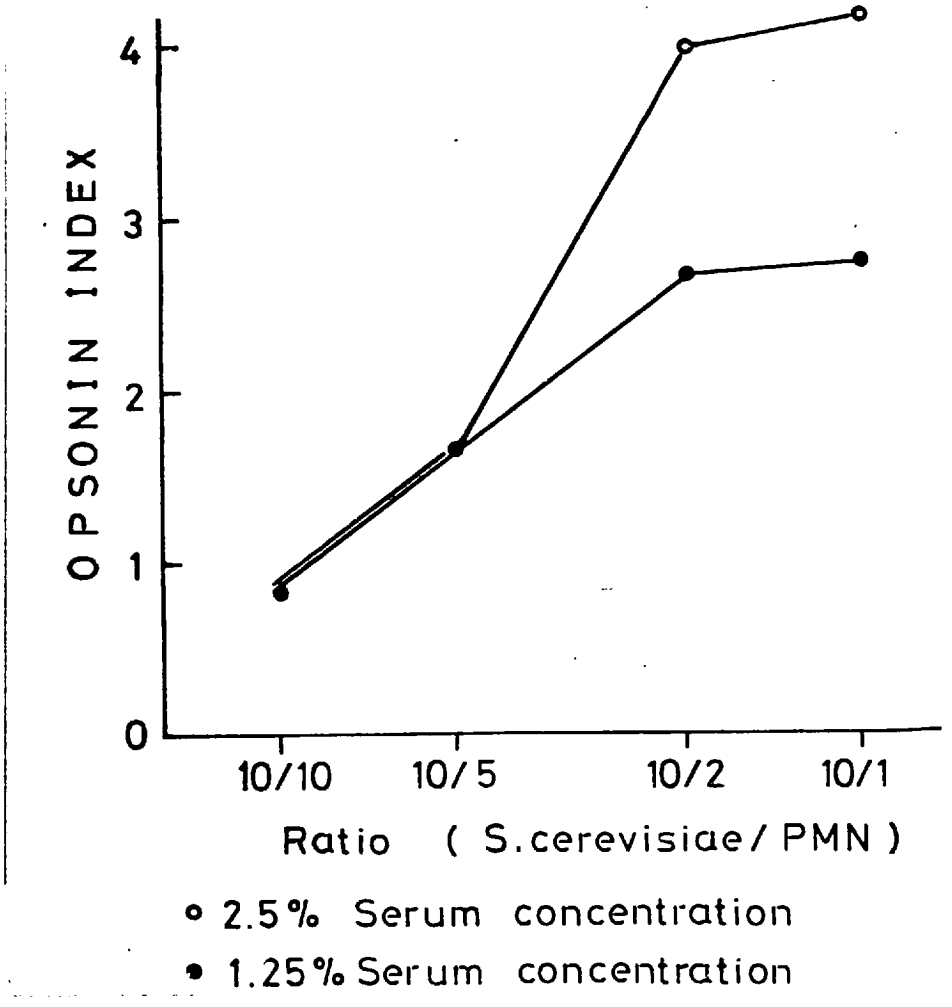
Opsonisation expressed as per cent inhibition of ^{3}H-uridine incorporation is dependent on the yeast / PMN ratio (Fig. 21). However, opsonin index if plotted against yeast / PMN ratio is only dependent on the concentration of serum as long as more than five S.cerevisiae are available for each PMN leukocyte. Hence the following standard assay conditions could be defined:

(i) 5×10^5 S.cerevisiae,

(ii) 5×10^4 and / or 10×10^4 PMN,

(iii) 1.25% and / or 2.5% serum.

Fig. 22 Effect of serum concentration & yeast/PMN ratio on opsonin index.



Comparison of the radiometric assay with a microscopical technique.

Six known opsonin deficient sera and three normal sera as determined by a microscopic technique (Soothill & Harvey, 1976) were obtained, through the courtesy of Professor Soothill & Mrs Harvey, at the Institute of Child Health. These sera were analysed blind by the radiometric assay. There was excellent correlation between the assays, except one control sample, which had normal opsonic activity as measured by microscopic examination but was found to be subnormal by the radiometric method (Table 12).

TABLE 12 Comparison of the radiometric assay with a microscopic assay.

	Radiometric assay	Microscopic assay*
	Opsonin index	Opsonin index
1	1.0	0.84
2	0.8	0.90
3	0.4	0.48
4	0.4	0.54
5	0.3	0.48
6	0.2	0.26
7	1.0	3.36
8	2.8	4.40
9	3.1	4.52

* This was done by Mrs B.A.H. Harvey at the Institute of Child Health.

The reproducibility of the radiometric assay.

To test the reproducibility of the assay sera from four healthy individuals, 2 with normal and 2 with defective opsonic activity and serum from one patient with recurrent infection, were tested repeatedly at different times. As shown in Table 13, the assay showed good reproducibility in this longitudinal study.

TABLE 13 Reproducibility of the assay in longitudinal studies.

Serum donors	No tests	OPSONIC INDICES 1.25% serum	2.5% serum
A	4	3.5, 2.7, 2.6, 3.2	4.1, 4.8, 3.9, 5.1
B	4	2.5, 2.3, 2.0, 2.3	4.5, 3.0, 3.1, 3.4
C	3	0.1, 0.5, 0	0.1, 0.5, 0
D	3	0, 0, 0	0, 0, 0
E	3	0.5, 0.2, 0.1	0.3, 0.3, 0.2

A & B = Normal healthy donors,

C & D = Healthy donors with opsonin deficiency,

E = Patient with recurrent infections.

Normal ranges.

Sera obtained from 20 healthy individuals were tested for opsonising capacity, using serum concentrations of 1.25% and 2.5%. The normal ranges are shown in Table 14.

TABLE 14 Normal range for opsonic activity of adult human sera.

	SERUM CONCENTRATION	
	1.25%	2.5%
Mean opsonin indices	2.7	4.0
S.D.	0.4	0.6
Range	1.9-3.5	2.8-5.2

n=20

Sera from two healthy individuals found to be deficient in opsonisation of S.cerevisiae were excluded.

Experimental error in this assay was found to be 5%, within an experiment and 10% between experiments.

Analysis of the opsonin activity.

Table 15 demonstrates that while C.albicans was effectively opsonised by heat inactivated serum, S.cerevisiae was not. Similarly, zymosan treated serum had strong opsonic activity for C.albicans but none for S.cerevisiae. Sera deficient in C2 had normal opsonic capacity for both yeast types, whilst C3 deficient serum only opsonised C.albicans and not S.cerevisiae. Contrary to Miller & Nilsson, (1970), C5 deficient serum had normal opsonic activity for S.cerevisiae. (Fig. 23). In order to test whether antibodies, although not essential, might nevertheless contribute to the opsonisation of S.cerevisiae, the yeast was preincubated in 20% heat-inactivated

(56°C, 30min) sera, washed and then tested in 1.25 and 2.5% of the R3 reagent. No opsonisation was detected in these experiments. It was therefore evident that under the assay conditions used, antibody was not involved in the opsonisation of S.cerevisiae (Table 15).

TABLE 15 Opsonisation of S.cerevisiae and C.albicans.

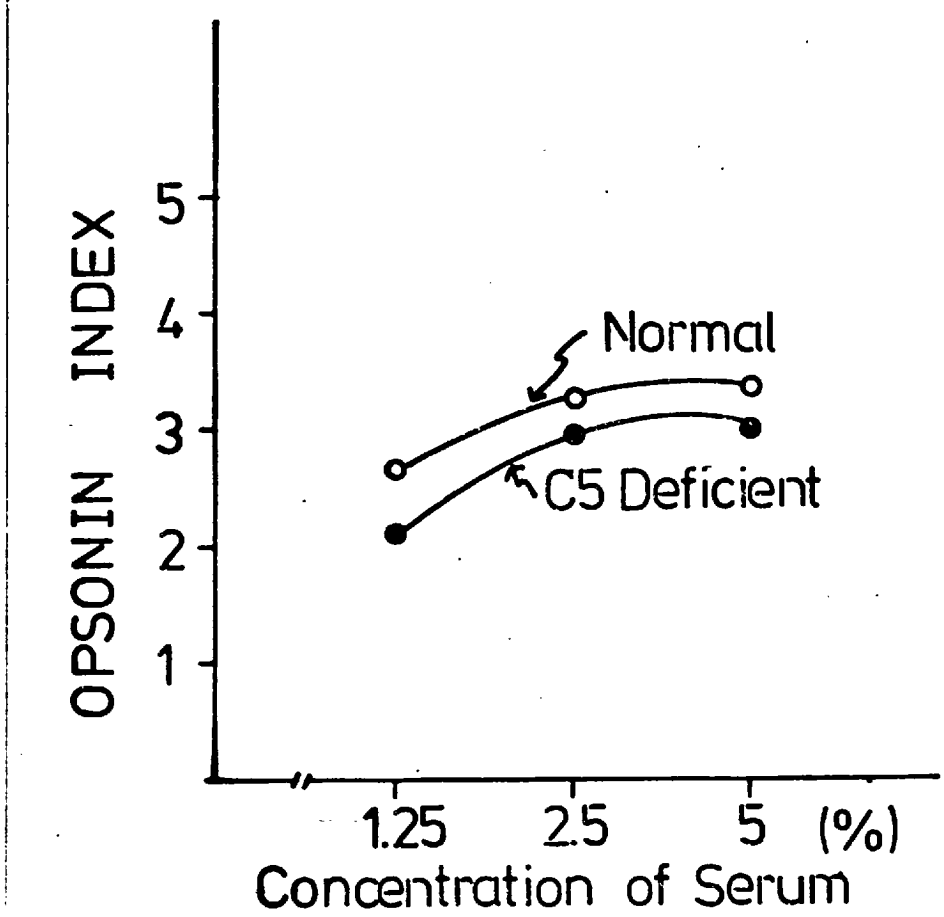
Serum used	S.cerevisiae		C.albicans
	1.25%	2.5%	2.5%
Normal	2.7±0.4	4.0±0.6	5.0±1.2
Heated (56°C, 30min)	0	0	5.6
Zymosan treated	0	0	4.6
Zymosan treated*	0.1	0.2	N.D.
Agammaglobulinaemic(50mg/l)	3.1	3.2	4.8
Agammaglobulinaemic(56°C,30min)	N.D.	N.D.	4.2
C5 Deficient	2.2	3.1	N.D.
C6 "	N.D.	N.D.	4.1
C2 "	0.5	3.0	4.9
C3 "	0.1	0.1	6.5
Heated (50°C, 15min)	0.5	1.2	N.D.

N.D. = Not done

*S.cerevisiae preopsonised by heat-inactivated (56°C, 30min) was used.

It was concluded from these experiments that under the assay conditions used, the opsonic activity for S.cerevisiae was exclusively derived from direct activation of the alternative pathway.

Fig. 23 Effect of C5 deficient serum on opsonisation of S.cerevisiae.

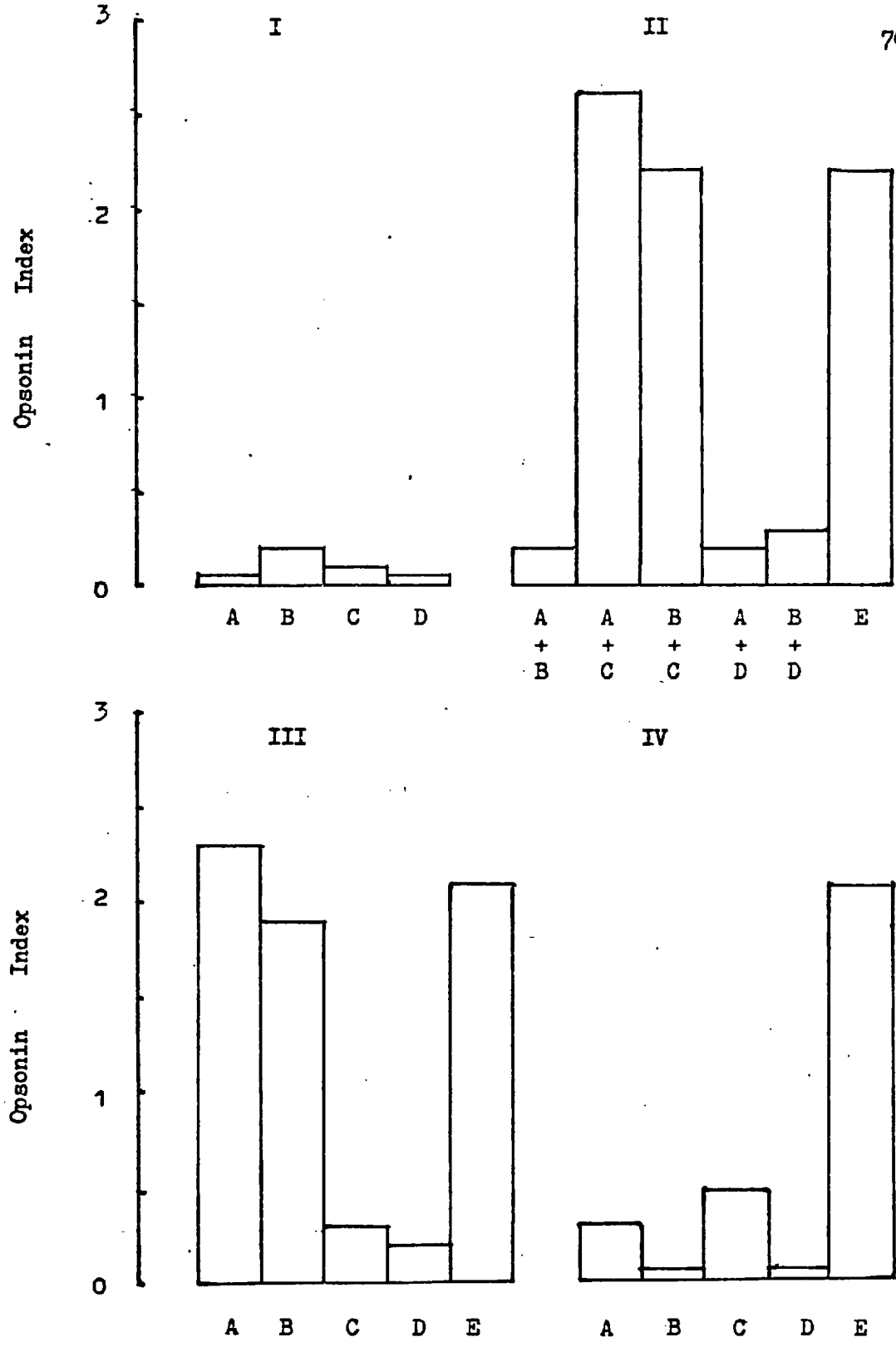


Opsonin defects in healthy individuals.

Sera from two apparently healthy laboratory workers (one male, one female) were found to have opsonin deficiency.

These sera had normal levels of total haemolytic complement, C1, C4, C2, C3 and C5. The alternative pathway and factor B activities were also normal in the haemolytic assay. Fig. 24 shows reconstitution experiments. The defect was corrected by factor B depleted serum (50°C, 15min), and also by using S.cerevisiae presensitised with factor B depleted serum (50°C, 15min). This indicates that the defective opsonisation is not due to abnormal factor B.

(See overleaf for Fig. 24)



A & B Opsonin deficient sera from healthy individuals.
C Normal serum heated 50°C, 15min.
D Normal serum heated 56°C, 30min.
E Normal serum.

I Opsonising capacity of the individual serum as indicated.
II Opsonising capacity of the mixed sera as indicated.
III Opsonising capacity of the individual serum, using preopsonised S.cerevisiae by heated (50°C,15min) serum.
IV Opsonising capacity of the individual serum, using preopsonised S.cerevisiae by Heated (56°C,30min) serum.

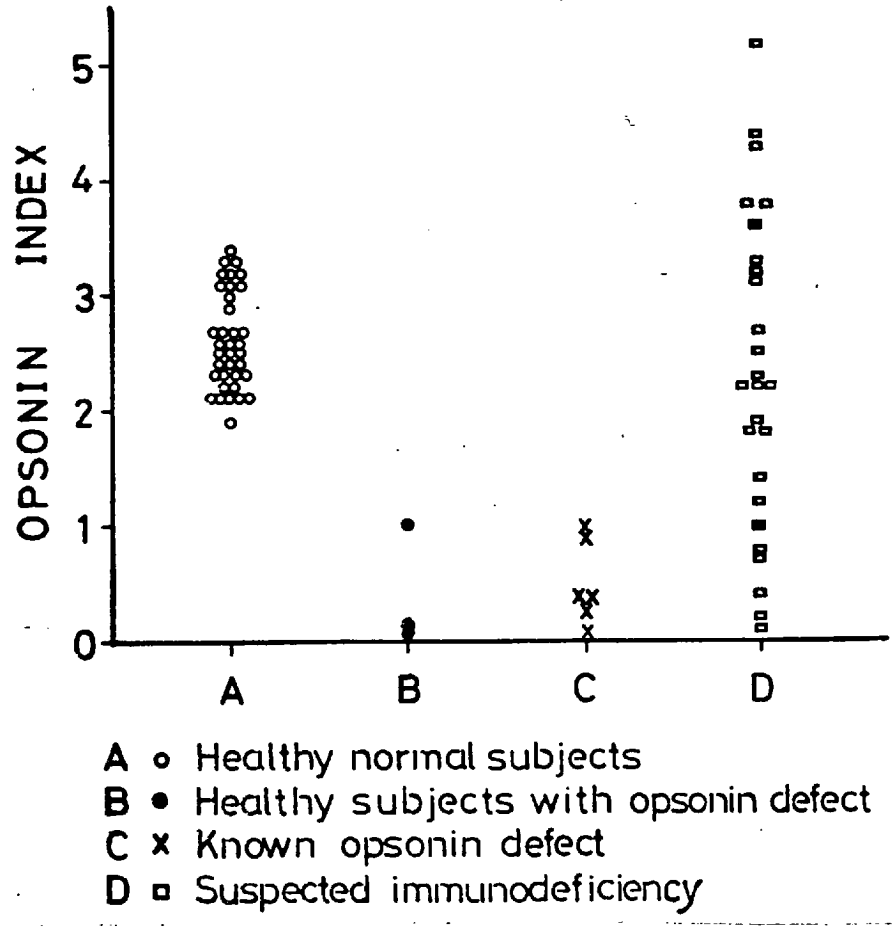
All experiments were done using 1.25% serum concentration.

Fig.24 Reconsitution of opsonin defective sera.

Preliminary clinical studies.

26 sera from patients with recurrent infection and 38 apparently healthy individuals were tested for opsonising capacity of the alternative pathway by the radiometric assay. The results are shown in Fig. 25. Sera from 8 out of 26 patients and 3 out of 39 apparently healthy individuals , have failed to generate opsonic activity. This is in good agreement with the data reported by Soothill & Harvey (1976).

Fig. 25 Summary of clinical studies.



DISCUSSION

The alternative pathway has hitherto only been analysed and measured by using haemolytic assays which in addition to the alternative pathway components also require the late classical pathway components (C5-C9). The radiometric assay described here measures directly the opsonising capacity of the alternative pathway, which is probably more relevant to microbial defence than the direct lysis of cell membranes.

In a preliminary study , sera which had normal alternative pathway and factor B activity as measured in haemolytic assays and C3 as measured immunochemically were found to have defective alternative pathway in terms of generation of opsonin activity. The defective factor(s) could be adsorbed from normal serum onto S.cerevisiae and zymosan. Preincubation of the yeast in normal serum heated at 50°C for 15 minutes suggests that the opsonin defect is not due to abnormal factor B. However, yeast preincubated in normal serum which had been heated at 56°C for 30 minutes was not ingested in the presence of the defective sera indicating that the defective factor(s) is heat labile .

The opsonin defect was not characterised further in this study. However the normal haemolytic activity of the alternative pathway in the defective sera suggests that the factor(s) involved, although activated with the alternative pathway, does not participate in the process leading to haemolysis. Alternatively the molecules involved could have two active sites, one participating in the haemolytic process and the other in opsonisation (Stossel et al., 1975).

Soothill and his colleagues have reported that their opsonin defect can usually be reconstituted by mixing sera of two unrelated opsonin deficient patients (Soothill & Harvey, 1976). This was not observed in this study. Several types of microorganisms have been shown to be capable of activating the alternative pathway directly (Bjornson & Bjornscn, 1977).

The specific immune mechanisms may be minimally involved in the elimination of such microorganisms if sufficient opsonin and chemotactic activities are generated for their rapid phagocytic elimination in the absence of specific antibody. Furthermore effective complement dependent opsonisation by the alternative pathway may be of critical importance especially in the neonatal period and also later in life during the early stages of infections, before effective levels of specific antibodies have been produced.

Chapter 7

General Discussion

GENERAL DISCUSSION

Immunity involves the elimination of foreign substances (e.g. microorganisms). If these are promptly eliminated from a host by non-specific defence mechanisms, specific immunity does not occur. Activation of the specific immune mechanisms results in the production of substances (e.g. antibodies, lymphokines) which enhance phagocytic elimination. The importance of phagocytic elimination is illustrated in patients suffering from agranulocytosis. These patients are more susceptible to infections than individuals with combined B- and T-lymphocyte deficiency. This clearly demonstrates that phagocytes play a central role in immunity. To evaluate phagocytic activity, one has to determine (1) the number of phagocytes, (2) the intrinsic functional capacity of phagocytes and (3) the ability of body fluids to provide substances which influence phagocytes' function. Phagocyte number in blood can be readily determined. Assessment of phagocytic activity requires objective and quantitative assays for the individual components of this activity. Previous assays are not satisfactory in this respect. Quote: "..... Before launching into evidence, some of the difficulties of evaluating the evidence require review. The first is the technical problem of measuring phagocytosis*..... " Thomas P. Stossel (1977).

The radiometric assays for measuring opsonisation, phagocytosis and intracellular killing described in this thesis are objective, simple and semi-quantitative. It is therefore possible to establish normal ranges for the various components of phagocytic activity in terms of these assays. Furthermore they require only small quantities of blood, which makes them suitable for clinical application, in particular for young children and patients with severe neutropenia.

Using these assays, I have demonstrated the following:

(1) opsonisation, procidation, phagocytosis and intracellular killing of

*underlined by (M.Y.)

fungal organisms can independently be measured.

(2) C.albicans is effectively opsonised in the absence of detectable antibodies and active complement.

(3) C3 directly participates in the intracellular killing of C.albicans. A new term "procidin" is suggested for substances such as C3, which render phagocytosed microorganisms more susceptible to intracellular killing.

(4) By using S.cerevisiae, it is possible to measure selectively the opsonising capacity of the alternative pathway. This biological activity is more directly relevant to microbial resistance than haemolytic activity generated by this pathway.

(5) Preliminary application of this opsonin assay suggests that a large proportion (30-40%) of patients with recurrent infection and 5-7% of apparently healthy individuals have defective alternative pathway in terms of opsonisation although this pathway was normal as measured with haemolytic assays.

These assays have been successfully adapted for measuring phagocytosis and intracellular killing of C.albicans by human blood monocytes (Cooper , Yamamura & Valdimarsson, 1978). Furthermore Dr.V.S.Sljivic has applied these assays for analysing phagocytic activity in mice(Sljivic, personal communication).

Experimental error in these assays is not more than 5% within an experiment and 10% between experiments. This error is accepted for most practical purposes. If one could use (a) standard phagocytes, (b) standardised opsonin and procidin and (c) standardised microorganisms, it should be possible not only to reduce the experimental error considerably but also to obtain comparable results between laboratories. Furthermore standard reagents may be essential for analysing phagocytic activity in terms of a three dimensional model. (see below)

(a) Standard phagocytes are cells which express a consistant function.

(b) Standardised opsonin and procidin might be obtained by isolating and purifying the substances involved. Alternatively one could use sera collected from healthy normal individuals as has been done for immunoglobulins assessment.

(c) Standardised microorganisms can be prepared in a lyophilised form.

Further consideration of a three dimensional model.

In chapter 1, I introduced a model to express phagocytic activity (see Diagram I and Diagram II). Since (1) chemotaxis occurs prior to phagocytosis and (2) since chemotactic substances are generated by the interaction of body fluids and microorganisms, which is already expressed on the X-axis of Diagram II, chemotaxis can be expressed on the negative axis of phagocytosis, that is (-Y) in Diagram III. Phagocytic activity can therefore be expressed within a three dimensional model (Diagram III).

Interaction of body fluids and microorganisms can be expressed as the function of x, i.e. $f(x)$.

Chemotaxis can be expressed as the function of x and -y, i.e. $f(x,-y)$.

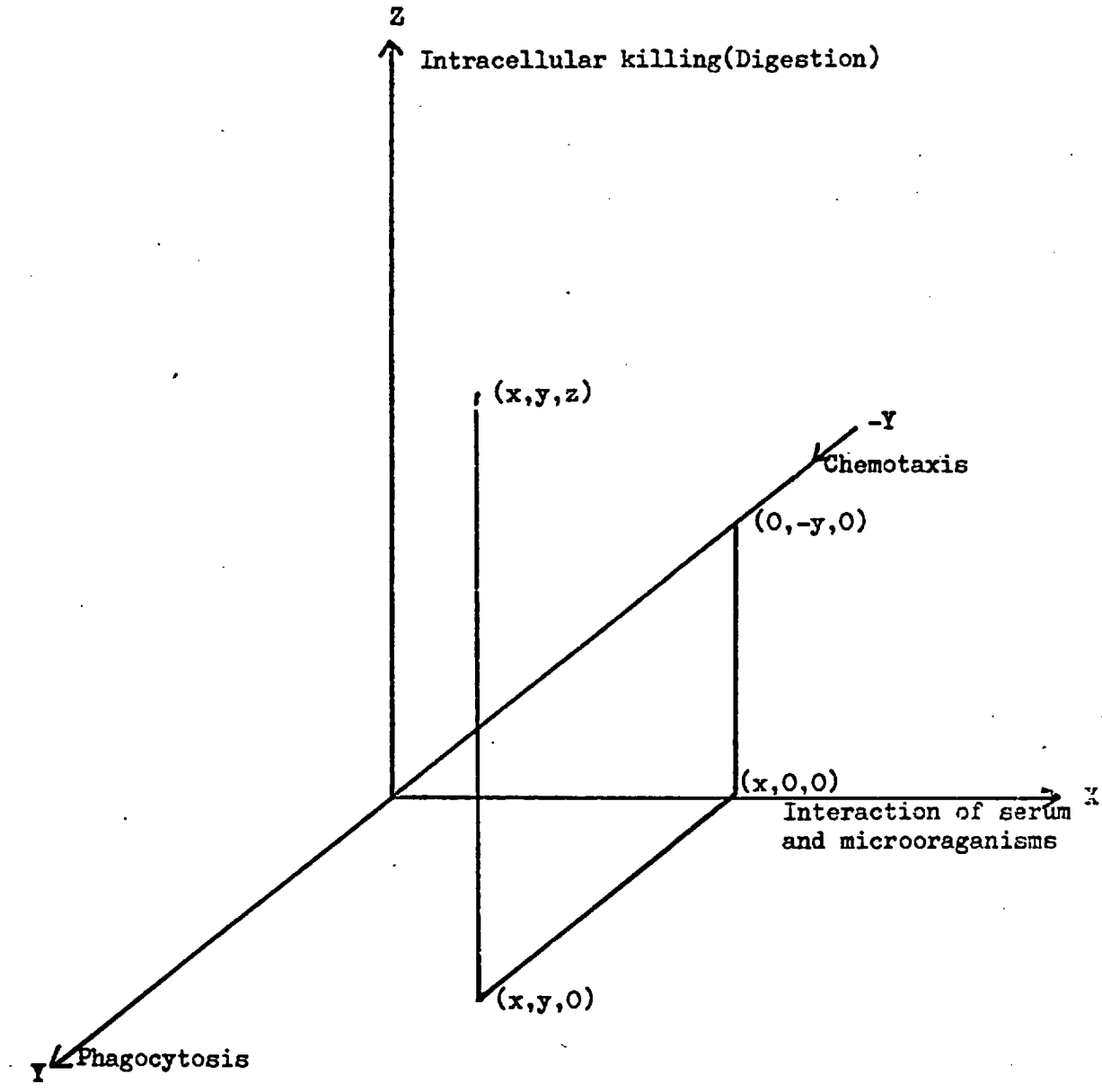
Phagocytosis can be expressed as the function of x and y, i.e. $f(x,y)$ & intracellular killing(& digestion) as the function of x,y and z, i.e. $f(x,y,z)$.

Phagocytic activity can therefore be expressed as the products of these functions multiplied by the number of phagocytes (N), provided that we are dealing with a homogeneous population of optimally activated phagocytes. Alternatively the model can also be used if the phagocytes express a normal distribution in their level of activity.

This can be expressed:

Phagocytic activity= $N \cdot f(x),(x,-y),(x,y),(x,y,z)$

Diagram III Expression of phagocytic activity.



Since the interdependence of these functions is unknown and can not be predicted, the above consideration can not be formulated mathematically. This is because phagocytic activity is undoubtedly affected by additional factors which remain to be identified. Once quantitative data on chemotaxis, phagocytosis, intracellular killing and digestion in the normal population have been accumulated, measurements of each parameter can then be plotted within Diagram III. The resultant curve can be analysed and a mathematical formula derived , which will enable the identification of the unknown factors and their role in phagocytic activity. This formula may then be extrapolated to in vivo phagocytic activity which would include the effect of specific immunity.

Once a formula for normal population has been established, the identification of defects in patients will become more precise and specific and their treatment thereby made more effective. Furthermore studies of defects in patients, may help to clarify the interdependence of phagocytic functions. The radiometric assays described in this thesis may contribute to a progress towards this objective.

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PHAGOCYTOSIS MEASURED AS INHIBITION OF URIDINE UPTAKE BY *CANDIDA ALBICANS*

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Inhibition of ^{3}H-uridine incorporation into *Candida albicans* can be used as a sensitive index of phagocytic function because: 1) there is a linear correlation between uridine incorporation and yeast number; 2) phagocytic cells do not incorporate significant amounts of uridine in short term cultures; and 3) *C. albicans* replicating inside phagocytic cells does not take up uridine from culture medium.

Appropriate conditions for measuring phagocytic capacity of human polymorphonuclear cells (PMNs) were 5×10^5 *C. albicans* and 5×10^4 PMNs in 0.5 ml of medium containing 2.5% AB serum. This mixture was incubated for 30 min at 37°C. Aliquots were then transferred into microtiter wells and incubated for a further 60 min in the presence of ^{3}H-uridine.

Under these conditions PMN leucocytes from 25 healthy individuals caused suppression of uridine incorporation ranging from 33 to 75% (50 ± 12).

INTRODUCTION

From the days of Metchnikoff (1905) techniques for assessing phagocytosis have mostly been based on microscopical counting of intra- and extracellular particles. An objective technique has been described by Stossel (1973), but the requirement for a large number of PMNs (10×10^6 per test) is a major drawback to applying this method in clinical studies.

Sensitised red cells labelled with ^{51}Chromium have also been used (Klaus, 1973) but this technique may not be suitable for measuring opsonizing activity in serum.

In this paper we describe a new assay for measuring phagocytosis. The technique is objective and easy to perform, requires less than 1 ml of blood and can be applied for measuring opsonizing activity in serum.

MATERIALS AND METHODS

^{3}H-uridine (specific activity 5 Ci/mmol) was obtained from the Radiochemical Centre, Amersham, England. LP3 plastic tubes (Lucham) were used for mixing *C. albicans* and PMNs during phagocytosis, while aliquots for measuring uridine uptake were transferred into microtiter plates (Cooke).

RPMI 1640 medium was used throughout in all experiments.

C. Albicans, strain UC 820 was maintained on 2% agar slope containing 1% peptone (Difco), and 2% glucose. Subcultures were made in 1% peptone and 2% glucose broth, for 3—4 days at room temperature. The broth cultures gave a homogeneous yeast form of *C. albicans* which maintained more than 98% viability when kept at 4°C for up to two weeks.

Leucocyte preparation

PMN leucocytes were prepared from blood, containing 20 units/ml of preservative free heparin, by sedimenting the red cells with Dextran 110 (6% w/v in 0.9% NaCl). The leucocytes were washed five times in medium containing 10 units heparin per ml and finally resuspended to 2.5×10^5 PMNs per ml.

Standard AB serum was kept in liquid nitrogen.

Assay procedure

Unless otherwise stated the standard assay procedure was as follows: *C. albicans* and PMNs were mixed in duplicates in LP3 tubes, each tube containing 5×10^5 *Candida* organisms and 5×10^4 PMNs suspended in 0.5 ml of medium with 2.5% AB serum. Duplicates of 5×10^5 *C. albicans* only in 2.5% serum were always included and other controls as appropriate. The tubes were incubated under constant rotation for 30 min at 37°C. Aliquots of 0.1 ml were then transferred to microtiter wells 5 µl of ^{3}H-uridine (10 µCi/ml) added, and the microtiter plates incubated for a further 60 min at 37°C. Cell associated radioactivity was then collected onto glass fibre discs with Skatron harvester, placed into toluene—PPO scintillation fluid and counted in a Packard spectrometer, model B2450.

Presentation of data

Replicate variability was 7%. Results were expressed as per cent inhibition of uridine incorporation by the following formula:

$$100 - \frac{\text{cpm } (C.\ albicans + \text{PMNs})}{\text{cpm } (C.\ albicans \text{ only})} \times 100$$

RESULTS

Can phagocytosed C. albicans incorporate ^{3}H-uridine from culture medium

For answering this question we used patients with chronic granulomatous disease (CGD), whose PMNs are known to have normal capacity to phagocytose but are not capable of killing or preventing germination of ingested *C.*

TABLE 1

Effects of PMN leucocytes from CGD patients on uridine incorporation by *C. albicans*.

PMN leucocyte donor	Per cent *Candida* killing		Per cent inhibition of uridine incorporation
	Methylene blue	^{51}Cr release *	
CGD patient (R.Y.)	7	1	45
CGD patient (P.Y.)	0	2	44
Mother and CGD patients	46	15	44
Healthy control	45	60	46
Normal ranges	ND	35—71	33—75

* Yamamura et al., 1976.

albicans (Lehrer, 1970). It was found that PMNs from 2 such patients, while unable to release ^{51}Cr from *C. albicans* organisms, had an inhibitory effect on their uridine incorporation to the same extent as did PMNs from healthy control subjects (table 1). It was concluded therefore, that phagocytosed *C. albicans* could not incorporate ^{3}H-uridine from the culture medium.

Incorporation of ^{3}H-uridine by C. albicans

Fig. 1 shows the incorporation of ^{3}H-uridine by varying numbers of *C. albicans* incubated at 37°C for 1 h. There was a linear rise in uridine uptake with increasing numbers of *C. albicans* organisms from 1×10^4 to 1×10^6 per well. The numbers of extra- and intra-cellular *C. albicans* could therefore be calculated from such a standard curve provided that between 1×10^4 and 1×10^6 organisms remained unphagocytosed. A mixture containing 1×10^5 *C. albicans* and 1×10^4 PMNs per microtiter-well incorporated uridine corresponding to approximately 5×10^4 unconsumed yeast organisms (50%). It should be noted that 2.5% serum significantly increased the uridine incorporation compared with serum free medium (fig. 1).

Serum requirements

C. albicans was optimally opsonized in 2.5% serum (fig. 2), and this concentration was therefore used in subsequent phagocytosis assays. There was no significant difference between the opsonizing activity of serum and heparinized plasma at any concentration. Phagocytosis, while abolished in 0.01 M EDTA, was present in serum heated at 56°C for 30 min, but minimal phagocytic killing was observed under these conditions (table 2).

Effects of varying PMN/C. albicans ratio

Fig. 3 shows curves for uridine incorporation in 2 experiments where increasing numbers of PMN leucocytes (4×10^3 to 4×10^5) were incubated

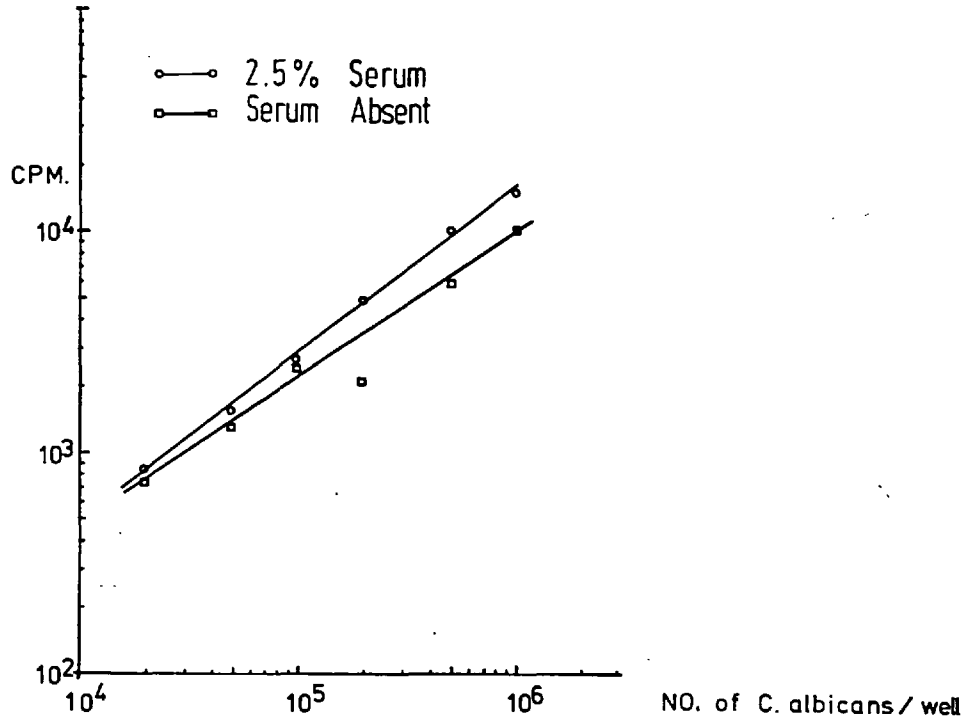


Fig. 1. Incorporation of ^{3}H-uridine by varying numbers of *C. albicans* incubated at 37°C for 1 h. □——□, Serum free medium. ○——○, 2.5% serum present.

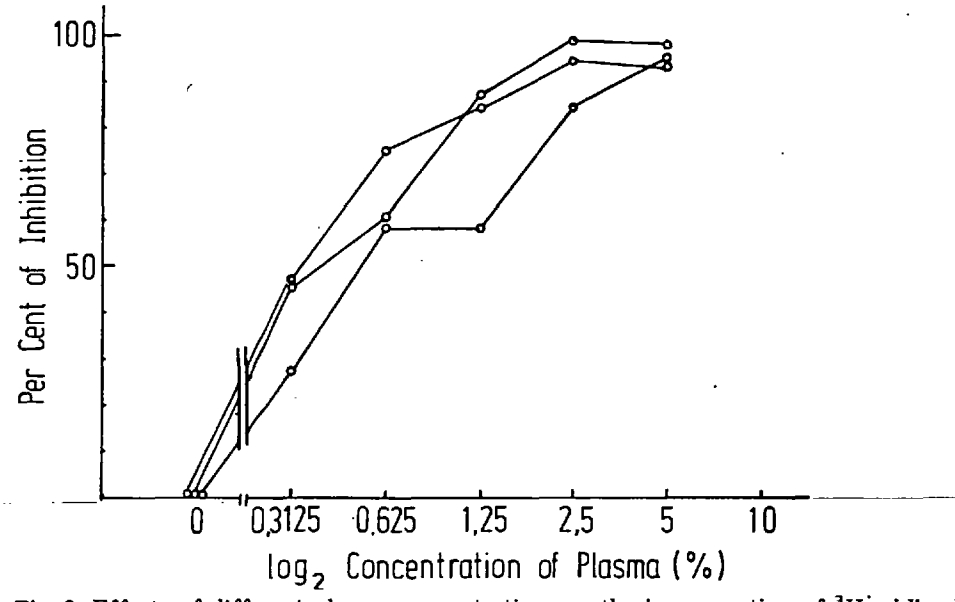


Fig. 2. Effects of different plasma concentrations on the incorporation of ^{3}H-uridine by *C. albicans* in 3 different experiments. The PMN/*C. albicans* ratio was 1/5.

TABLE 2

Serum requirements for phagocytosis and killing of *C. albicans*.

	Per cent inhibition of uridine incorporation		Per cent chromium release *	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
Untreated serum	55	67	60	69
Heated serum (56° C for 30 min)	46	56	1	2
Serum + 0.01 M EDTA	2	1	2	3
Medium without serum	3	7	2	1

* Yamamura et al., 1976.

with 4×10^5 *C. albicans*. There was approximately 50% inhibition of uridine uptake at a PMN/*C. albicans* ratio of 1/10 and this ratio was therefore subsequently used in the standard assay for phagocytic capacity of PMN leucocytes.

Normal ranges

PMN leucocytes from 25 healthy individuals were tested for phagocytic capacity in the presence of 2.5% standard AB serum. At a PMN/*C. albicans*

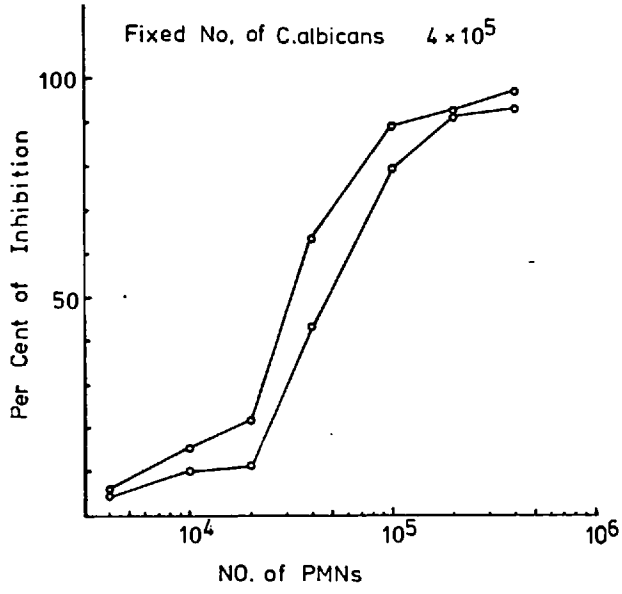


Fig. 3. Two experiments in which PMN/*C. albicans* ratios were varied from 1/100 to 1/1. There was approximately 50% inhibition of uridine incorporation at a PMN/*C. albicans* ratio of 1/10.

ratio of 1/5, a range of 74 to 99% inhibition was observed with a median of 92%. A ratio of 1/10 gave a Poisson distribution ranging from 33 to 75% with a mean of 50%.

DISCUSSION

This paper describes a new method for objective assessment of phagocytosis. It is based on the observation that *C. albicans* replicating inside phagocytic cells cannot incorporate extracellular uridine, while there is a linear relationship between the number of non-phagocytosed yeast and uridine uptake. It is therefore possible to use a standard curve for estimating the number of *C. albicans* phagocytosed and thereby to calculate the average number of yeasts ingested by each PMN leucocyte. At a PMN/*C. albicans* ratio of 1/10 and a serum concentration of 2.5%, approximately 50% of the yeast is ingested, corresponding to an average intake of 5 organisms per phagocyte.

This assay principle can probably be used for measuring phagocytosis of other micro-organisms. It has several distinct advantages such as sensitivity and objective end point. The small amounts of blood required makes the method particularly suitable for clinical use.

We are currently using this technique together with a previously reported method for measuring candidacidal activity (Yamamura et al., 1976) in order to analyse the various factors involved in opsonization and phagocytic killing of *C. albicans*.

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A ^{51}CHROMIUM RELEASE ASSAY FOR PHAGOCYTIC KILLING OF *CANDIDA ALBICANS*

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Intracellular killing of *Candida albicans* was measured by a chromium release technique. Appropriate conditions were equal numbers of polymorphonuclear leucocytes (PMN) and ^{51}Chromium labelled *C. albicans* (10^6/ml), fresh plasma at a final concentration of 2.5%, incubated at 37°C for 60 min. Using normal PMNs, 35—71% of releasable chromium was liberated into the supernatant under these conditions. This assay is easy to perform, requires a small amount of blood and offers an objective measurement of intracellular killing of *C. albicans*.

INTRODUCTION

Most techniques for assessing microbiocidal capacity of phagocytes require the removal or killing of extracellular organisms followed by lysis of the phagocytic cells. The surviving intracellular organisms are then enumerated by colony counting. Methods for assessing phagocytic killing of *Candida albicans*, without employing colony counting, have been developed but estimation of the killing is dependent on subjective microscopical examination (Lehrer and Cline, 1969; Lehrer, 1970).

This paper describes a method in which release of radioactive chromium was used for assessing candidacidal function of human leucocytes. This assay is suitable for routine clinical use because it is objective and easy to perform.

MATERIALS AND METHODS

Candida albicans, strain UC 820, was maintained on 1% peptone and 2% glucose agar slopes. Subcultures were made in 1% peptone (Difco), 2% glucose broth, for 3—4 days at room temperature. These broth cultures gave a homogeneous yeast form of *C. albicans* which maintained 99% viability if kept at 4°C for up to two weeks.

Sodium chromate ($Na_2\ ^{51}Cr_2\ O_7$) was obtained from the Radiochemical Centre, Amersham, England. *DNase* was purchased from Sigma and *sodium deoxycholate* from BDH. *RMPI 1640 medium* was used throughout.

Leucocyte preparation

Leucocytes were prepared from blood, containing 20 units/ml of preservative-free heparin, by sedimenting the red cells with Dextran 110 (6% w/v in 0.9% NaCl). If plasma was required, this was first removed by centrifugation and the blood then reconstituted with medium to its original volume. The leucocytes were washed 3 times by centrifugation at 300 *g* for 7 min in medium containing 10 units heparin per ml and finally resuspended to 2.5×10^6 PMNs per ml.

Chromium labelling of Candida albicans

The suspension of *C. albicans* was centrifuged at 700 *g* for 10 min and mixed gently with 100 μCi ^{51}Cr (1 mCi per ml). After 60 min incubation at 37° C the yeast was washed three times in 0.9% saline, counted and resuspended in medium to a concentration of 2.5×10^6 per ml. Under standard labelling conditions, 5×10^5 *C. albicans* organisms gave 8000 ± 3000 cpm. Labelled yeast could be used for up to 2 weeks if kept at 4° C.

Assay procedure

The following parameters were investigated: 1) correlation between chromium release and *Candida* viability as determined by the methylene blue dye exclusion; 2) effects of varying PMN — *Candida* ratio; 3) plasma/serum requirements; 4) effects of heat inactivation and EDTA treatment of the plasma; 5) time course of chromium release; 6) normal ranges. Unless otherwise stated, standard assay conditions were 5×10^5 *C. albicans* in duplicate, and 5×10^5 PMNs in a final volume of 0.5 ml medium containing 2.5% plasma. This mixture was incubated for 60 min at 37° C under constant rotation in LP3 plastic tubes. After incubation, 0.2 ml of DNase and 0.1 ml of sodium deoxycholate was added to a final concentration of 20 μg and 2.4 mg respectively. This mixture was incubated for a further 15 min at 37° C. The tubes were then centrifuged at 2500 *g* for 10 min 0.2 ml of the supernatants collected and counted in a Packard gamma counter. Variability of replicates was 11%.

The results were expressed by the following formula: $100(X - S)/(T - S)$), where (X) is the radioactivity released in the presence of PMNs and (S) the spontaneous release after 60 min incubation in medium. The total releasable chromium (T) was determined by incubating *C. albicans* with PMNs for 60 min followed by sodium deoxycholate-DNase treatment and boiling for 30 min. This resulted in the release of approximately 60% of the total chromium label, while only 40% release was achieved by boiling the organisms without prior incubation with PMNs.

In both instances the boiling resulted in 100% killing as determined by the

methylene blue exclusion method. It was therefore concluded that partial phagocytic digestion of *C. albicans* allowed more chromium to be released.

RESULTS

Phagocytosed *C. albicans* did not release significant amount of chromium even after prolonged incubation followed by lysis of the leucocytes in 0.24 per cent sodium deoxycholate (table 1). Thus, treatment with DNase was necessary for releasing the chromium into the supernatant.

Correlation of ^{51}Cr release and Candida viability

It has already been established that assessment of *Candida* viability by the methylene blue exclusion test correlates closely with the colony counting method, provided an adjustment is made for the effects of yeast clumping in the latter (Lehrer and Cline, 1969). We have confirmed this observation, and therefore decided to compare the chromium release assay with the methylene blue exclusion test.

Table 2 shows the correlation between these tests when used simultaneously on PMNs in 20 per cent autologous plasma from 13 individuals.

Effects of varying PMN-Candida ratio

Fig. 1 shows chromium release curves from 2 experiments in which 5×10^4 to 2×10^6 PMN leucocytes were incubated with a constant number (5×10^5) of *C. albicans*.

There was a linear increase in the amount of chromium released with increasing numbers of PMN leucocytes up to a PMN/*C. albicans* ratio of 1/1.

TABLE 1

Conditions for ^{51}Cr release from *C. albicans* *.

	Treated with:		CPM in supernatants
	Sodium deoxycholate	DNase	
C. albicans only	—	—	371
C. albicans only	+	—	389
C. albicans only	+	+	401
C. albicans + PMN	—	—	378
C. albicans + PMN	+	—	382
C. albicans + PMN	+	+	631

* Plasma at 2.5% used throughout.

TABLE 2

Comparison between the methylene blue exclusion test and ^{51}Cr release.

Subjects tested	Methylene blue test (% *C. albicans* killed)	^{51}Cr release (%)
1	39	66
2	42	64
3	32	62
4	42	58
5	35	60
6	30	42
7	44	52
8	34	48
9	44	68
10	45	60
11 *	7	1
12 *	0	2
13 **	46	15

* Patients with chronic granulomatous disease (CGD).
** Mother of 2 boys with CGD.

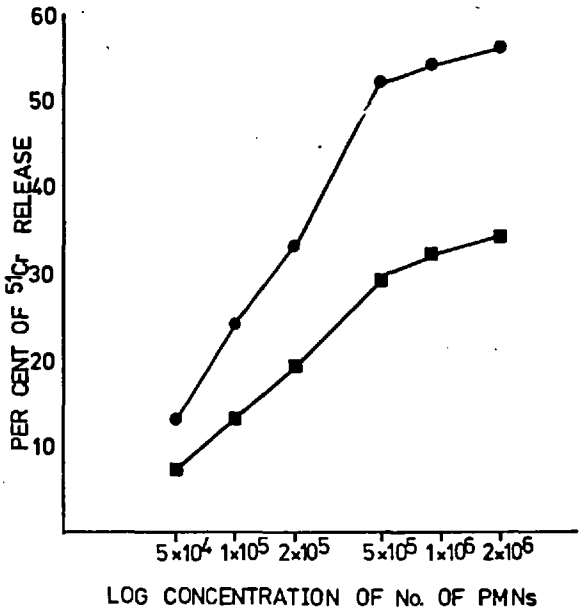


Fig. 1. Chromium release curves from 2 experiments in which increasing numbers of PMNs were incubated with 5×10^5 *C. albicans*.

In subsequent tests equal numbers (5×10^5) of PMNs and *C. albicans* were therefore used.

Plasma requirements

Experiments in which inhibition of ^{3}H-uridine incorporation into *C. albicans* was used to measure phagocytosis, demonstrated that plasma concentration between 1 and 2.5% was sufficient for optimal opsonization of this organism (Yamamura et al., in preparation). Preliminary studies showed that in the absence of PMN leucocytes, plasma concentrations of 2.5% or less did not induce significant chromium release from *C. albicans*, whereas linear increment of chromium release was observed when these organisms were incubated in plasma alone at a final concentration exceeding 5% (fig. 2). It was therefore decided to use 2.5% AB plasma in the standard assay for candidacidal capacity of PMN leucocytes. Fig. 2 demonstrates the chromium release produced by PMN leucocytes from 3 individuals incubated in doubling dilutions of autologous plasma ranging from 20 to 0.625%. Plateaux were reached at plasma concentrations of 2.5 to 5%. It should be noted that in this experiment, the descent of the curves between 5 and 10% plasma concentrations is partly due to the increase of background release caused by the higher plasma concentration.

The standard AB serum gave a consistent optimal phagocytic release at a concentration of 2.5%.

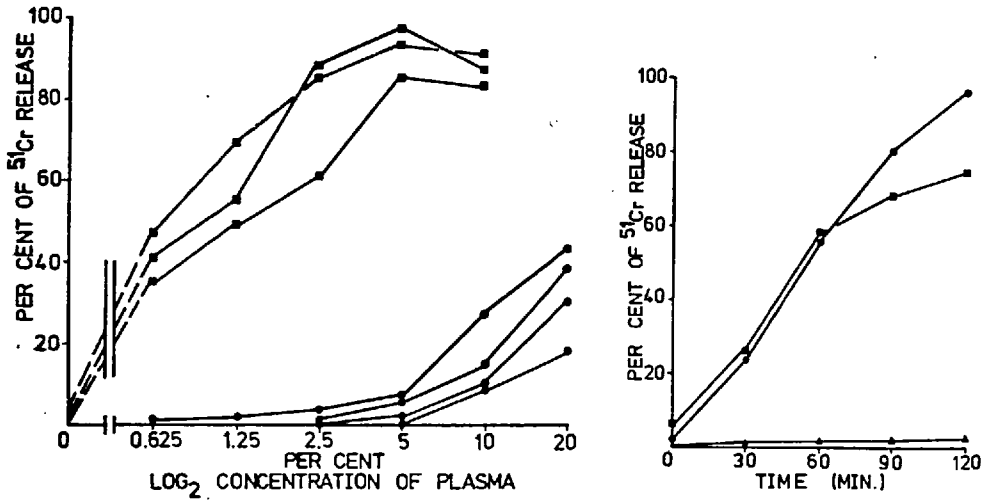


Fig. 2. Effects of plasma concentration on the chromium release. ●——●: plasma only; ■——■: PMN and plasma.

Fig. 3. Time course of chromium release. ▲——▲: 2.5% plasma only; ●——● and ■——■: PMN and 2.5% plasma.

TABLE 3

Plasma or serum requirements for phagocytic killing of *C. albicans* *.

	^{51}Cr release (%)		
	Exp. 1	Exp. 2	Exp. 3
Untreated heparin plasma	61	76	67
Untreated serum	60	69	61
Serum heated 56°C for 30 min	1	2	ND
EDTA 0.01 M	2	3	ND
No serum or plasma	2	1	ND

* 2.5% serum or plasma used in all experiments.

As shown in table 3, serum and plasma containing 20 units heparin/ml gave comparable results in the standard assay. Thoroughly washed PMN leucocyctes, when tested in the absence of plasma, did not induce chromium release, reflecting the minimal phagocytic uptake of the yeast under these conditions. Similarly, in the presence of 2.5% heat inactivated or EDTA treated plasma, PMN leucocytes were unable to induce significant chromium release (table 3).

Time course of chromium release

Fig. 3 demonstrates 2 experiments in which supernatants were harvested at 30 min intervals. In all such experiments chromium release increased with time in a linear fashion up to 60 min.

The amount of chromium released after 60 min incubation was large enough for accurate measurement and this incubation period was therefore subsequently used in the standard assay.

Normal range

PMN leucocytes from 20 healthy individuals were tested in the presence of 2.5% AB serum. A range of 35 to 71% chromium release was observed with a median of 45%.

DISCUSSION

Methods hitherto used for measuring candidacidal activity of leucocytes or serum depend on counting colony-forming units on agar or microscopic evaluation of stained versus unstained organisms. In the first yeast clumping is a major problem (Chilgren et al., 1968), whilst the latter is liable to subjective errors.

In this paper we describe a new approach in which *C. albicans* is labelled

with ^{51}Chromium and the release of this radio-isotope is used for measuring candidacidal activity. The amounts of chromium released from these organisms reflect their viability as determined by other methods. It should be noted that release of chromium from phagocytosed yeast cannot be detected in the supernatants until the leucocytes have been lysed with sodium deoxycholate and the cell lysates treated with DNase. This treatment does not affect the viability nor does it induce chromium release from non-phagocytosed yeast (table 1).

Ranges for the linear relationship obtained between chromium release and each of the following parameters were established: PMN/*C. albicans* ratio, incubation time and plasma or serum concentration. Maximal sensitivity for measuring the competence of intracellular killing mechanisms is achieved with the first 2 parameters within the linear range, whilst serum must be used in a plateau concentration ($\geqslant$2.5%) at which the candidacidal activity is no longer limited by opsonization (Yamamura et al., in preparation).

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Participation of C3 in Intracellular Killing of *Candida albicans*

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Using new objective methods for measuring, independently, phagocytosis and killing, it was demonstrated that *Candida albicans* opsonized by C3-deficient serum was ingested but not killed in vitro by human polymorphonuclear leukocytes. Killing could be induced by adding purified C3 to the C3-deficient serum. It is concluded that C3 participates directly in the intracellular process leading to phagocytic killing of *C. albicans*.

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The complement system has been extensively studied in relation to extracellular microbiocidal activity and opsonic adherence (1, 2). There is also some evidence that thermolabile serum factors may be required for effective phagocytic killing (6, 7), but direct participation of complement in the processes leading to intracellular killing of pathogens has not been conclusively demonstrated. Techniques suitable for independent but simultaneous assessment of extracellular killing, opsonic adherence, ingestion, and intracellular microbiocidal activity have not been available so far. We have now developed methods whereby these activities can be measured independently in relation to *Candida albicans* (8, 9). In the studies to be reported here, these methods were used to evaluate the role complement may have in contributing to candidacidal activity of human polymorphonuclear (PMN) leukocytes.

MATERIALS AND METHODS

Reagents and equipment. Sodium chromate ($Na_2\ ^{51}Cr_2O_7$) and [^{3}H]uridine (specific activity, 5 Ci/mmol) were obtained from the Radiochemical Centre, Amersham, England; DNase (DN100) from Sigma Chemical Co.; and sodium deoxycholate and 2.5-diphenyloxazole (PPO) from British Drug House Ltd; medium RPMI 1640 (Flow Laboratories) was buffered with *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (Hepes) and used throughout. LP_3 plastic tubes, 7 × 65 mm (Luckam), were used for mixing *C. albicans* and polymorphonuclear leukocytes (PMNs) during phagocytosis, but aliquots for measuring uridine uptake were transferred into microtitre wells (Cooke). Cell-associated activity was collected onto fibreglass discs with a Skatron harvester, placed in toluene-PPO scintillation fluid, and measured in a Packard spectrometer, Model B2450.

Candida albicans. Strain UC 820 was cultured in 1% peptone (Difco) and 2% glucose broth for 3 days at 28°C. These cultures gave a homogeneous yeast form of *C. albicans* that maintained more than 98% viability for 2 weeks when kept at 4°C.

Leukocyte preparation. PMN leukocytes were isolated from blood containing 20–40 units/ml of preservative-free heparin by sedimenting the

erythrocytes with a Dextran 110 (6% wt/vol in 0.9% NaCl). The leukocytes were washed five times in medium and adjusted to 2.5×10^6 PMNs per ml.

Sera and purified C3. Standard human serum was obtained from healthy AB donors. The standard AB serum was treated with zymosan and tested for whole complement (CH50), C4, C2, C3, and Factor B (3). Human sera deficient in C2, C3, and C6 and purified C3 were kindly supplid by Dr. P. J. Lachmann. These reagents were kept in aliquots at −190°C.

The phagocytosis assay. This assay was based on the inability of phagocytosed *Candida* to take up uridine from culture medium. The details of the procedure have been published (8). Duplicates of 5×10^5 *Candida* organisms and 5×10^4 PMNs were mixed in 0.5 ml of medium containing 2.5% or 5.0% of human serum. Duplicates of 5×10^5 *C. albicans* in 2.5% or 5.0% of the appropriate serum without PMNs were always included together with other controls as indicated. After incubation at 37°C for 30 min, aliquots from these mixtures were transferred to microtitre wells and 0.05 μCi of [^{3}H]uridine added to each well. The plates were then kept at 37°C for 60 min before the cells were harvested and the radioactivity measured. The data are presented as PMN-induced inhibition of uridine incorporation by the following formula:

$$100 - \left\{ \frac{\text{Cpm } (C.\ albicans + \text{PMN})}{\text{Cpm } (C.\ albicans \text{ only})} \right\} \times 100$$

The assay for intracellular candida killing. Details of this assay have already been published (9). *C. albicans* was incubated at 37°C for 60–120 min with 100 μCi of ^{51}Cr. After being washed, the chromium-labelled yeast was resuspended in RPMI 1640 medium to a concentration of 2.5×10^6 organisms per ml. Labelled yeast could be used for up to 2 weeks when kept at 4°C. Candida killing was measured by mixing duplicates of 5×10^5 PMNs and 5×10^5 chromium-labelled *Candida* in 0.5 ml of medium containing 2.5% or 5.0% human serum. These mixtures were incubated for 60 min at 37°C under constant rotation in LP_3 plastic tubes. The release of ^{51}Cr into the supernatant after dissolving the PMNs with sodium deoxycholate, followed by 15 min treatment with 20 μg DNase, has been found to be a reliable index of candida killing in this system (9). After centrifugation at 2500 *g* for 10 min, 0.2 ml of the supernatants was collected and radioactivity counted in a Packard gamma counter. The results are expressed by the formula: $\frac{X-S}{T-S} \times 100$, where X is the radioactivity released in the presence of PMNs, S the spontaneous release after 60 min of incubation in medium, and T the radioactivity released from the cells by heating. T was consistently found to be approximately 60% of the total chromium taken up by the *Candida* organisms.

Preopsonization of C. albicans. In some experiments (Table I) phagocytosis and killing

Table I. Phagocytic killing of preopsonized *Candida albicans**

Candida preopsonized in	Percentage ^{51}Cr release			
	Undiluted serum		2.5% serum	
	Expt. 1	Expt. 2	Expt. 3	Expt. 4
Fresh, untreated serum	28	45	10	9
Serum heated at 56°C for 30 min	26	44	1	0
Serum + 0.01M EDTA	19	36	1	1
Medium (standard assay controls)**	43	71	46	43

* *Candida* preincubated in undiluted or 2.5% serum for 30 min; killing assays carried out in the absence of serum.

** Each experiment included a standard assay control in which unopsonized *Candida* was mixed with polymorphonuclear leukocytes in the presence of 2.5% fresh, untreated serum.

were tested in serum-free medium using *Candida* organisms that had been preincubated with undiluted and 2.5% serum at 37°C for 30 min and then washed.

RESULTS

Table I shows the results of experiments in which *C. albicans* was preopsonized in undiluted and in 2.5% untreated, heated, or EDTA-treated sera. Whereas complement was not required for intracellular killing of *C. albicans* that had been preincubated in undiluted serum, organisms preopsonized in 2.5% serum, although normally phagocytosed, were not susceptible to phagocytic killing unless intact serum was used.

Table II summarizes the results of several experiments in which phagocytosis and intracellular killing of *Candida* were measured by the standard assay in the presence of untreated, heat-inactivated, C2-, C3-, or C6-deficient sera at a concentration of 2.5% and 5.0%. The various complement-deficient sera all had a normal opsonizing activity for *C. albicans*, whereas intracellular killing was not observed unless intact C3 was present. Thus, serum deficient in C3 did not induce phagocytic killing even at a concentration of 5%. However, this serum did induce killing when purified C3 was added to a final concentration of 25 μg/ml.

Serum deficient in C2 had minimal phagocytic candidacidal activity at a 2.5% concentration, whereas normal killing was observed at a concentration of 5.0% or more. C6-deficient serum had strong phagocytic candidacidal activity at a 2.5% concentration. Similarly, zymosan-treated human serum, depleted of C5–9 but, to judge from two-dimensional immunoelectrophoresis, retaining approximately 50% of intact C3, also induced phagocytic killing when used at a concentration of 2.5%.

DISCUSSION

It has previously been reported that *C. albicans* organisms preopsonized in *undiluted* heat-inactivated serum are killed by human PMNs

Table II. Complement requirements for phagocytic killing of *Candida albicans**

Type of serum used	Percentage serum concentration	Phagocytosis (% inhibition of uridine incorporation)		Intracellular killing (% chromium release)	
		Expt. 1	Expt. 2	Expt. 1	Expt. 2
Normal, untreated	2.5	51	40	54	63
Normal, untreated	5.0	56	45	48	50
Heated 56°C for 30 min	2.5	56	46	2	1
Heated 56°C for 30 min	5.0	N.D.	N.D.	5	2
C2-deficient	2.5	49	62	2	12
C2-deficient	5.0	N.D.	N.D.	42	48
C6-deficient	2.5	41	50	38	38
C6-deficient	5.0	43	37	38	34
Zymosan-treated	2.5	46	51	48	49
Zymosan-treated	5.0	N.D.	N.D.	41	42
C3-deficient	2.5	65	62	0	0
C3-deficient	5.0	N.D.	N.D.	0	N.D.
C3-deficient + C3	2.5	N.D.	N.D.	25	N.D.
Normal ranges**	2.5	33–75		33–71	

* Each serum was tested simultaneously for phagocytosis and killing in at least two experiments. The complement-deficient sera were not all tested on the same day and the table therefore summarizes data from several experiments, each including 2.5% untreated serum as a control. N.D. = not done.

** Untreated control sera, $n = 20$.

(4). We have confirmed this observation. However, *Candida* preopsonized in 2.5% heat- or EDTA-treated serum is ingested but not killed.

The experiments presented in this paper demonstrate that C3, while not essential for the ingestion of *C. albicans* by human PMN leukocytes, is directly involved in the processes leading to phagocytic killing of this organism in vitro.

Our observation that C2-deficient serum did not have a significant phagocytic candidacidal activity at a concentration of 2.5% whilst 5% of such serum induced normal phagocytic killing indicates that *C. albicans* is capable of directly activating the altemative complement pathway. We have previously demonstrated that 2.5% of intact serum gives optimal intracellular killing of *C. albicans* in our standard assay system (9). It is well established that a higher serum concentration is required for direct activation of C3 via the alternative pathway than is required for its 'classical' activation by $\overline{C42}$ convertase.

The peroxidase-hydrogen-peroxide system is the principal pathway for intracellular killing of *C. albicans* in human PMN leukocytes (5), but it is not clear whether or to what extent other microbiocidal mechanisms may be involved. The exact role played by C3 in these intracellular processes remains to be elucidated.

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