

The development of a radiometric assay for ingestion and killing of fungal blastospores by human phagocytes, with special reference to it's analytical and clinical application.

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ABSTRACT

A radiometric assay for the simultaneous, but independent, measurement of phagocytosis and killing by human mononuclear and polymorphonuclear phagocytes was developed. The main advantage of this technique is an objective discrimination between impaired ingestion and defective killing by phagocytes. The method is based upon the observation that viable ingested fungal blastospores are unable to incorporate uridine from the culture medium unless released from phagocytes by sodium deoxycholate treatment.

Optimal assay conditions were defined for the organism/phagocyte ratio, serum concentration, and incubation time. Thus standardised, this method is sensitive and eminently suitable for distinguishing the relative contributions of cellular and humoral elements to overall phagocytic activity.

The chief observations made with this assay were:

1. Optimal killing by polymorphonuclear leucocytes (PMNs) was achieved in 5-10% serum which, in terms of protein concentration, corresponds to the extravascular environment.
2. The third component of complement (C3) was necessary for killing but not ingestion of Candida blastospores by PMNs. It was found that defective killing, due to the absence of C3 or complement, was associated with reduced microbial iodination and failure to induce the respiratory burst.

A similar decrease in phagocytic killing was apparent if either heparin, which prevents C3 conversion, or trypan blue, which blocks C3b receptors, were present. Taken together these findings indicate that oxidative microbicidal processes in PMNs are preferentially activated via C3b surface receptors. Mononuclear phagocytes, unlike PMNs, did not require extrinsic C3 for killing Candida.

3. Most antibiotics, including penicillin, gramicidin, gentamicin, tetracycline, and metronidazole had no untoward effect on PMN function. In contrast, co-trimoxazole diminished phagocytic killing without affecting phagocytosis, whereas erythromycin prevented phagocytic killing by inhibiting ingestion. The antifungal drug ketoconazole, which is effective in vivo at lower concentrations than in vitro, increased the susceptibility of Candida blastospores to phagocytic killing.

4. PMNs from 112 patients with suspected immune deficiency were tested. Intrinsic impairment of PMN candidacidal activity was found in 25 patients while sera from an additional 18 patients failed to support killing by intrinsically normal phagocytes. Therefore, cellular or humoral defects of phagocytic function were observed in 37% of the patients with abnormal infections.

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'Nature has provided, in the white corpuscles as you call them—— in the phagocytes as we call them—— a natural means of devouring and destroying all disease germs. There is at bottom only one genuinely scientific treatment for all diseases, and that is to stimulate the phagocytes.'

Sir Ralph Bloomfield Bonnington in *The Doctor's Dilemma* by G.B.Shaw, 1906.

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CHAPTER 1

GENERAL INTRODUCTION

Section A: Immunity to infection

Section B: The phagocyte in host defence

SECTION A: IMMUNITY TO INFECTION

In higher animals the protection of the individual from potentially pathogenic organisms can be assigned to two types of immunity: specific and non-specific. Specific immunity is characterised by the production of antibody or reactive lymphocytes to particular antigens. It is a relatively modern evolutionary invention which has been superimposed on older non-specific defence mechanisms. Non-specific defence is active against a wide range of microorganisms and makes only crude distinctions between different antigens. It has been pointed out that both forms of immunity are interdependent (Manning, 1979). However, for convenience of presentation the two types of immunity will be discussed separately.

1-1 Non-specific immunity

Non-specific immunity is chiefly concerned with preventing entry of microorganisms into the body, although it is also effective in preventing their establishment when the primary defences have been breached.

1-2 Factors preventing host invasion by microbes.

The skin is the foremost barrier to the entry of microorganisms as well as presenting an inhospitable environment to organisms insufficiently adapted to survival on

it's surface. Commensal microbes which have adapted to this austere lifestyle are not normally a threat to their host, achieving an extremely successful symbiotic relationship which does not incur a vigorous immune response. Despite this the skin flora is continually removed by desquamation thus maintaining a constant and acceptable microbial load for the host. Correspondingly organisms are also removed from mucosal surfaces e.g. from the oropharynx and conjunctiva by the lavaging action of secretions; from the urethra by the flushing action of urination; from the gut by defaecation; and from the respiratory tract by the sweeping motion of cilia.

Commensal organisms colonise their host early in life and occupy specific environments, for example Lactobacilli are found in the vagina and anaerobic genera are present in the gut (Savage, 1972). Members of the indigenous flora can defend their existence by interference with other indigenous microbes (Savage, 1969), and with pathogens (Meynell, 1963; Hentges and Maier, 1970; Abrams and Bishop, 1966). Therefore, the commensal flora, when successfully defending itself, incidentally protects the host. However, commensal organisms are also capable of causing opportunistic disease when the host is immunocompromised (von Graevenitz, 1977; Williams, 1973). This emphasises the existence of a dynamic relationship between host defence and microbial infection, especially that between the host and his indigenous microbial flora which is normally in a state of balance.

In order to initiate disease a successful pathogen must have properties comparable to those of indigenous microbes. First, the ability of adhering to mucous surfaces and avoiding detachment by moving lumen contents, muscular or mucociliary action. Second, to withstand pH changes and antimicrobial substances in host secretions. Third, to compete on equal terms or better with other microbes on the host surface (Smith,1976).

Not only commensals (Brooker and Fuller,1975; Savage, Dubos, and Schaedler,1968) but also pathogens adhere selectively mucous surfaces. For example, Streptococcus mutans adheres to teeth by means of an extracellular polysaccharide glyco-calyx but it is not able to attach to gums or buccal epithelium (Gibbons and vanHoute,1971;Gibbons and vanHoute,1975; Hamada and Slade,1980). Similarly, the ability of enteropathogenic Escherichia coli to attach to the brush border of piglet small intestine is conferred by the protein antigen K88 (Sellwood et al.,1975;Smith and Linggood,1971;Hohmann and Wilson,1975), and gonococci adhere selectively to the epithelium of the urinogenital tract (Ward and Watt,1972).

At present the way in which pathogens overcome non-specific antimicrobial substances in secretions and compete successfully with indigenous organisms is poorly understood (Smith, 1977).

1-3. Factors preventing establishment of microbes after host invasion.

Some pathogens overwhelm the primary defences and cause

disease without significant penetration of the host. For example, Vibrio cholerae attaches to epithelial cells and cause morbidity by exotoxins (Craig,1972; Craig and Pierce, 1970). Organisms of this type probably avoid a concerted immune response simply because antigen is not 'seen' by the immune system.

On the other hand organisms which do penetrate host tissue are subjected to the non-specific secondary defences of lysozyme which is found in mucus, blood, and lymph (Fleming, 1922; Muschel, 1963); iron-chelating proteins which deny bacteria essential iron for growth (Weinberg,1978); and basic polypeptides (Hibbit and Benians,1971). However more important defences than the aforementioned are interferon, the complement system, and phagocytes.

a. Interferon.

The antiviral activity of interferon was first reported in 1957 (Lindenmann, Burke, and Isaacs,1957) and since that time a number of other effects have been discovered which include depression of cell division, inhibition of non-viral intracellular pathogens, enhancement of lymphocyte cytotoxicity, and enhancement of phagocytic activity (reviewed by Stewart, 1979). It is active against a wide range of viruses and intracellular parasites including malaria (Jahiel, Vilcek, and Nussenzweig, 1970), and therefore should be included in a description of non-specific immunity.

Interferon is released from virally infected cells and by interaction with the plasma membrane of uninfected cells

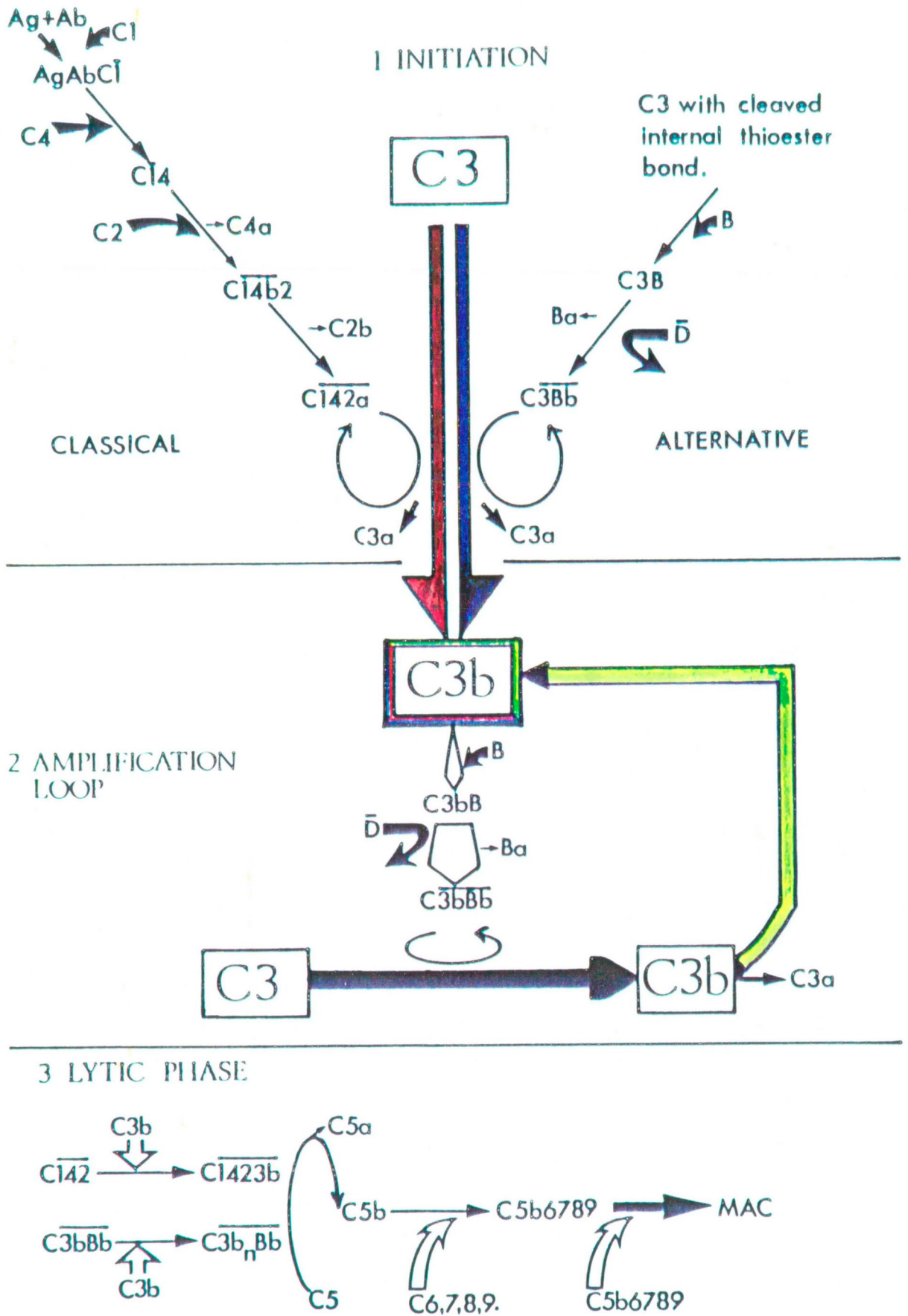
renders the latter resistant to infection (Friedman, 1967; Vengris, Stoller, and Pitha, 1975). The binding of interferon to the cell surface appears to result in the production of an inactive intracellular virus inhibitor which becomes active when that cell is infected (Friedman, 1979). The exact level at which virus replication is affected, whether at uncoating, transcription, translation, or protein synthesis remains unclear (Friedman, 1977).

b. The Complement System.

Complement consists of approximately twenty plasma proteins eleven of which, upon sequential activation, give an amplified response to an original stimulus. This type of system is called an enzyme cascade and can be compared to the clotting and fibrinolytic systems present in plasma (MacFarlane, 1969). Biologically active components of the complement cascade are responsible for opsonisation, promotion of phagocyte chemotaxis, vascular permeability, and membrane lysis.

Activation of complement proceeds via two pathways: first, the classical pathway which is started by antibody binding to antigen, and second, the alternative pathway which is initiated by microbial surfaces in the absence of antibody. The classical pathway can be regarded as an interface between specific and non-specific immunity whereas the alternative pathway is truly non-specific in function. However, both routes revolve around the conversion of C3 and are common in the later stages which lead to membrane lysis (Fig.1.1).

FIGURE 1.1. The Complement System.



Nomenclature

Components of the classical pathway are named C1, C2, C3, C4, C5, C6, C7, C8, and C9. The three subcomponents of C1 are called C1q, C1r, and C1s. Fragments of an enzymatically cleaved component are distinguished by small letters eg. C5a and C5b. An enzymatically active component or complex has a bar above it, eg. $\overline{C42}$, whereas a fragment in it's inactivated form is identified by the subscript 'i', eg. C3b_i.

The nomenclature of the alternative pathway is given in Table 1.1. The rules obtaining in the classical pathway for activated components and fragments are also applied in the alternative pathway.

TABLE 1.1. Nomenclature of components in the alternative complement pathway.

Description of component	Symbol
Third component of complement	C3
C3 Proactivator	B
C3 Proactivator convertase	D
C3b Inactivator (Factor I)	C3b INA
C3b Inactivator accelerator (Factor H)	β 1H

The Classical Pathway

The classical pathway of complement is activated when IgM or IgG antibodies are bound to antigen (Muller-Eberhard, 1968; Spiegelberg, 1974). This is thought to produce conformational changes in the antibody molecule allowing binding of C1q to previously hidden sites in the C_H2 domain of IgG and the C_H4 domain of IgM (Colomb and Porter, 1975; Burton et al., 1980; Brown and Koshland, 1977; Hurst et al., 1974; Yasmeen et al., 1976; Bubb and Conradie, 1978; Siegel and Cathon, 1980).

The native C1 molecule consists of one C1q, two C1r, and two C1s subunits in a Ca²⁺-dependent macromolecular complex (Lepow et al., 1963; Ziccardi and Cooper, 1973 a and b). The C1q molecule is made up of six protein structures, each consisting of three polypeptide chains A, B, and C. Disulphide bridges hold the A and B chains together within a single structure whilst the C chain forms a disulphide bridge with a C chain of another structure forming a doublet. The three doublets in C1q are linked by non-covalent forces, the whole structure resembling a 'bunch of tulips' (Figure 1.2.; Porter and Reid, 1979).

Limited proteolysis suggests that the immunoglobulin recognition sites of C1q reside in the globular C terminal ends of the molecule (the flower heads) while the C1s binding site is in the collagen-like N terminal region (the stalks) (Knobel et al., 1975; Knobel et al., 1974; Reid et al., 1977; Wautier et al., 1977). Each C1q molecule can bind twelve to eighteen IgG molecules (Schumaker et al., 1976). Therefore it seems likely that

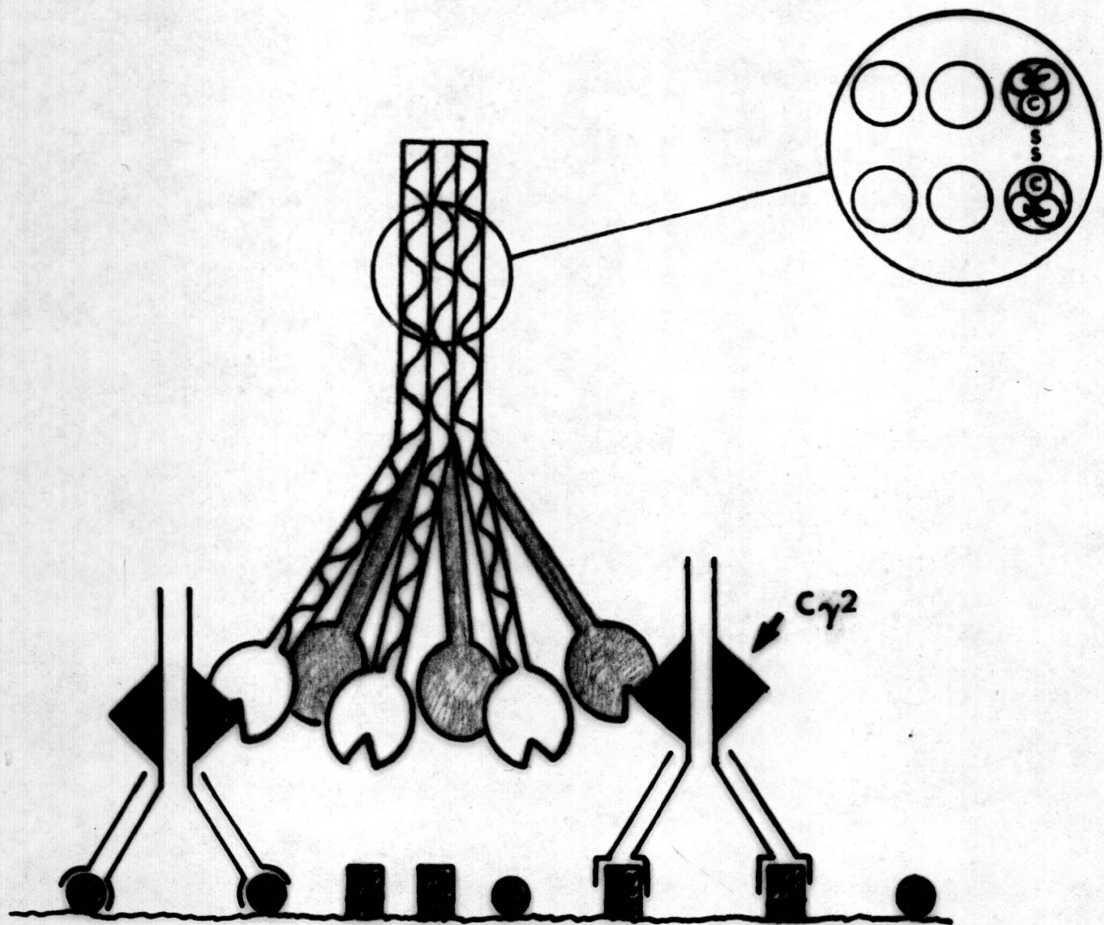


FIGURE 1.2. The Structure of C1q. Schematic representation of C1q, after Reid and Porter (1976), showing attachment to immunoglobulins of different specificity. See text for details.

C1q, as well as acting on C1r to activate complement, can also increase the avidity of bound IgG to make a 'IgM-like' antigen binding complex without the restriction of Ig variable region monospecificity.

Activation of complement by IgG requires two closely adjacent antibody molecules (probably at a distance apart not much greater than the diameter of the circle described by the globular heads of C1q), whilst a single molecule of IgM is sufficient (Borsos and Rapp, 1965; Howard et al., 1978; Humphrey and Dourmashkin, 1965 and 1969).

C1r and C1s are incapable of binding to antigen-antibody complexes in the absence of C1q (Müller-Eberhard and Kunkel, 1961). It is therefore thought that activation of C1 to C1 $\bar{r}$ is the result of a conformational change in C1r originating from a signal transmitted along the collagen-like tail of C1q as it binds to antibody with its globular C terminal region. The change causes the exposure of a catalytic site on C1r which is active upon other C1r molecules causing their irreversible change to C1 $\bar{r}$. C1 $\bar{r}$ then acts on C1s to form C1 $\bar{s}$, which unlike C1 $\bar{r}$, is able to activate the next component of the pathway, C4 (reviewed by Porter and Reid, 1979).

C4 is split by the esterase activity of C1 $\bar{s}$ to its fragments C4b and C4a. C4a is lost into solution but C4b possesses a labile hydrophobic binding site which allows it to attach to the cell surface and perhaps the Fab region of the bound Ig molecule (Goers and Porter, 1978). C4b has no enzymatic activity but allows the binding of C2 which is then split by C1 $\bar{s}$ (

Muller-Eberhard et al.,1966). The $C142$ complex is unstable; $C2$ is readily lost into the fluid phase as an inactive product leaving $C14$ free to generate further $C142$ (Borsos, Rapp, and Mayer,1961).

$C3$ is cleaved into $C3b$ and $C3a$ by the action of $C142$ which is the classical pathway $C3$ convertase. The $C3b$ portion has a labile binding site for membranes and the $C142$ complex, but $C3a$ is lost into the fluid phase. The $C142$ complex, like the $C1$ complex upon binding $C4$, alters it's specificity when $C3b$ becomes attached. In this instance the change in enzymatic specificity occurs in the $C2$ part of the complex which now attacks the α chain of $C5$ (Cooper and Muller-Eberhard,1970).

The $C3d$ portion of $C3b$ appears to be responsible for the binding of $C3b$ since this part of the molecule is left on the cell surface when $C3c$ is removed by the action of $C3INA$ (vide infra) (Ruddy and Austen,1971). Recently it has been suggested that $C3b$ is bound to the surface of particles by a covalent bond formed between a carbonyl group in the α portion of $C3b$ and a hydroxyl group on the target surface (Law and Levine,1977; Law, Lichtenberg, and Levine,1979).

Control of the classical pathway

$C1$ -inactivator acts non-enzymically on $C1$ inhibiting it's esterase activity (Harpel and Cooper,1975). The inhibition appears to be due to dissociation of $C1r$ and $C1s$ from antigen-antibody- $C1$ complexes leaving $C1q$ intact (Arland et al.,1979; Sim, Arland, and Colomb,1979; Ziccardi and Cooper,1979).

C3b-inactivator (C3bINA) modulates the classical and alternative pathways by cleaving C3b into a nicked inactive form, C3b_i (Pangburn, Schreiber, and Muller-Eberhard, 1977; Nagasawa and Stroud, 1977; Harrison and Lachmann, 1978). The nicked inactive form, which has a cut in the α chain, precedes the appearance of fragments C3c and C3d. C3bINA requires a cofactor called β 1H for production of C3b_i (Nilsson and Müller-Eberhard, 1965; Whaley and Ruddy, 1976; Weiler et al., 1976). Recently a second scission has been found in the α chain of C3b which releases a small 3K fragment to form C3b_i (Harrison and Lachmann, 1980). The identity of the responsible protease is not clear although the available evidence favours the participation of C3bINA and β 1H. The production of C3c and C3d from C3b_i requires another protease, possibly plasmin (Pangburn et al., 1977; Nagasawa and Stroud, 1977) (Figure 1.3.).

C4 binding protein (C4bp). Both Harrison and Lachmann (1980) and Pangburn et al. (1977) hold the view that β 1H is the cofactor required in the nicking of C3b. However, other workers initially attributed this activity to a C4 binding protein different from β 1H (Fujita, Gigli, and Nussenzweig, 1978; Scharfstein et al., 1978). C4bp with C3bINA acts on both C3b and C4b (Nagasawa and Stroud, 1977; Shiraishi and Stroud, 1975) but this combination is much less effective on C3b than β 1H and C3INA (Fujita and Nussenzweig, 1979). It therefore appears that C4bp is primarily involved with control of the classical pathway (Gigli, Fujita, and Nussenzweig, 1979).

It is not surprising that 'b' fragments of C4 and C5 are broken

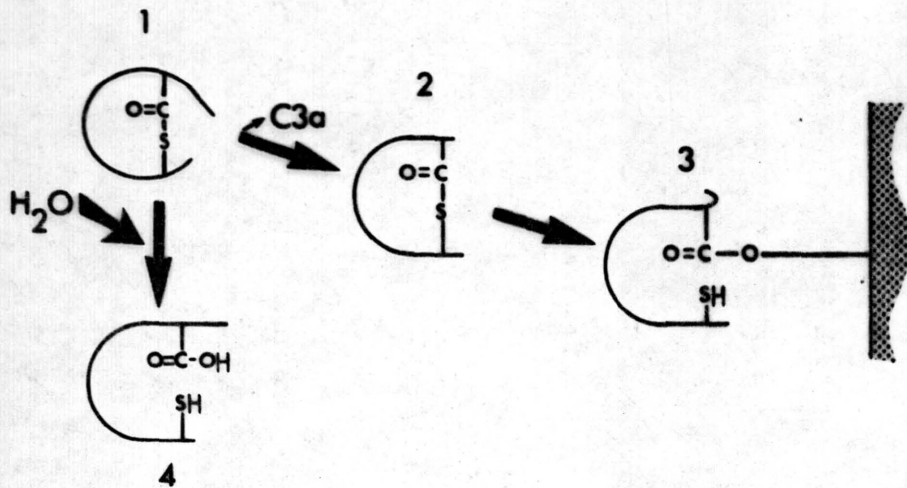
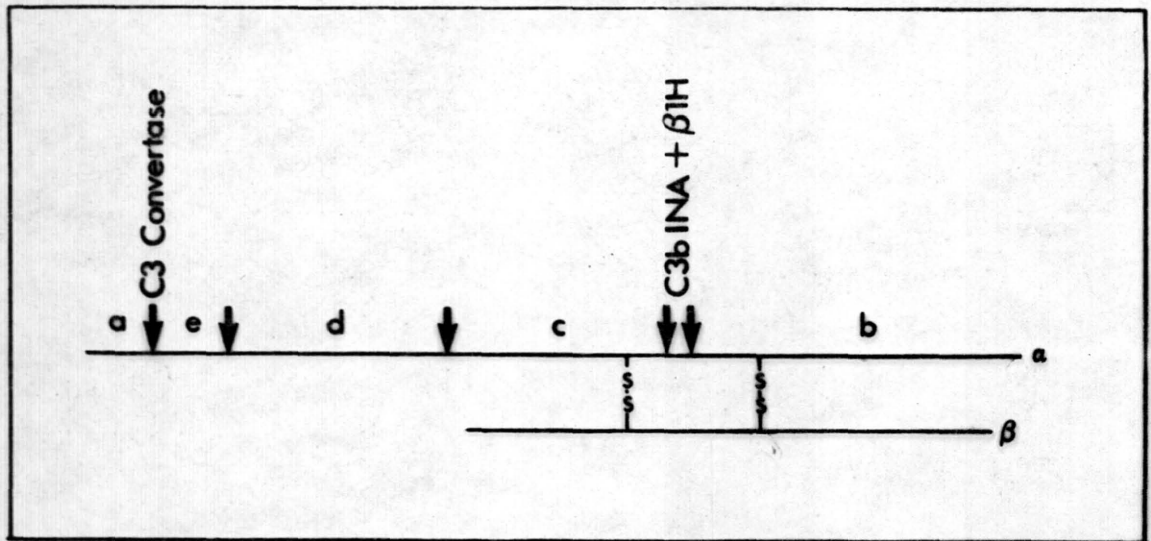


FIGURE 1.3. The metastable binding site of C3b. It has been proposed that native C3 (1) has an internal thioester bond which becomes exposed after C3 convertase-mediated removal of C3a (2). The metastable C3b so formed is then capable of covalent attachment to surfaces (3). The nucleophilic attack of water on intact C3 breaks the internal thioester bond and produces functionally "C3b-like" C3 without removal of C3a (4). After Pangburn and Müller-Eberhard (1980). See text for further details.

INSET shows proposed sites of enzyme cleavage of C3. Arrows without labels indicate cleavage sites of unidentified serum proteases. After Harrison, RA and Lachmann, PJ (1980) *Molec. Immunol.* 17, 219.

down by C3bINA since they share structural homology with C3b (see Porter and Reid, 1979). Interestingly, it has been reported that a nicked inactive form of C4b (C4b') appears before breakdown of C4b into C4c and C4d fragments (Nagasawa, Ichihara, and Stroud, 1980).

The Alternative Pathway

The alternative pathway is activated directly by bacteria, viruses, fungi and parasites without the aid of antibody (reviewed by Gotze and Müller-Eberhard, 1976; Müller-Eberhard and Schreiber, 1980). As a consequence of this the alternative pathway is considered to be of great importance in the early stages of infection when inadequate quantities of specific immunoglobulin are available.

Current theory argues that the alternative pathway is constantly cycling so that small quantities of C3b are continuously deposited on the cell surface (Lachmann and Nicol, 1973). However, the surface bound C3b is subject to prompt inactivation by C3bINA and β 1H unless certain conditions of the cell surface structure are satisfied. Significant activation of the pathway is therefore only seen when bound C3b escapes the control of C3bINA/ β 1H and is able to form the alternative pathway C3 and C5 convertases.

Initiation of the alternative pathway

The alternative pathway is started by the interaction of C3

and Factor B in the presence of magnesium ions and Factor D (Schreiber et al.,1978). After binding to C3 Factor B becomes susceptible to the action of Factor D which cleaves off the Ba fragment (Lesavre and Muller-Eberhard,1978). The resulting complex of C3Bb then cleaves other C3 molecules removing the C3a fragment and producing a metastable binding site which enables C3b to attach to particle surfaces (Law and Levine, 1977; Law et al.,1979). The C3 moiety in the initial C3 convertase appears to be of equal molecular weight to the native structure (Schreiber et al.,1975). Furthermore, the experiments of Pangburn and Muller-Eberhard (1980) suggest that the C3 of the initial enzyme complex, although whole in terms of molecular weight, has a cleaved internal thioester bond. This bond when cleaved produces the reactive carbonyl group responsible for the covalent attachment of C3b to cell surfaces. The production of the first C3 convertase may therefore depend upon the breakage of this bond by the nucleophilic attack of water (Figure 1.3.).

The C3b, having become bound to a surface, is able to form another C3 convertase (C3bBb) with the aid of Factors B and D in the presence of Mg^{2+} . The C3b fragment is unique in that it is both a product and a subunit of C3 convertase and it is this functional duality which serves to amplify both complement pathways and constitutes the C3-dependent positive feedback loop (Figure 1.1.)(Mak, Lachmann, and Majewski,1977; Mak, Majewski, and Lachmann,1977; Muller-Eberhard and Gotze, 1972)

Control of the alternative pathway

Both C3b and C3bBb are controlled by $\beta 1H$ and C3bINA: $\beta 1H$ prevents formation of the alternative pathway convertase, or, with C3bINA, causes it's active disassembly and degradation (Pangburn and Müller-Eberhard,1978; Whaley and Ruddy,1976). Properdin is implicated in the stabilisation of C3bBb (Fearon and Austen,1976; Medicus, Götze, and Müller-Eberhard,1976; Götze, Medicus, and Müller-Eberhard,1977) but it's role in the initiation of the pathway remains controversial.

The ability of the nascent C3 convertase to develop fully is dependent on the nature of the surface to which it is attached. A surface which restricts the regulation exerted by $\beta 1H$ and C3INA, by preventing interaction of C3b with $\beta 1H$, is said to be an activator of the alternative pathway (Pangburn and Müller-Eberhard,1978; Fearon and Austen,1977 a and b; Pangburn et al., 1980). Although the exact property of $\beta 1H$ interference is unknown for most activators, it has recently been shown that removal of sialic acid from sheep erythrocytes (Pangburn and Müller-Eberhard,1978; Kazatchkine et al.,1979a), or introduction of E.coli lipopolysaccharide into the membranes of sheep red cells (Pangburn et al.,1980) converts them from non-activators to activators. On the other hand it has been possible to convert activators of the pathway to non-activators by the introduction of heparin or cyanogen bromide (Kazatchkine et al.,1979b). It is unclear how surface structures dictate activation or control of the alternative pathway (Schreiber et al.,1978; Pangburn et al.,1980). There are however two theories: the ' $\beta 1H$ -antagonist' theory proposes that surface

structures bind to discriminatory sites on C3b which in turn reduces β 1H attachment and allows activation (Pang'urn et al., 1980)(Figure 1.4.). Conversely, the ' β 1H-protagonist theory' suggests that surface structures prevent activation by interacting with C3b thus causing an increase in β 1H binding (Kazatchkine et al., 1979a).

Activation of components C5-C9.

The addition of C3b to the C142 complex of the classical pathway or to the C3bBb complex of the alternative pathway allows the cleavage of the α chain of C5 (Cooper and Müller-Eberhard, 1970; Medicus, Götze, and Müller-Eberhard, 1976). The cleavage of C5 into C5a and C5b is considered to be the last enzymatic step of both complement pathways. C5a is lost into the fluid phase whilst C5b has a transient binding site for C6 and C7.

It has been difficult to demonstrate C5 convertase activity in purely fluid phase systems (Dias da Silva and Lenow, 1967; Medicus, Götze, and Müller-Eberhard, 1976) but not if surfaces have been present (Daha, Fearon, and Austen, 1976). These observations have been explained by Vogt and his coworkers (1978) who showed that C3b fixed to particles binds C5 in such a way that surface bound or fluid phase C3 convertase can act as a C5 convertase.

The assembly of C6 to C9 proceeds upon cleavage of C5 without any further enzymatic activity (Müller-Eberhard, 1975). Part of the C5b-C9 complex is inserted into the membrane (Kolb and Müller-Eberhard, 1976) producing the characteristic lesions of

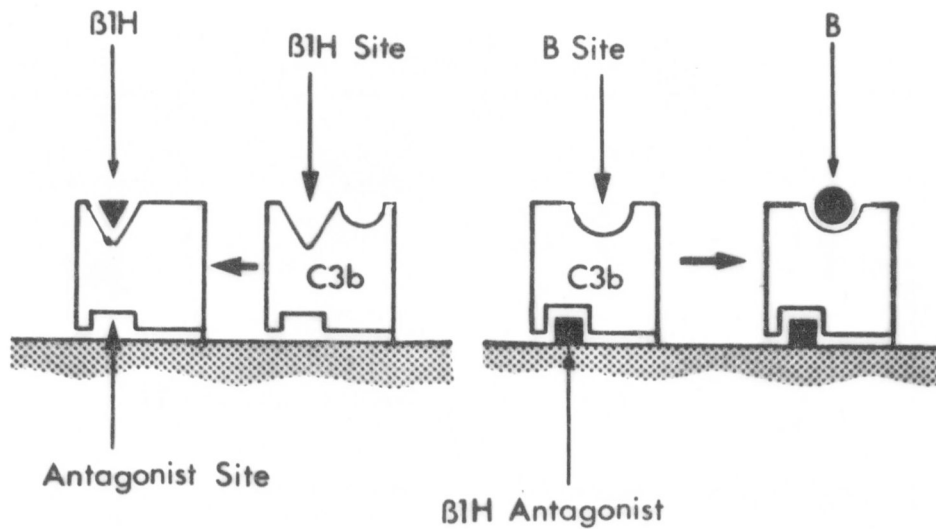


FIGURE 1.4. The antagonist theory of alternative pathway control.

Complement activation is restricted when $\beta 1H$ binds to C3b on the surface of a non-activator (LEFT). The putative $\beta 1H$ antagonist of an activating surface removes the $\beta 1H$ binding site on C3b and allows the uncontrolled formation of alternative pathway C3 convertases (RIGHT).

After Müller-Eberhard and Schreiber (1980).

complement (Humphrey and Dourmashkin,1969). C6 combines with C5b which in turn binds C7 (Lachmann and Thompson,1970; Podack, Kolb, and Müller-Eberhard,1978; Podack, Biesecker, Kolb, and Müller-Eberhard,1978). The $\overline{\text{C5b67}}$ complex has a metastable binding site for membranes (Lachmann, Munn, and Weissmann,1970; Podack et al.,1978), which can be blocked at the C5b6 stage by the S-protein, a newly recognised molecule (Podack, Kolb, and Müller-Eberhard,1978). The trimolecular complex binds C8 and, finally, C9 (Podack, Biesecker, Kolb, and Müller-Eberhard,1978). The whole complex then dimerizes to form the membrane attack complex (MAC)(Biesecker et al.,1979; Podack et al.,1979).

The effector functions of complement

1.Membrane lysis. Activation of C5 to C9 produces a membrane attack complex (vide supra). The MAC becomes inserted into lipid membranes (Hammer, Nicholson and Mayer,1975; Bhakdi et al.,1975; Hammer et al.,1977; Giavedoni, Mason, and Dalmasso, 1978; Bhakdi et al.,1978) producing channels which can be detected by electrical conductance (Michaels et al.,1976). These channels are of differing sizes; the size apparently increasing with complement dose (Boyle and Borsos,1979; Boyle, Gee, and Borsos,1979; Ramm and Mayer,1980).

The lytic action of complement can work in conjunction with lysozyme to kill Gram negative organisms (Wilson and Spitznagel, 1968; Muschel, Carey, and Baron,1959) or with antibody alone against parasites (Pearson and Steigbigel,1980). Although some parasites can eliminate potentially lytic complement-activating antibodies and so resist destruction (Balber et al.,

1979). Also, it seems that the lytic action of complement is manifested by lymphocytes which manufacture MAC components and therefore may contribute towards antibody-dependent cell-mediated cytotoxicity by these cells (Sundsmo and Müller-Eberhard, 1979; Simone and Henkart, 1980).

2. Kinin activity. C2 kinin is thought to be a cleavage product of C2 which increases vascular permeability. It is believed to be responsible for the kinin activity seen in hereditary angioneurotic oedema (Klemperer, Donaldson, and Rosen, 1968).

3. C3a anaphylatoxin activity. C3a causes the release of histamine from mast cells and thereby produces associated phenomena of smooth muscle contraction and vascular permeability (Dias da Silva, Eisele, and Lepow, 1967; Bokisch, Müller-Eberhard, and Cochrane, 1969; Cochrane and Müller-Eberhard, 1968).

4. C3b opsonin activity. The term 'opsonin' for substances in normal serum which promote microbial ingestion by phagocytes was coined by Wright and Douglas (1903). In addition, they showed that opsonins acted on the microorganism and that some of the opsonic activity was heat labile.

Gigli and Nelson (1968) and Stossel et al. (1975) established that microbial surface bound C3b was responsible for the heat labile opsonic activity in serum. Further work has indicated that antibody and complement have differing roles in opsonization: C3 appears to be responsible for the attachment of

microbes to the phagocytic cell membrane whereas Ig triggers ingestion (Mantovani, 1975; Scribner and Fahrney, 1976; Mantovani, Rabinovitch, and Nussenzweig, 1972). However, other workers claim that C3 promotes particle ingestion at low Ig levels (Wellek, Hahn, and Opferkuch, 1974) and is absolutely necessary for phagocytosis of IgM coated particles (Wellek et al., 1974; Huber et al., 1968). Conversely, it has been suggested that it is the strength of particle binding, rather than the nature of a particular ligand-receptor interaction, which triggers phagocytic ingestion (Capo et al., 1978).

The non-phagocytic stimulation of phagocytes by complement components including C3 has been the subject of recent attention (Goldstein and Weissman, 1979). This aspect of phagocytic activity will be discussed in the second section of the General Introduction, 'The phagocyte in host defence'.

5. C3e leucocytosis-inducing activity. It has been reported that C3e mobilises leucocytes from isolated rat bone marrow and causes an increase in circulating levels of leucocytes in rabbits after intravenous injection (Ghebrehiwet and Müller-Eberhard, 1979).

6. C5a anaphylatoxin activity. Fragment C5a possesses anaphylatoxin activity (Cochrane and Müller-Eberhard, 1968). This activity is subject to inactivation by a carboxypeptidase enzyme which removes an arginyl residue from the C terminal region. The product of inactivation, C5 des arg, has no anaphylatoxin activity (reviewed by Goldstein, 1979).

7. C3 and C5 derived chemotactic activity. A C3 derived fragment structurally related to C3a has been reported to be chemotactic (Ward, 1967; Bokisch, Müller-Eberhard, and Cochrane, 1969; Ward, Cochrane, and Müller-Eberhard, 1965). However studies on purified C3a have thrown these initial observations into question and doubt has arisen as to whether C3a has any significant function as a chemoattractant (Fernandez et al., 1978; Snyderman, Phillips, and Mergenhagen, 1971).

C5a is chemoattractant for polymorphonuclear leucocytes (Ward and Newman, 1969; Fernandez et al., 1978; Chenoweth and Hugli, 1978). The anaphylatoxin inactivation of C5a also abolishes its chemotactic properties (Fernandez et al., 1978), but it remains active in the presence of small amounts of normal serum. Other studies have revealed reversible inactivators of C5 derived chemotactic activity. The presence and levels of these inactivators can be correlated with clinical disease (reviewed by Goldstein, 1979). The trimolecular complex C5b67 is also regarded as a chemoattractant.

Complement deficiencies

The basis and effect of various complement defects will now be examined. For detailed reviews the reader is directed to Lachmann and Rosen (1979) and Whaley (1978).

C2 deficiency. Human C2 deficiency is inherited in an autosomal recessive manner (Klemperer et al., 1966). It is the

most common complement deficiency however it's clinical significance is unclear; approximately half the patients described are healthy. Factor B and C2 are thought to be derived from the same ancestral gene. It's therefore possible that in some circumstances Factor B can compensate for the absence of C2. Some patients with C2 deficiency have immune complex associated diseases which may indicate that this defect is causally related to abnormal antigen clearance. However, immune complex-like disorders are associated with other unrelated complement deficiencies and therefore no firm conclusions can be drawn.

Hereditary angioneurotic oedema is due to C1-inactivator deficiency. Individuals suffering from this disease have unrestrained consumption of C1-C4 which gives rise to fragments affecting vascular permeability. C3 and other components are at normal levels.

C3 deficiency. Complete absence of C3 has been reported in five subjects (Alper et al.,1976; Ballow et al.,1975; Davis et al.,1977; Grace et al.,1976; Osofsky et al.,1977). Partial C3 deficiency has also been described (Alper et al.,1969; Hoppe et al.,1978; McLean et al.,1980).

There are two dominant C3 allotypes present in the population designated C3 Fast and C3 Slow (C3F and C3S). C3S has a higher gene frequency which is reflected in the population phenotypically (Alper and Rosen,1976). There are no functional differences between various allotypes although C3FF serum supports

greater C3-mediated rosettes with monocytes than does C3SS (Arvilommi,1974).

Patients with complete C3 deficiency are subject to life-threatening infections by pyogenic microbes. Patients with partial deficiency may be predisposed to immune complex diseases resembling Systemic Lupus Erythematosus.

A primary deficiency of C3bINA causes a secondary deficiency of native C3 and Factor B due to uncontrolled formation of alternative pathway C3/C5 convertases (Abramson et al.,1971; Thompson and Lachmann,1977; Ziegler et al.,1975). Clinically patients with this disorder resemble those with C3 deficiency.

C5 deficiency. This defect has been described in three families (Rosenfeld, Kelly, and Leddy,1976; Snyderman et al.,1979; Haeney et al.,1980). Homozygotes suffer from Gram negative bacterial infections and their serum fails to support in vitro chemotaxis by normal phagocytes. Later components in the lytic stage of complement (C6-C9) are also necessary for efficient defense against Gram negative bacteria.

1-4 Specific Immunity

In most instances non-specific defence is sufficient to protect the host. It is only when these mechanisms fail to eliminate microbes that specific immunity comes into play.

Specific immunity is the acquired ability of the host to respond to microbial infection. It is characterised by the production of antibody and reactive lymphocytes which leads to an increased state of immunity and memory specific for the infecting agent.

Specific immunity is dependent upon the clonal selection of lymphocytes bearing receptors for antigenic determinants on foreign material (Burnet, 1959; Goodman, 1977). Antibody to antigenic determinants is produced by B lymphocytes in collaboration with helper T lymphocytes and macrophages (reviewed by Miller, 1972; Parkhouse, 1977; Roelants, 1977; Rosenthal, 1978).

The principal antibodies of the immune response are IgG and IgM (Spiegelberg, 1974). IgM is produced during the primary response when its high avidity and complement fixing properties are crucial to the host's resistance to early stages of infection. High affinity IgG, produced in the secondary response, is, as well as activating complement, opsonic in its own right. This class of antibody also has regulatory properties. Both IgG and IgM are found in mucous secretions but they are probably of functionally greater importance in interstitial fluid. Maternal IgG can cross the placenta and therefore helps to defend the foetus and neonate before its

own immune system is fully operational.

IgA, acting like a protective 'paint', is found on mucous surfaces, eg. the gut where it is derived from bile and lymphoid areas of the intestine (Hall and Andrew, 1980). It is also found in colostrum thus serving the newborn in a similar way to maternal IgG.

IgD is found as a membrane protein on a proportion of small lymphocytes. Its function is unknown but it has been suggested that it acts as a regulator of lymphocyte activation.

IgE, the remaining immunoglobulin class, causes histamine release from mast cells upon binding antigen. It is also associated with immunity to parasites.

In addition to antibody, specific cellular immunity is practised by cytotoxic T cells which can recognise and destroy virally infected cells (Shearer and Schmitt-Verhulst, 1976; Zinkernagel and Doherty, 1978; Cerottini and Brunner, 1977; McMichael, 1979). T cells also produce lymphokines which activate macrophages turning them into cytotoxic effector cells (Evans and Alexander, 1972).

Historically, the relative importance of cellular and humoral immunity was hotly debated (Silverstein, 1979). However, the integrated nature of the immune system is now realised since it is known that antibody and complement, although to some extent effective on their own, also work in conjunction with phagocytes and lymphocytes to opsonise and kill target cells (Sissons and Oldstone, 1980).

SECTION B: THE PHAGOCYTE IN HOST DEFENCE

Phagocytic function research was initiated by Metchnikoff with his classical experiments on inflammation in starfish larvae during the early 1880s (Karnovsky, 1981). Almost a hundred years has passed and in that time a significant impetus for further study was the recognition of chronic granulomatous disease and its attribution to a primary phagocytic defect (Good et al., 1968).

Over the last twenty years research has been concentrated on many areas of the phagocyte's life history and although much is known about the role of opsonins and chemotaxins, relatively little work has been devoted to the effect of serum on phagocytic killing. Since much of this thesis is given over to the effect of serum on phagocyte microbicidal activity the following description of phagocyte biology will emphasise microbial killing mechanisms and deal with other aspects only briefly.

1-5. Development.

Polymorphonuclear and mononuclear phagocytes (PMNs and MNP's respectively) develop in the bone marrow where they are derived from committed stem cells (Territo and Cline, 1975; Walker and Willemze, 1980). In this environment neutrophil PMNs acquire their armoury of primary (azurophil) and secondary (specific) granules (Bainton, Ulliyot, and Farquhar, 1971; Bainton, 1977). The azurophil granules, which contain myeloperoxidase, cationic proteins and acid hydrolases, are the

first to appear, occurring at the promyelocyte stage. The azurophil granules are considered to be true lysosomes, unlike the specific granules which contain lysozyme and alkaline phosphatase and do not appear until the myelocyte stage. Both quantitative and qualitative granule defects can occur (Bainton,1981).

The maturation time from myeloblast to segmented form is approximately one to two weeks. Therefore, to offset the disadvantage of a slow maturation time a large pool of granulocytes, called the marrow granulocyte reserve, is available for sudden needs. Cells enter the circulation from the marrow and do not return to their sites of proliferation and differentiation. Instead they marginate on vessel walls and eventually leave the circulation. The normal daily turnover in man between marrow and tissue accounts for approximately 10^{12} cells.

Mononuclear phagocytes are released into the circulation where they are known as monocytes. Monocytes are then thought to become tissue macrophages which are highly phagocytic and do not normally divide (Langevoort et al., 1969).

1-G. Leucocyte movement

There have been several excellent reviews on phagocyte locomotion and it's pathology in recent years (Zigmond,1977; Callin et al.,1980; Wilkinson,1974; Wilkinson,1980; Snyderman

and Pike,1977). I therefore shall not attempt a comprehensive account here especially since 'the time when the whole field could be covered realistically in a short review has passed forever' (Wilkinson,1980). Instead I merely mention that PMNs and MNP's move in response to chemokinetic and chemotactic factors released from inflammatory sites or cell damage. The principal chemotactic peptide appears to be C5a which, to simplify, forms a concentration gradient along which the leucocyte travels to meet invading microbes. Movement is accomplished by means of the so called 'motor' which is comprised of actin and myosin (Stossel et al.,1980).

1-7. Phagocytosis

Phagocytes possess receptors which enable them to recognise and attach particles opsonised with immunoglobulin and complement (Stossel,1974; Stossel,1975; Rabinovitch,1968), although they can also bind and ingest certain microbes in the absence of immunoglobulin (van Oss,1978; Weir and Ogmundsdottir, 1977). Ingestion is achieved by enveloping pseudopodial processes which fuse on the distal side of the microorganism causing the latter, enclosed in a vacuole, to be pinched off from the inner side of the cytoplasmic membrane. The increasing apposition of phagocyte membrane and microbial surface, which terminates with the formation of a phagocytic vacuole or phagosome, has been likened to a zipper being fastened (Griffin et al.,1975; Griffin, Griffin, and Silverstein,1976; Michl et al.,1979). The coup de grace is delivered when granules fuse with the phagosomal membrane and discharge their contents

into the vacuolar space. Degranulation results in the formation of secondary phagosomes containing dead and degraded microorganisms (Elsbach, 1980).

1-8. Antimicrobial activity

The respiratory burst.

The 'respiratory burst' is a collective term used to describe the biochemical changes accompanying phagocyte stimulation. Oxygen consumption is the cardinal feature of the respiratory burst but amongst other changes are superoxide anion formation and hydrogen peroxide production (Babior, 1978).

A sharp increase in oxygen consumption by phagocytosing cells was first noted by Balldridge and Gerard (1933) using canine leucocytes. Later experiments gave similar results with guinea pig granulocytes (Stahelin, Suter, and Karnovsky, 1956; Stahelin, Karnovsky, Farnham, and Suter, 1957). It was initially thought that oxygen uptake was necessary for the energy requiring process of particle engulfment. However, this idea was soon quashed when it was realised that ingestion, oxygen consumption and hexose monophosphate shunt activation, were all unaffected by inhibitors of mitochondrial respiration (Sbarra and Karnovsky, 1959). Moreover, phagocytosis could take place in anaerobic conditions.

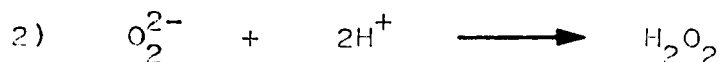
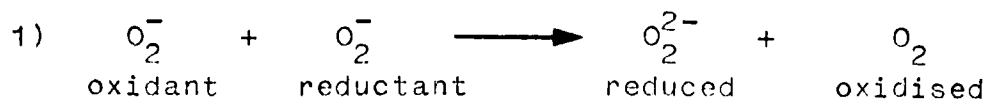
The true purpose of oxygen consumption, namely to arm the phagocytes against microbes, was brilliantly foretold by Iyer, Islam, and Quastel (1961) who postulated that peroxide

production from oxygen was involved in phagocytic killing. More direct measurements subsequently confirmed that peroxide was produced by phagocytes (Paul and Sharra, 1968; Root, Metcalf, Oshino, and Chance, 1975; Homan-Muller, Weening, and Roos, 1975), although it is now recognised that peroxide works antimicrobially in conjunction with myeloperoxidase (Klebanoff, 1968; Klebanoff, 1967; Cech et al., 1979a; Lehrer and Cline, 1969b).

Biochemistry of the respiratory burst.

The one electron reduction of oxygen to form the superoxide anion is considered the first of the reactions following initiation of the respiratory burst (Babior, Kipnes, and Curnutte, 1973; Drath and Karnovsky, 1975; Johnston, Keele, and Misra et al., 1975; Tauber, Goetzl, and Babior, 1976; Johnston, 1978). It has been estimated that all the oxygen consumed by phagocytes is converted to the superoxide anion, approximately 80% of the latter being used for the production of H_2O_2 (Root and Metcalf, 1977).

Ground state molecular oxygen has two unpaired electrons in its outer orbitals. These electrons occupy separate orbitals and have parallel spins thus satisfying Hund's rules (see Coulson, 1973). As the orbitals are incomplete each can accept another electron. Addition of one electron to the molecule results in the formation of the superoxide anion (O_2^-), whilst filling both orbitals produces the peroxy anion (O_2^{2-}). Peroxide can form from the spontaneous dismutation of two superoxide anions since the latter can act as oxidant or reductant:



The dismutation reaction is slower at neutral pH than at acid pH because of electrostatic repulsion between unprotonated anions. To overcome this oxygen utilising cells possess superoxide dismutase (SOD) which can promote dismutation at a very fast rate in the pH range 5-9 (Fridovich, 1975). A rise in phagosomal pH (Segal et al., 1981) would appear to correlate with SOD favoured dismutation.

The source of electrons for synthesis of O_2^- , and the enzyme system responsible for this reaction, remains a subject of considerable controversy (Badwey, Curnutte, and Karnovsky, 1979; DeChatelet, 1973), not least because of the variety of sources used for phagocytes and the different experimental conditions employed. I shall briefly outline the evidence for the strongest candidates which may act as electron donors.

'NADPH oxidase'

This enzyme activity was first reported by Iyer and Quastel (1963) who studied homogenates of guinea pig granulocytes. NADPH or NADH could act as electron donors to produce H_2O_2 . However, maximal activity was achieved with NADPH at low pH in the presence of Mn^{2+} . This enzyme system was present in the granule fractions (Rossi and Zatti, 1964; Patriarca et al., 1973) and was found to be diminished in the granule fraction

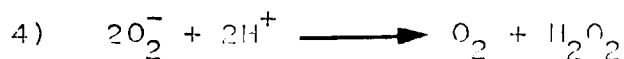
of cells from individuals suffering from chronic granulomatous disease (Hohn and Lehrer, 1975).

Unfortunately, all of the foregoing work was done at acid pH in the presence of Mn^{2+} which was later shown to promote a non-enzymic chain reaction oxidising NADPH in an O_2^- dependent manner (Curnutte, Karnovsky, and Babior, 1976). The enzyme activity was then studied again in the absence of Mn^{2+} and found to have a broad pH optimum at neutrality (Gabig and Babior, 1979).

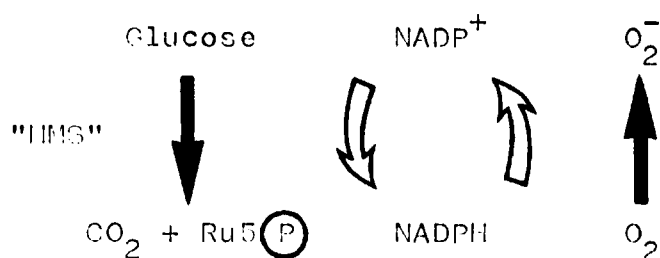
Sensitive isotopic and fluorometric assays were developed and found to substantiate the earlier findings of SOD inhibitable NADPH oxidation in leucocyte granule fractions (Iverson, DeChatelet, Spitznagel, and Wang, 1977; McPhail, De Chatelet, and Shirley, 1976). The work of Babior and his associates, using O_2^- production as opposed to NADP formation, largely agreed with the above (Babior, Curnutte, and McMurrich, 1976), although a flavin requirement was also reported (Babior and Kipnes, 1977). NADPH is considered an attractive candidate for the cofactor in O_2^- formation because it was possible to confirm that there is reduced NADPH oxidase activity in granule fractions of CGD leucocytes (McPhail, De Chatelet, Shirley, and Wilfert et al., 1977; Curnutte, Kipnes and Babior, 1975).

The postulated sequence of reactions for the reduction of oxygen if NADPH was the electron donor are as follows (Babior et al., 1976):

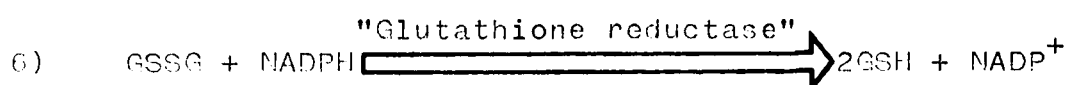
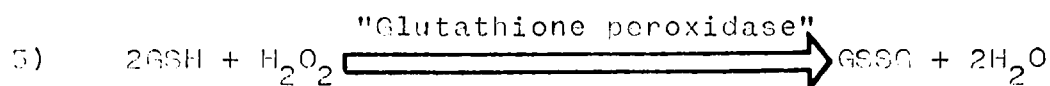




The production of NADP^+ from NADPH drives the hexose mono-phosphate shunt (HMS) and accounts for it's activation during the respiratory burst (Sbarra and Karnovsky, 1959). The HMS regenerates NADPH which would then be available for further oxygen reduction:



Another potential source of NADP^+ for the HMS is the detoxification of H_2O_2 in the cytosol by the glutathione peroxidase-glutathione reductase system (Reed, 1969; Voetman, Loos, and Roos, 1980):



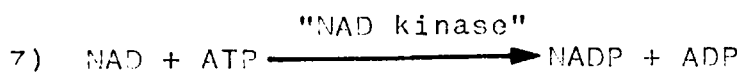
'NADH oxidase'

An NADH oxidase system has been reported in guinea pig leucocytes (Cagan and Karnovsky, 1964) which produces both O_2^- and H_2O_2 (Badwey and Karnovsky, 1979). A plasmalemma bound NADH oxidase has also been found in human PMNs (Briggs, Drath, Karnovsky, and Karnovsky, 1975). The latter is decreased in patients with CGD (Briggs, Karnovsky, and Karnovsky, 1977).

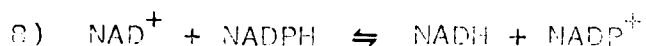
Recently, considerable interest has arisen because a b-type cytochrome has been identified in human granulocytes (Segal and Jones, 1979 a and b). However, it has not been possible to show that this surface bound cytochrome (Segal and Jones, 1979a) is reduced by NADH or NADPH (Cross et al., 1981).

Similar cytochromes have been found in horse (Hattori, 1961) and rabbit granulocytes (Shinagawa et al., 1966). Cytochrome b₂₄₅ studied by Segal and his associates is absent in CGD phagocytes (Segal et al., 1978). Not all individuals with CGD lack this cytochrome (Borreagaard et al., 1979; Segal and Jones, 1979b) which implies that the superoxide anion generating system is made up of several components. Indeed, this would agree with the genetic basis of CGD which is manifested in an X-linked or autosomal recessive manner (Good et al., 1968; Winthorst et al., 1968; Azimi et al., 1968; Ament and Ochs, 1973; Dujak et al., 1973; Clark and Klebanoff, 1973).

The paradox of diminished NADPH and NADH oxidase activity in CGD can perhaps be explained by the observation that in human leucocytes there exists an NAD kinase (De Chatelet et al., 1972) which could synthesise NADP:



Therefore, NADH oxidation could be linked to formation of NADP and stimulation of the HMS. It is also conceivable that a transhydrogenation reaction between NAD^+ and NADPH could take place (see Bhagvan, 1978):



Site and initiation of the respiratory burst

The relationship between PMN metabolism and stimulation by various surface acting agents has recently been reviewed (Goldstein and Weissmann, 1979; Baehner and Boxer, 1979). Because much of the material presented in this thesis is concerned with PMN activation it will also be covered here.

1. Membrane oxidative systems.

Phagocytosis is not strictly necessary for initiation of the respiratory burst since materials which perturb the plasma membrane in the absence of ingestion are active in this phenomenon. These include Concanavalin A, phospholipase C, non-phagocytosable particles, and detergents (Graham et al., 1967; Romeo, Zabucchi, and Rossi, 1973; Patriarca et al., 1971; Simberkoff and Elsbach, 1971). Conversely, other surface active agents such as p-diazobenzenesulphonic acid (DBSA) appear to inhibit oxidative metabolism (Goldstein, Cerqueira, Lind, and Kaplan, 1977). These findings strongly suggest that the O_2^- generating system is, at least in part, an ectoenzyme system. This is supported by various morphometric and cytochemical lines of evidence. First, the redox dye nitroblue tetrazolium, which is reduced to blue formazan by O_2^- (Segal, 1974; Baehner, Boxer, and Davis, 1976), has disclosed that respiratory burst activity is produced at the point of membrane contact with particles (Nathan, Baehner, and Weaver, 1969). Second, the detection, on the surface of stimulated PMNs, of H_2O_2 with

cerous ions (Briggs, Drath, Karnovsky, and Karnovsky, 1975) or diaminobenzidine (Briggs, Karnovsky, and Karnovsky, 1975). (Using both of these techniques Briggs et al., (1977) showed that there was considerably less H_2O_2 produced in CGD cells.) Third, NAD(P)H dependent O_2^- forming activity has been located in the plasma membrane of human (De Wald et al., 1979) and guinea pig leucocytes (Cohen, Chovaniec, and Davies, 1980). Fourth, the cytochrome activity found by Segal and associates is present in plasma membrane fractions (Segal and Peters, 1977; Segal and Jones 1979a).

Evidence against a plasma membrane location for the oxidase activity has been presented (Patriarca et al., 1973; Iverson, et al., 1977), but its interpretation is, at present, confused (Babior et al., 1978). However, it is perfectly logical to assume that the location of oxidase activity would shift from the membrane to phagosome after ingestion.

2. Stimulation of oxidative metabolism.

Mention has already been made of agents which can stimulate oxidative metabolism. This section, therefore, will be restricted to the action of immunoglobulins and complement components.

Adherence of PMNs to non-phagocytosable surfaces coated with aggregated IgG produces an increase in their oxygen metabolism and release of lysosomal contents (Henson and Oades, 1975; Johnston and Lehmeyer, 1976). This phenomenon has been called 'reverse endocytosis' or 'frustrated phagocytosis'. The latter

description is perhaps more accurate since phagocytic behaviour is normal bar the obvious effect of presenting the cell with an opsonised area greater than that of the phagocyte itself.

Discharge of granule contents into the medium can also occur with phagocytosable particles, heat-aggregated IgG or soluble C5a if cytochalasin B is present (Zurier, Hoffstein, and Weissmann, 1973; Goldstein et al., 1975). Since cytochalasin B affects microfilaments and prevents ingestion the net effect is the same as the above (Zigmond and Hirsch, 1972).

Nevertheless, physiological release of granule contents to the cell exterior during phagocytosis would appear to be possible if the stimulus by phagocytosable particles is sufficiently large (Weissmann et al., 1971).

Both heat-aggregated IgG on millipore filters (Johnston and Lehmeyer, 1976; Johnston, Lehmeyer, and Guthrie, 1976) and monomeric IgG or IgA attached to polystyrene (Kiyotaki et al., 1978) can stimulate phagocyte oxidative metabolism. Free, soluble immunoglobulins or Fc and Fab fragments bound to plastic are unable to promote changes in phagocyte metabolism, which implies that conformational changes in the immunoglobulin molecule are the most likely cause of this activity.

Natural and synthetic chemotactic factors also stimulate O_2^- production, HMS, and glycolysis (Anderson, Glover, and Rabson, 1978; Simchowicz and Spilberg, 1979). Esterase activity and calcium ions are apparently required for optimal stimulation (Simchowicz and Spilberg, 1979; Simchowicz, Mehta, and Spilberg, 1979). Furthermore, the calcium ionophore A23187 can induce

superoxide generation in PMNs which, with the above findings, would suggest that changes of intracellular Ca^{2+} follow interaction of stimulants with the cell surface (Becker, Sigman, and Oliver, 1979). Putative Ca^{2+} fluxes may be responsible for the observed changes in membrane potential when granulocytes are treated with the same agents ie. C5a and synthetic chemotaxins (Seligmann et al., 1980; Whittin et al., 1980). However, potassium was the dominant ion in the latter studies.

It has been postulated that secretion of granule enzymes may play a role in substrate guided chemotaxis (Wilkinson and Bradley, 1981). The function of esterase activity in the generation of superoxide (Kitagawa, Takaku, and Sakamoto, 1980) and ingestion of antigen-antibody complexes (Nagai, Nakamura, and Koyama, 1978) on the other hand is unclear. It is conceivable that the requirement for this protease activity in the stimulation of oxidative metabolism could be extended to enzymes on microbial surfaces. In which case microbially associated complement could interact enzymatically with the phagocyte surface and not merely act in a ligand-receptor manner.

Goldstein and his associates have confirmed that aggregated IgG and C5a stimulate superoxide production in PMNs (Goldstein, et al., 1975). Serum treated zymosan, but not unsonosed zymosan, also stimulated O_2^- production (Goldstein et al., 1975). There appeared to be no correlation between stimulation of oxidative metabolism and release of granule enzymes. This was particularly true of C5a which suggested that the signals for production of these responses were different. Furthermore, the effects of C3 and immunoglobulin appeared to be independent

since oxidative metabolism could be stimulated by C3 coated senharose particles in the absence of immunoglobulin (Goldstein, et al.,1976). Lysosomal enzyme release did not occur under these conditions, but if IgG was also present then there was enhanced oxidative metabolism and release of granule enzymes. Metabolic stimulation could be blocked by $F(ab')_2$ anti-C3, whereas $F(ab')_2$ anti-IgG prevented enzyme release but did not completely abolish superoxide production in the presence of C3b.

The above findings suggest that stimulation of oxidative metabolism is preferentially mediated by complement components C5 and C3. This agrees with the observations of others using similar techniques (Tedesco et al.,1975). However, from these studies it is impossible to identify which of these two components has the greater activity.

The presence of large amounts of IgG appears to cause both superoxide and granule enzyme release, although it remains to be proven if immunoglobulin is merely increasing a metabolic response to C3b. This could be resolved by attaching IgG to non-phagocytosable particles in the absence of complement. Obviously, a particle which has no inherent ability to stimulate metabolism should be used.

Fusion of granules with the phagosome would appear to be a secondary event following stimulation of oxidative metabolism. However, the kinetics of granule fusion in the presence of particles coated only with C3b versus particles coated only with IgG are unknown.

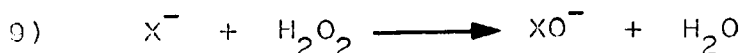
Oxygen-dependent microbial killing

1. The myeloperoxidase-halide-H₂O₂ system.

Peroxide formed in the respiratory burst interacts with myeloperoxidase (MPO) and halide to create a phagocytic killing mechanism which is the best described to date (Klebanoff, 1975; Klebanoff and Clark, 1977).

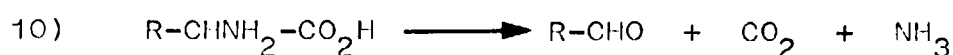
MPO is a haem protein of approximately 160,000 mw (Zgliczynski et al., 1968; Saunders et al., 1964) and is present in azurophilic granules of mammalian monocytes and neutrophils but not those of avians (Brune and Spitznagel, 1974). It has been reported that it undergoes conformational changes during phagocytosis (Wever et al., 1976) although this is unconfirmed (Blum et al., 1978). MPO is delivered to phagosomes where it catalyses the halogenation of microbes in an H₂O₂ dependent manner (Root and Stossel, 1974; Leffel and Spitznagel, 1974). The physiologically relevant halide in this reaction would appear to be chloride since it is present in sufficient quantity, whereas other halides are probably not.

MPO catalyses the oxidation of halide to hypohalite ions with H₂O₂:

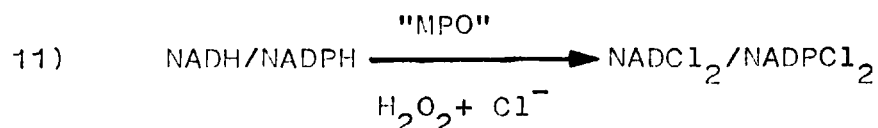


The exact mechanism whereby microorganisms are killed by this system is unresolved. Incorporation of halide into the microbial

cell wall resulting in damage and loss of membrane integrity has been put forward as one possibility (Klebanoff, 1967; Zgliczynski and Stelmazynska, 1975). However, halogenation per se as a microbicidal mechanism is difficult to prove when other potentially damaging reactions are taking place. These include decarboxylation of amino acids resulting in damage to macromolecules and secondary production of toxic aldehydes (Zgliczynski et al., 1968; Strauss, Paul, and Jacobs, 1970):



Even these possibilities are subject to criticism as it appears that efficient killing by the MPO-halide-H₂O₂ system can take place in the absence of decarboxylation (McCall et al., 1971). It is also believed that insufficient quantities of aldehyde are produced (Zgliczynski et al., 1968). Recently, a third mechanism has been proposed (Selvaraj et al., 1980). This involves the chlorination of bacterial NADH or NADPH by the MPO-halide-H₂O₂ system to catalytically inactive coenzymes:



This mechanism will be of little significance if MPO is shown to be incapable of penetrating microbes or cells. Nevertheless, this reaction could work physiologically by limiting indefinite continuation of the respiratory burst. Indeed, increased O₂ consumption and H₂O₂ formation in MPO deficient leucocytes

compared to normal cells does occur (Rosen and Klebanoff, 1976; Klebanoff and Pincus, 1971; Jandl et al., 1978).

2. Toxic oxidising species.

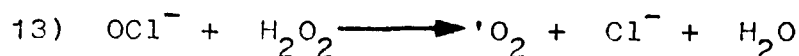
It has been shown that SOD or catalase can inhibit the bactericidal properties of the xanthine/xanthine oxidase system which produces both O_2^- and H_2O_2 (Babior, Curnutte, and Kipnes, 1975) and can also prevent phagocytic killing when attached to latex particles (Johnston et al., 1975). These observations indicate that a product of the reaction between O_2^- and H_2O_2 is microbicidal and present in leucocytes. Recent evidence suggests that the hydroxyl radical, formed by the Haber-Weiss reaction (Beauchamp and Fridovich, 1970), is the toxic product involved since it appears to be produced by both monocytes and neutrophils (Weiss, King, and LoBuglio, 1977; Tauber and Babior, 1977):



Unfortunately, ethylene, the end product of the assay system used to detect $OH^\bullet$ is produced by cells in an MPO-dependent manner, a finding which obviously complicates the above observations (Klebanoff and Rosen, 1978).

Singlet oxygen has the same molecular formula as atmospheric oxygen but possesses a distorted electron cloud which makes it highly reactive (Kearns, 1971). Singlet oxygen (1O_2) is thought to be formed both in the Haber-Weiss reaction and in the spontaneous dismutation of O_2^- to H_2O_2 (Kellog and Fridovich,

1975; Khan,1970). It is also formed as a product of the reaction between hypochlorite and H_2O_2 (Kasha and Khan,1970):



Thus ${}^1\text{O}_2$ is an indirect product of the MPO-halide- H_2O_2 system (Rosen and Klebanoff,1977).

3. Toxic properties of $\text{OH}^\bullet$ and ${}^1\text{O}_2$.

Hydroxyl radicals are considered one of the most reactive species known to organic chemistry (Halliwell,1978). For example, they hydroxylate DNA bases causing mutation (Myers, 1973). This may explain why the recA^+ gene product in E.coli K-12 has better survival value than catalase or SOD when these organisms are exposed to H_2O_2 (Carlsson and Carpenter,1980).

Hydroxyl radicals and singlet oxygen also cause lipid peroxidation (see Halliwell,1978; Badwey and Karnovsky,1980), a process which once initiated is autocatalytic and leads to widespread cellular damage. Lipid peroxidation could be linked indirectly to prostaglandin metabolism in activated phagocytes (Goldstein et al.,1978; Wentzell and Epand,1978; Hirata et al., 1979; Hsueh, Kuhn, and Needleman,1979; Morley et al.,1979).

Oxygen-independent killing by phagocytes

The purpose of this section is to list antimicrobial substances of phagocytes other than products of oxygen metabolism.

1. Organic acids and protons.

It is possible that lactic acid produced by increased glycolysis diffuses into microbial cells where it dissociates and reduces the pH to microbicidal levels. Organic acids are more effective as antimicrobial agents when the proton concentration outside the organism is high enough to suppress dissociation of the acid (Freese et al., 1973; Dubos, 1953). This is probably due to higher diffusion across the microbial membrane when the acid is undissociated.

2. Lactoferrin.

Lactoferrin is an iron chelating protein found in specific granules which can inhibit microbial growth. Its immediate importance in phagocytic killing is unclear.

3. Lysozyme.

Lysozyme is a potent antimicrobial agent against susceptible bacteria like Micrococcus lysodeikticus. Other bacteria, for example Gram negative organisms such as E.coli, are lysed by lysozyme only when the outer membrane of their cell wall is disrupted by the action of complement. It is likely that lysozyme inside phagocytes is more important to digestion

rather than killing.

4. Cationic proteins.

These proteins so named because of their electrophoretic mobilities have been found in the granules of PMNs from several species (see Avila, 1979).

Human cationic proteins are optimally toxic at high pH and are antimicrobial even after heating. Moreover, cationic proteins are highly specific in their antimicrobial properties ie. a given protein will attack a limited range of microbes (Zeya and Spitznagel, 1968).

MPO and oxygen independent antimicrobial activity has been described in PMNs and monocytes (Lehrer, 1972; Lehrer, 1975) and studies with Candida parapsilosis have attributed this to cationic proteins (Lehrer, Ladra, and Hake, 1975; Drazin and Lehrer, 1977).

Recently it has been found that there is an increase in pH following stimulation of normal but not CGD leucocytes (Segal et al., 1981). Segal and his associates argue that the sequential rise and fall in vacuolar pH favours killing by granule proteins (high pH) followed by digestion of organisms by hydrolytic enzymes (low pH). If this is confirmed then the relatively neglected antimicrobial properties of cationic proteins may assume a greater significance in microbial killing. In this regard the purpose of the respiratory burst

would be to raise vacuolar pH briefly by consuming protons in the reduction of oxygen.

Mononuclear phagocytes

Mononuclear phagocytes are the central cells of the immune system. Not only do they phagocytose and kill microorganisms but they also produce complement components, present antigen, secrete monokines, and destroy tumour cells.

The origin and kinetics of MNPs in man is essentially the same as the mouse: promonocytes in the bone marrow divide to form monocytes which enter the circulation and eventually become tissue fixed macrophages (van Furth, Raeburn, and van Zwet, 1979).

Monocytes, like PMNs, show an increase in oxygen metabolism during phagocytosis. However, oxygen uptake in monocytes is only partially channelled to oxygen dependent killing, the rest seemingly directed to providing energy for phagocytosis (Reiss and Roos, 1978; Sagone, King, and Metz, 1976). Human blood monocytes, unlike PMNs, can be cultured in vitro for several months. In this environment they undergo changes such that they resemble tissue macrophages and have increased powers of digestion (Ødegaard, Viken, and Lamvik, 1974; Zuckerman, Ackerman, and Douglas, 1979).

Macrophages also produce toxic oxygen products (Johnston et al., 1978) which can kill susceptible parasites (Murray et al., 1979) and tumour cells (Nathan, Silverstein, Bukner, and Cohn, 1979).

Macrophages have a wide spectrum of metabolic activity: MNPs from immunised animals releasing twenty-five times more H_2O_2 than MNPs from normal animals (Murray et al., 1979). Increased metabolism in cells from immune-boosted animals is most likely due to lymphokine activation (Nogueira and Cohn, 1978; Nathan, Nogueira, Juangbhanich, Ellis, and Cohn, 1979).

Stimulation of monocyte oxidative metabolism occurs when cells are in contact with IgG bound to substrate (Johnston, Lehmyer, and Guthrie, 1976). Moreover, lysis of IgG coated red cells by normal but not CGD monocytes is partially dependent on oxygen (Boregaard and Kragballe, 1980). It therefore appears that monocytes are in many respects similar to PMNs with regard to stimulation and execution of oxygen dependent killing. However, monocytes, when compared to macrophages, are functionally immature. It is therefore possible that monocytes are only cells in transit, fulfilling their dedicated role when they have fully developed into tissue fixed macrophages.

Phagocyte defects

Phagocyte killing defects will now be described. Other phagocyte related defects eg. chemotaxis will not be discussed.

Chronic granulomatous disease (CGD). This disorder is pre-eminent amongst other defects of phagocyte function. The disease manifests itself in early childhood with repeated pyogenic infections. The infections are slow to heal often progressing to granuloma formation (Good et al., 1968).

Virtually any organ is susceptible but common sites are the liver, lungs, lymph nodes, and skin. Patients previously undiagnosed often present with splenomegaly and scars which bear witness to surgical drainage of abscesses. CGD is an X-linked or autosomal recessive disorder (Windhorst et al., 1968; Azimi et al., 1968; De Chatelet et al., 1976). In the X-linked form female carriers can be detected (Mills, Rholl, and Quie, 1980).

CGD phagocytes do not reduce oxygen and therefore all signs of the respiratory burst are absent (Holmes, Page, and Good, 1967; Baehner and Nathan, 1967; Curnutte, Whitten, and Babior, 1974; Mandell and Hooke, 1969a; Nathan, Baehner, and Weaver, 1969; Stjernholm et al., 1973). Myeloperoxidase-mediated iodination is also defective because H_2O_2 is not produced (Klebanoff and White, 1969). Other aspects of phagocytic function including ingestion (Quie et al., 1967; Stossel, Root, and Vaughn, 1972) and degranulation (Baehner, Karnovsky, and Karnovsky, 1969; Gold et al., 1974) are usually normal. Disorders of chemotaxis however have been noted in some patients (Clark and Klebanoff, 1978). Unusually small, tight phagosomes have also been noted (Segal et al., 1981).

CGD phagocytes fail to kill catalase positive microbes, eg. Staph. aureus and members of the enterobacteriaceae, but can destroy organisms like Strep. pneumoniae which excrete H_2O_2 . Catalase negative organisms therefore commit suicide by providing the phagocyte with H_2O_2 which can partake in MPO-mediated killing (Mandell and Hooke, 1969b).

Since CGD phagocytes do not form O_2^- upon stimulation it is thought that the biochemical lesion lies in the oxidase responsible for the reduction of oxygen— this has already been examined in previous sections. However, it is worth reiterating here that the 'oxidase' is probably a system made up of several components any one of which is potentially abnormal or absent in CGD.

Glucose-6-phosphate dehydrogenase deficiency (G-6-PD Def.).

Phagocytes from patients with severe G-6-PD deficiency fail to mount a respiratory burst and appear unable to kill catalase positive microorganisms (Cooper, De Chatelet, McCall et al., 1972; Baehner, Johnston, and Nathan, 1972; Gray et al., 1973). Glucose-6-phosphate dehydrogenase is the enzyme responsible for the second step in the HMS, the conversion of glucose-6-P to 6-phosphogluconolactone. Consequently this pathway is inoperative in total G-6-PD deficiency and fails to provide NADPH. Therefore the simple answer to the cause of the microbicidal defect in these patients' cells is the failure to give electron donors for oxygen reduction (see Babior, 1978). However, although clinically similar to CGD, G-6-PD deficiency does not fully reflect the severity of CGD as expected (see Segal, 1979).

Myeloperoxidase deficiency. MPO is a relatively common disorder which is usually undetected. The incidence has been estimated at 1/2000 (Kitahara et al., 1981). In vitro phagocytes from MPO deficient individuals display delayed but otherwise normal killing of bacteria (Kitahara et al., 1981; Cech et al.,

1979a). In contrast MPO appears to be an absolute requirement for phagocytic killing of C.albicans (Cech et al.,1979a; Lehrer and Cline,1969a). Patients with MPO deficiency and diabetes mellitus are susceptible to fungal infections (Lehrer and Cline,1969a; Cech et al.,1979 a and b). On the other hand completely MPO deficient subjects without diabetes do not have fungal infections (Kitahara et al.,1981; Lehrer and Cline,1969a). This suggests that diabetes makes an important contribution to defective phagocytic function in MPO deficiency. The majority of individuals with MPO deficiency do not suffer from undue susceptibility to infection. It must therefore be inferred that oxygen-dependent (without MPO), or oxygen-independent (Mandell,1974) killing provides the host with sufficient protection.

Disorders of the glutathione reductase-glutathione peroxidase system. Glutathione reductase deficiency has been reported (Loos et al.,1976; Roos, Weening, and Loos,1979). Patients with this defect do not suffer from infections, and, although their phagocytes kill normally in vitro they cannot sustain a prolonged respiratory burst. It is therefore concluded that H_2O_2 produced during the respiratory burst eventually causes damage to the phagocyte. However before this happens, during the short period when the respiratory burst is functioning (presumably due to intracellular pools of GSH) the intracellular organisms are killed.

Glutathione peroxidase deficiency has been noted in some patients with CGD (Quie et al.,1968; Holmes et al.,1970; Matsuda et al.,1976). It must be established if glutathione peroxidase deficiency in these patients is the primary cause of their disease or whether defective oxidase activity is concurrent with this condition. If glutathione peroxidase deficiency is the sole defect which gives rise to a disease which phenotypically resembles CGD then adequate reasons must be forthcoming (see Roos et al.,1979).

Chediak-Higashi syndrome (CHS). Phagocytes of patients with CHS have abnormally large granules which fail to fuse with phagosomes (reviewed by Mills and Quie,1980). The respiratory burst is normal but resting and active HMS as well as MPO-mediated iodination are both raised. Patients suffer from severe infections and malignancy, often not surviving the first decade of life.

CHAPTER 2

THE DEVELOPMENT OF A RADIOMETRIC PHAGOCYTOSIS AND KILLING ASSAY

2-1. Introduction

In 1903 Wright and Douglas published a paper which attempted to resolve the heated debate between proponents of cellular and humoral theories of immunity (Wright and Douglas, 1903). With hindsight it can be said that this paper assisted the marriage of both theories and helped to establish the integrated two-armed theory of immunity which is recognised today. Wright and Douglas's work is also significant in that it laid the foundations of modern phagocytic activity measurement, namely to distinguish the relative contributions made by phagocytes and serum in the recognition, attachment, engulfment, intracellular killing and subsequent digestion of microorganisms by phagocytic cells.

More recent methods used to determine phagocytosis and killing are dictated by the type of organisms used as prey, eg. bacteria, fungal blastospores, or parasites. Further distinction in methodology can be made on the basis of culture conditions, that is to say suspension assays in which phagocytes and microorganisms are actively mixed, or monolayer assays in which the phagocytes are attached to substrate. In the following review I have chosen the nature of the target microbe as the primary criterion in classifying the principal methods available for measurement of phagocytic activity. Assays which have used unphysiological/synthetic target particles but, despite that, have contributed to a better understanding of phagocytic function will also be mentioned.

Assays using bacteria as the target particles.

Measuring phagocytosis by monolayer assays involves exposing glass or plastic adherent cells to phagocytosable particles, removing the free particles after a prerequisite time by washing, and finally preparing the culture for light microscopy. Phagocytic assays which use monolayer culture conditions readily lend themselves to examination by light microscopy and therefore the majority of such techniques rely on this instrument (Axline and Reaven, 1974; Griffin, Bianco, and Silverstein, 1975), although radiometric measurement is possible (Mantovani, Rabinovitch, and Nussenzweig, 1972). However, a serious drawback to microscopical assessment of phagocytosis is the inability of the observer to distinguish intracellular particles from those attached extracellularly to the phagocyte membrane. Dilworth et al. (1975) were able to overcome this by using a fluorescein conjugated antiserum to N.gonorrhoeae which reacted with extracellular organisms but not ingested gonococci. Measurement of bacterial killing in monolayer culture systems is achieved by disrupting phagocytes and counting the number of viable organisms by their ability to form colonies on solid media (Baughn and Bonventre, 1975).

More sophisticated techniques have replaced microscopical evaluation of phagocytosis the most popular of which are based on the suspension culture assay introduced by Maaløe (1946). This method employs differential centrifugation to separate phagocytosed bacteria from those remaining outside the phagocyte. The outline of the technique is as follows: mixtures of

phagocytes and opsonised bacteria are incubated in tubes at 37°C with constant agitation. At intervals the mixtures are diluted and centrifuged at approximately 150g to pellet phagocytes and phagocyte associated bacteria leaving the remaining bacteria in the supernatant. Aliquots of supernatant are then plated on solid media and each colony so formed has, in theory, arisen from a single bacterium or colony-forming unit (CFU). The number of CFU remaining in the supernatant is inversely correlated to ingestion. This basic technique has been modified to make an estimation of killing as well as phagocytosis (Cohn and Morse, 1959; Quie, White, Holmes, and Good, 1967).

Verhoef and his co-workers have developed a radiometric method for measuring phagocytosis using a protocol based upon Maaløe's technique (Verhoef, Peterson, and Quie, 1977). In this modification Staphylococcus aureus was prelabelled with ³H-thymidine and then placed in mixtures containing phagocytes and serum. Aliquots were removed at intervals and centrifuged. The resulting pellet was washed several times before solubilisation in scintillation fluid and counted in a spectrometer. The percentage of radioactivity associated with phagocytes was proportional to ingestion. Verhoef et al. assessed killing by lysing phagocytes from a duplicate tube and plating the released organisms for CFU. A false measurement of ingestion due to phagocyte-mediated release of label is a possible disadvantage to this technique.

Antibiotics have been used instead of differential centrifugation to separate phagocytosed bacteria from those

remaining outside (Holmes, Quie, Windhorst, and Good, 1966; Alexander and Good, 1968). However their use is controversial as it has been reported that some antibiotics can penetrate phagocytes (Easmon, 1979). Lysostaphin, a muralytic enzyme, may be used in the same way as antibiotics to remove extracellular Staph. aureus and appears not to penetrate phagocytes (Tan et al., 1971; Verhoef et al., 1977). Both the use of antibiotics and lysostaphin have the additional advantage of giving a true value for phagocytosis because they remove microorganisms attached to the outside of phagocytes.

Vray and his associates used the muralytic enzyme lysozyme to remove extracellular fluorescently labelled Micrococcus lyso-deikticus in a novel ingestion assay (Vray et al., 1980). In this technique fluorescence associated with lysozyme treated and washed phagocytes was directly proportional to quantity of phagocytosed bacteria. Unfortunately, this method required the use of dead organisms and consequently no simultaneous measurement of bacterial killing was possible. On the other hand, a microassay has been developed for measuring phagocyte bactericidal activity (Phillips, Shelton, and Hosking, 1979). The number of organisms killed is evaluated by statistical methods and, although the number of bacteria in the phagocytes available for killing is determined, no measurement of ingestion is made in terms of the original number of organisms presented to the phagocyte.

Assays using fungal blastospores as target particles.

The Lehrer and Cline assay for measuring phagocytosis and killing of Candida albicans, like the bacterial assay of Maaløe, has become a classical technique upon which many newer ones have been founded (Lehrer and Cline, 1969b). The original method of Lehrer and Cline was as follows: phagocytes and C.albicans were mixed in equal numbers at 37°C for one hour. The volume of the reaction mixture was 1ml and contained 25% serum. After incubation the phagocytes were lysed with sodium deoxycholate and the released blastospores exposed to methylene blue. The concentration of methylene blue used stains only non-viable organisms and killing can therefore be determined by microscopical examination of phagocyte lysates.

In a modification of the original Lehrer and Cline assay phagocytes and organisms were allowed to interact as before but methanol fixed cytocentrifuged preparations were stained with Giemsa (Lehrer, 1970). Organisms stained deep blue were considered viable whilst unstained or pale pink organisms were regarded as killed. The advantage of this assay over the methylene blue dye exclusion technique is that a simultaneous measurement of phagocytosis and killing by different types of phagocyte is possible. A serious disadvantage of this assay however is that organisms, although killed, may not be scored as dead because the pale pink staining is indicative of digestion rather than killing.

Brune et al. (1973) using phagocytes attached to glass were able to use the original method of Lehrer and Cline with intact

phagocytes. Thus killed and live organisms could be distinguished with methylene blue when inside phagocytes. However, as pointed out earlier, it is notoriously difficult to distinguish between attached and ingested organisms with the light microscope. Therefore, unstained organisms could be extracellular, and although attached, might not be killed.

Another assay using microscopical examination has been designed which distinguishes attachment and ingestion (Hed,1979). This uses the phenomenon of crystal violet quenching fluorescein-isothiocyanate (FITC). FITC conjugated yeast are allowed to interact with phagocytes for 15 mins at 37°C whereupon crystal violet is added; crystal violet quenches the fluorescence of attached uningested organisms leaving the fluorescence of intracellular organisms intact.

Fluorescence was also used by Schuit in a phagocytosis and killing assay which used Saccharomyces cerevisiae, C.albicans, and C.tropicalis (Schuit,1979). Whilst the assay of Hed has the advantage of distinguishing attachment and ingestion it was not designed to measure killing. Conversely, the assay of Schuit is not capable of differentiating between attachment and ingestion but can make a measurement of killing by taking advantage of the different fluorescing colours of live and dead organisms.

Micro-methods for measuring phagocytosis and killing of fungal blastospores have been published (Oseas and Lehrer,1973; Wood and White,1978). These techniques require fewer phagocytes than other assays and are therefore useful in clinical settings

where obtaining large volumes of blood is often impossible. These assays are based upon the the original methods of Lehrer and Cline (1969b) and Lehrer (1970). A significant advance was made by Yamamura and his co-workers who developed a radiometric micromethod for measuring phagocytosis which was able to distinguish surface attachment and ingestion (Yamamura, Boler, and Valdimarsson, 1977). The further development of this assay enabling measurement of intracellular killing in addition to phagocytosis will be described in this thesis.

Assays for phagocytosis using synthetic particles as targets.

Stossel introduced a method for the phagocytosis of albumin coated oil droplets stained with a red dye (Stossel, 1973). The advantages of this system are 1) the particle is well defined, and 2) phagocytosis is assessed quantitatively with a spectrometer using the absorbance of oil red O after extraction. Stossel, using this assay, was able to investigate the kinetics of phagocytosis (Stossel, 1973; Stossel, Mason, and Hartwig et al., 1972) as well as granule fusion (Stossel, Mason, and Pollard et al., 1972)

Other techniques have used polystyrene or polyvinyl latex particles in a similar manner since they can be extracted from cells in a soluble form and quantitated spectrophotometrically (Roberts and Quastel, 1963; Weisman and Korn, 1967). Obviously synthetic particles cannot be used to give values for phagocytic killing although they may be used to analyse metabolic activity associated with killing (Baehner and Nathan, 1968).

2-2. The design of phagocytosis and killing assays: some remarks.

Definitions

- 1) 'Phagocytic activity' is defined as all events in the interaction of phagocytes, serum and microbes. The different, temporally related aspects of phagocytic activity (PA) are, in sequence, a. opsonisation, b. generation of chemoattractants, c. chemotaxis of phagocytes, d. attachment and ingestion of microbes, e. intracellular killing, and f. digestion.
- 2) Intrinsic PA is defined as the cellular component of phagocytic function. Example: the ingestion of optimally opsonised microorganisms.
- 3) Extrinsic PA is defined as the humoral support of phagocytic function. Example: the production of opsonins by activation of the complement system.

Measurement of intrinsic PA demands the use of non-limiting conditions in terms of serum concentration. It is also necessary to provide a quantity of microbes known to saturate the engulfment and killing capacity of healthy control cells. Moreover, when studying the enhancement of intrinsic PA, eg. in the presence of drugs, high organism/phagocyte ratios should be employed. The importance of this premise is emphasised by the work of Palmblad and Engstedt (1979) who reported enhanced killing by phagocytes in the presence of levamisole.

Carefully chosen high organism/phagocyte ratios were used in their experiments as lower organism/phagocyte ratios in this assay did not readily disclose levamisole induced stimulation of PA.

In practice maintaining precise organism/phagocyte ratios during the course of an assay is difficult, especially when bacteria are used as the prey. Hooke and associates (1978) overcame this problem by selecting for temperature sensitive mutants of E.coli which were unable to divide at the assay temperature of 37°C but grew into colonies on solid media when plated out at a lower temperature. This technique should be extremely useful for methods employing other species of bacteria. However, it is debatable whether organisms unable to elaborate virulence properties at 37°C fully reflect the dynamic interaction between phagocytes and micro-organisms.

Assessment of extrinsic PA requires the use of suboptimal serum concentrations.

Titration of serum in relation to phagocytic activity produces a peak or plateau of PA at a given serum concentration which is defined as optimal. Limiting serum dilutions below the peak or plateau are defined as suboptimal, whereas serum concentrations beyond optimum may be inhibitory (see Figure 2.1).

Ideally, extrinsic phagocytic activity should be measured using suboptimal serum concentrations because at limiting dilutions phagocytic activity is linearly related to serum concentration.

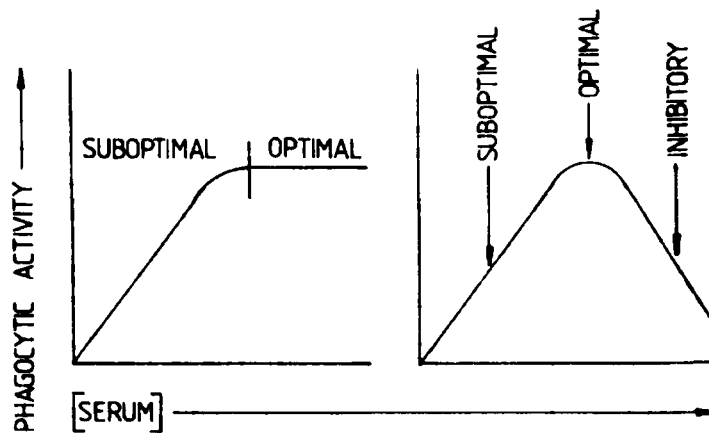


FIGURE 2.1. Properties of extrinsic PA.

Several suboptimal serum concentrations should be used to construct a graph the gradient of which can be measured and expressed as units of PA per unit of serum concentration (Figure 2.2a). Titration of several normal sera in this manner can be used to build up a normal range for the slope. The steeper the slope the more efficient the serum in supporting phagocytic activity over the concentration range chosen.

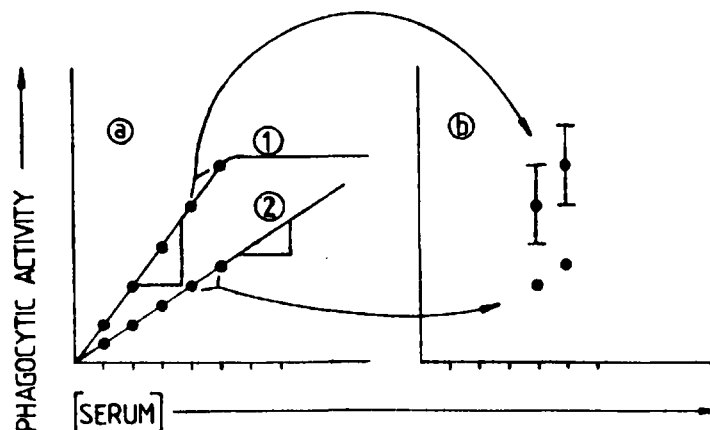


FIGURE 2.2. Measurement of extrinsic PA I.

Alternatively, the ability of serum to support phagocytic activity can be expressed as that serum concentration which produces 50% of the total PA (PA_{50}) (Figure 2.3.). This method is useful because it takes into account sera which behave like serum B in Figure 2.3.

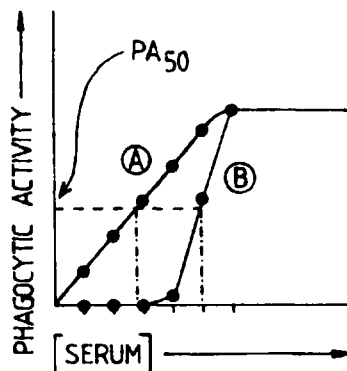


FIGURE 2.3. Measurement of extrinsic PA_{50} .

However, for clinical use it is more convenient to use one or two serum concentrations per test as opposed to several. In this case a gradient or PA_{50} cannot be calculated but values of phagocytic activity at a given serum concentration may be compared to a normal range (Figure 2.2b.).

Additional considerations of existing assays

Many published phagocytosis and killing assays are unsatisfactory. Monolayer culture conditions are unacceptable because consistent organism/phagocyte ratios cannot be maintained from experiment to experiment, or within an experiment if cells are detaching from the substrate. Monolayer cultures also measure the additional variables of chemotaxis. Assays which use the

active mixing of phagocytes and microbes are therefore better in this respect because particle contact is related to the probability of collision (Fenn,1922) and unrelated to chemotaxis. However, it should be remembered that phagocytes in vivo probably trap organisms against surfaces in confined intercellular spaces.

Using metabolic changes as indications of phagocytosis or killing is highly questionable since this approach assumes the one is always a direct reflection of the other. For example, nitroblue tetrazolium should not be used as an indicator of phagocytosis because in CGD ingestion can take place in the absence of NBT reduction (Good et al.,1968). It has been reported that closely related organisms differ in their susceptibility to microbicidal mechanisms of phagocytes (Lehrer,1972). Measuring the biochemical changes associated with killing without recourse to simultaneous assessment of microbe death is therefore unsatisfactory. Furthermore, quantitative measurements of microbial killing should be accompanied by a simultaneous assessment of phagocytosis. This is because of the temporal relationship between intracellular killing and ingestion which would make evaluation of killing in isolation invalid.

The use of CFU as an endpoint for assays of phagocytic activity is unsuitable because bacteria and C.albicans tend to clump in high serum concentrations (Koch,1974; Chilgren, Hong, and Quie, 1968). Microscopical analysis is also unsatisfactory because it is subjective, time-consuming, and laborious.

The assay described in this thesis measures phagocytosis and killing simultaneously using a radiometric suspension culture method which is standardised for extrinsic and intrinsic PA.

2-3. Introduction to method

It has previously been demonstrated that incorporation of ³H-uridine into C.albicans is a sensitive indicator of phagocytic function because 1) there is a linear correlation between uridine incorporation and yeast number, 2) Candida organisms surviving within phagocytic cells do not take up uridine from the culture medium, and 3) phagocytic cells do not incorporate significant amounts of uridine in short term cultures (Yamamura, Boler, and Valdimarsson, 1977). Phagocytosis can therefore be determined by comparing the uridine incorporation into a fixed number of C.albicans in the presence and absence of phagocytes.

It was reasoned that it should be possible to determine intracellular killing in a similar manner by liberating ingested surviving organisms with sodium deoxycholate. This substance disrupts the phagocyte cell membrane without affecting the Candida. Ingested, killed microorganisms, although released, would be unable to incorporate label. Therefore, the reduction in uridine uptake in mixtures with phagocytes would give a direct measure of phagocytic killing (Figure 2.4.).

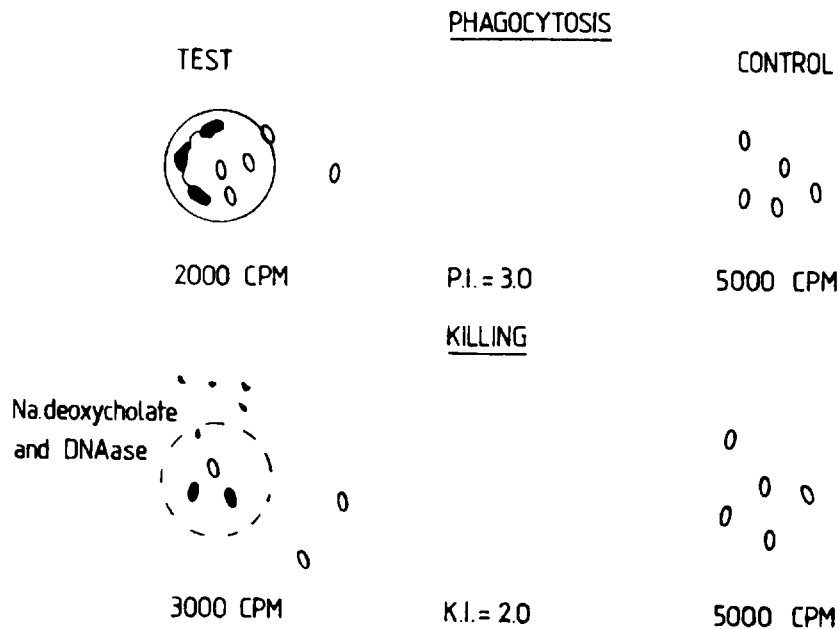


FIGURE 2.4. Principle of radiometric phagocytosis and killing assay. Coloured organisms are those which have taken up radiolabel. Black organisms are killed. Clear organisms have not had the opportunity to incorporate radiolabel. See text for details.

2-4. MATERIALS and METHOD

RPMI 1640 tissue culture medium buffered with 20mM Hepes was used throughout this study and is referred to as 'medium' in the text.

Preparation of phagocytes:

Polymorphonuclear leucocytes (PMNs) were prepared from heparinised (20 units/ml) human venous blood by sedimenting the erythrocytes with dextran. Two volumes of blood were mixed with one volume of dextran 110 (dextran 6% w/v in

0.9% NaCl) and allowed to stand for 30 mins at 37°C, whereupon the leucocyte-rich supernatant was removed, centrifuged at 200g for 5 mins, and the cells washed four times in medium before final resuspension to the appropriate working concentrations. On occasion further purification was achieved by differential centrifugation on a Ficoll-Hypaque gradient (density=1.077g/ml). Two volumes of leucocyte-rich supernatant, obtained from dextran sedimentation, were layered on one volume of gradient and the resulting preparation subjected to centrifugation for 40 mins at 20°C with a force of 400g at the plasma/gradient interface (Böyum, 1968). The PMN enriched pellet was then removed and the cells washed four times in medium before final resuspension to the appropriate working concentrations. For PMN oxygen consumption studies red cells were removed by hypotonic lysis using ice-cold distilled water. After a short period of agitation the tonicity was returned to normal with an equal volume of 1.8% saline.

The cell suspension obtained from dextran sedimentation contained 60-80% PMNs, the remainder comprising lymphocytes. Monocytes were removed during the washing procedure. The suspension of PMNs obtained with Ficoll-Hypaque centrifugation were not less than 95% pure and had a viability greater than 95% as evidenced by trypan blue dye-exclusion and acridine orange-ethidium bromide fluorescence (Parks et al., 1979).

Mononuclear phagocytes (MNPs) were obtained from either cell-separator residues (anticoagulated with CPD), or heparinised venous blood. Both sources of MNPs were sedimented with dextran as described above and the resulting supernatant if obtained from cell-separator residues diluted 1+3 in medium, or if obtained from peripheral venous blood diluted 1+1 in medium. Two volumes of the diluted supernatant were layered on one volume of Ficoll-Hypaque gradient and centrifuged as described above. After centrifugation MNPs were taken from the plasma/gradient interface, washed three times in medium using siliconised glassware, and finally resuspended to the appropriate working concentrations.

The MNP suspension thus procured had 75-90% lymphocyte contamination and less than 1.5% contamination with PMNs. Viability was greater than 95%.

Preparation of microorganisms

Candida albicans (strain UC 820) was maintained on 2% agar slopes containing 1% peptone and 2% glucose. Subcultures were made in 1% peptone-2% glucose broth and incubated at 28°C for 2-3 days.

Candida parapsilosis (strain 46392) and Candida guilliermondii were both provided by the Mycology Dept., St.Mary's Hospital Medical School. They were maintained on agar slopes and subcultured as described above.

Saccharomyces cerevisiae (strain NCYC 373) was maintained on

agar slopes as described above. Subcultures were made in RPMI 1640 and incubated at 28°C for 2-3 days.

All of the above organisms from subcultures had a viability greater than 98% and a homogeneous yeast form. They may be kept for 1-2 weeks at 4°C without loss in number or viability. In the experiments presented here the subcultured organisms were not more than 1 week old at the time of assay.

Pseudohyphal formation was negligible in suspensions of C.albicans incubated in serum (5%) for up to two hours. The other organisms do not form germ tubes.

Sera

Human sera genetically deficient in C3 and C2 were provided by Professor P.J.Lachmann, Cambridge, UK. Additional C3 deficient serum was provided by Dr.A.Rabson, Capetown, SA. C5 deficient serum was obtained from B10 D2 old strain mice. These sera together with normal sera and standard AB serum were kept in 0.5 and 0.1ml aliquots in liquid nitrogen prior to use. Serum from freshly clotted blood was also used.

⁵¹Chromium ($\text{Na}_2^{51}\text{Cr}_2\text{O}_7$)(specific activity 250-500 $\mu\text{Ci}/\mu\text{gCr}$) was used to label C.albicans in the ⁵¹Cr release killing test of Yamamura et al.(1976). This was done by centrifuging a suspension of Candida, removing the supernatant and gently adding 0.2ml of ⁵¹Cr solution taking care not to disperse the cell pellet over the walls of the tube when mixing. The Candida were then incubated for two hours at

28°C before washing four times in phosphate buffered saline.

^{125}I iodine (as sodium iodide, specific activity $\sim 15\text{mCi } ^{125}\text{I}/\mu\text{g}$ iodine) was used for MPO-mediated microradioiodination of C.albicans by PMNs. ^{125}I was diluted in medium to appropriate working concentrations.

^3H -uridine (5,6, ^3H -uridine, specific activity 45Ci/mMol uridine) was diluted in phosphate buffered saline to 100 $\mu\text{Ci}/\text{ml}$. Working concentrations of ^3H -uridine were made by further dilution in medium.

Sodium deoxycholate was used as a 1.25% solution in distilled water.

Deoxyribonuclease (DNAase) was dissolved in phosphate buffered saline to a concentration of 1mg/ml and stored at -20°C in 0.2 and 0.5ml aliquots. Further dilution in medium to working concentration was carried out shortly before use.

a. Standard assay procedure.

Unless otherwise stated the assay procedure was as follows: microorganisms and phagocytes were mixed in triplicate in 2ml flat-bottomed plastic tubes, each containing 1×10^6 blastospores and 2×10^5 phagocytes suspended in a total of 0.5ml medium with 5% serum. Control tubes without phagocytes containing 1×10^6 blastospores and 5% serum were also set up in triplicate. The tubes were incubated at 37°C with constant end-over-end rotation (30 rpm) for 40 minutes.

Phagocytosis was measured by transferring a single 0.1 ml aliquot from each tube into a corresponding U-bottom microtitre well containing 0.2 μCi ^3H -uridine in 110 μl medium.

Intracellular killing was measured by transferring a single 0.1 ml aliquot from each tube into a corresponding U-bottom microtitre well containing 0.2 μCi ^3H -uridine in 10 μl medium, 625 μg sodium deoxycholate in 50 μl distilled water, and 5 μg DNAase in 50 μl medium.

Phagocytosis and killing was measured in the same microtitre plate (Sterilin M24AR or Linbro Titertek) which was incubated for 60 mins at 37°C to allow microorganisms to incorporate radiolabelled uridine. Cell associated radioactivity was then collected onto glass fibre discs with a Skatron harvester. Finally, the discs were dried, placed in toluene-PPO (4 g/litre) scintillation fluid, and counted in a Packard β -counter.

Thus both phagocytosis and killing were assessed under identical conditions regarding time, organism/phagocyte ratio, serum and ^3H -uridine concentration. Moreover, aliquots removed for measuring phagocytosis and killing contained the same number of organisms (2×10^5 / microtitre well).

A schematic outline of the assay procedure is given in Figure 2.5.

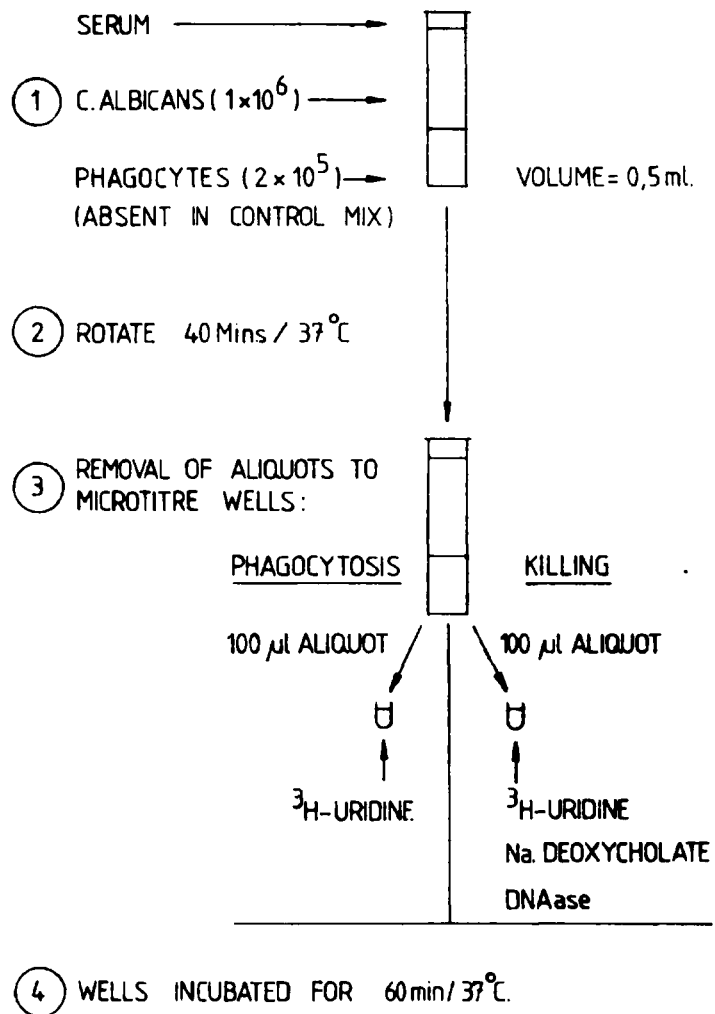


FIGURE 2.5. Schematic outline of assay procedure

b. Presentation of data

The results for phagocytosis and killing were calculated with the same basic formula—the difference being that killing was calculated with radioactive counts obtained from wells containing disrupted phagocytes whereas phagocytosis was evaluated with counts obtained from wells containing intact phagocytes. Results are expressed as an index; the phagocytic index (PI) is the average number of organisms ingested per phagocyte and the killing index (KI) is the average number of organisms killed per phagocyte.* The indices were calculated with the following formula:

$$PI \text{ or } KI = \left[1 - \frac{\text{cpm } \underline{\text{Candida}} + \text{phagocytes}}{\text{cpm } \underline{\text{Candida}} \text{ alone}} \right] \times R$$

where cpm = counts per minute, R = organism/phagocyte ratio.

The killing efficiency is determined by the following formula:

$$(KE)(\%) = \left(\frac{KI}{PI} \right) \times 100 = \text{Killing efficiency}$$

The overall killing efficiency (OKE) should be used when values of PI fall below the normal range. The OKE is derived thus:

$$OKE (\%) = \left(\frac{KI}{R} \right) \times 100$$

Values of OKE are not presented in this thesis.

* See Figure 2.4.

c. Is there a correlation between Candida viability and the uptake of uridine from the culture medium?

Before standardising the assay it was essential to know whether the number of Candida killed could be equated with a KI derived from uridine incorporation. To investigate this live and heat killed organisms were mixed in suitable proportions and 0.1 ml aliquots, each containing 2×10^5 organisms and 5% serum, placed in microtitre wells containing 0.2 μ Ci of ^3H -uridine. The microtitre plates were then incubated for 60-90 mins at 37°C whereupon cell associated radioactivity was collected and measured as described previously. Thus this procedure faithfully followed conditions used in the assay.

Figures 2.6 and 2.7 show the results for C.albicans and C.parapsilosis respectively. The KI is calculated from the radioactive counts and plotted against the percentage of dead organisms in the original mixtures. It can be seen that there is an excellent correlation between the two for both organisms. This correlation could be demonstrated for organisms in all serum concentrations referred to in the results. It was also proved that the sodium deoxycholate and DNAase at concentrations used in the assay did not affect the uridine incorporation by Candida (Table 2.1.).

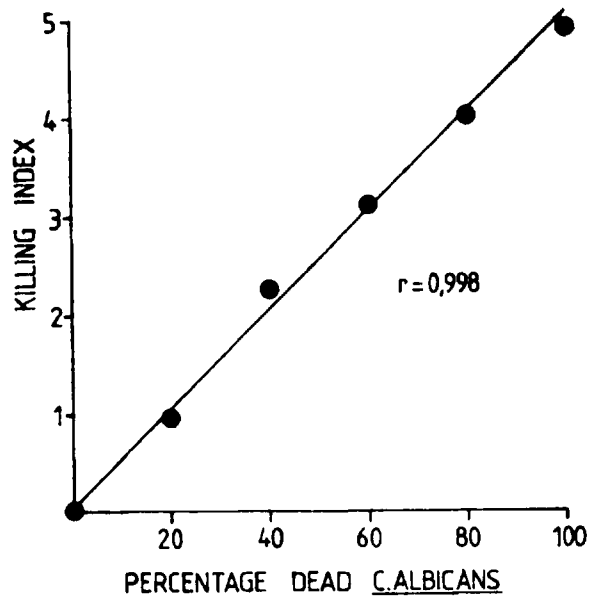


FIGURE 2.6. Relationship between *C. albicans* viability and ^3H -uridine uptake.

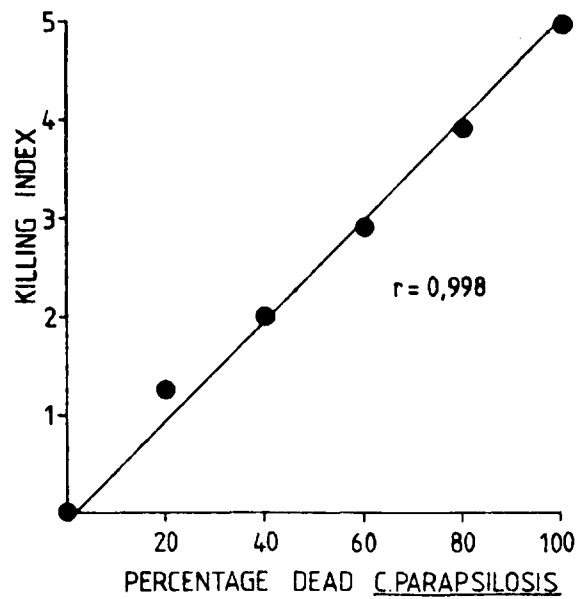


FIGURE 2.7. Relationship between *C. parapsilosis* viability and ^3H -uridine uptake.

TABLE 2.1. Effect of DNAase and sodium deoxycholate on
³H-uridine incorporation by C.albicans.

Agents present*	Correlation coefficient, r.**	slope
Na deoxycholate	0.997	0.864
DNAase	0.999	1.0
Both of above	0.998	0.967

* Agents present in concentrations corresponding to assay conditions, ** r is derived from regression calculations. Protocol is the same as for results presented in FIG. 2.6. and 2.7. See text for details.

RESULTS

2-5. Standardisation of the assay to measure phagocytosis and killing of C.albicans by polymorphonuclear leucocytes.

Time course. It was established that the ingestion of C.albicans was complete after approximately 15 minutes. Figure 2.3. demonstrates time course of Candida killing. Each point represents the mean of not less than 7 and not greater than 14 experiments conducted with PMNs from healthy volunteers in the presence of 5% normal AB serum. Killing increased in a linear fashion up to 40 mins when a plateau was reached. A standard incubation time of 40 mins was therefore used for the combined phagocytosis and killing assay.

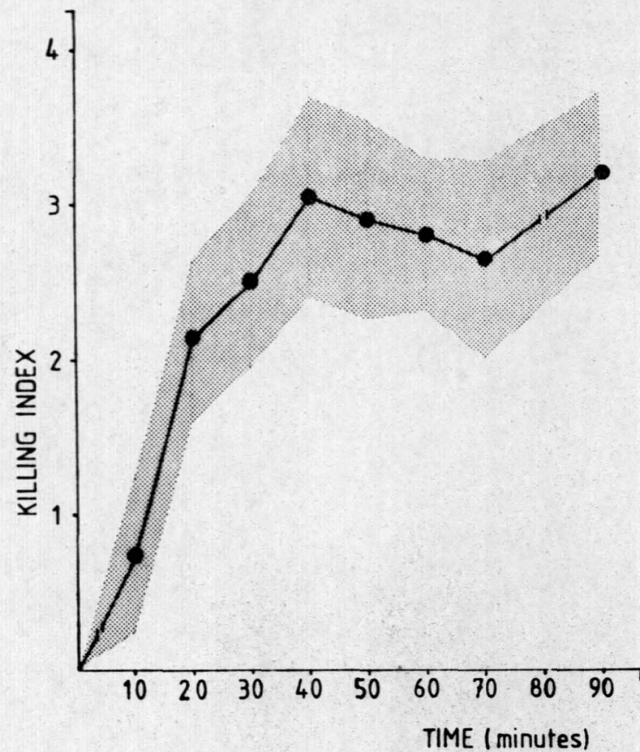


FIGURE 2.8. Killing index versus incubation time for PMNs and C.albicans. Standard organism/PMN ratio and serum concn. Shading represents one standard deviation either side of mean.

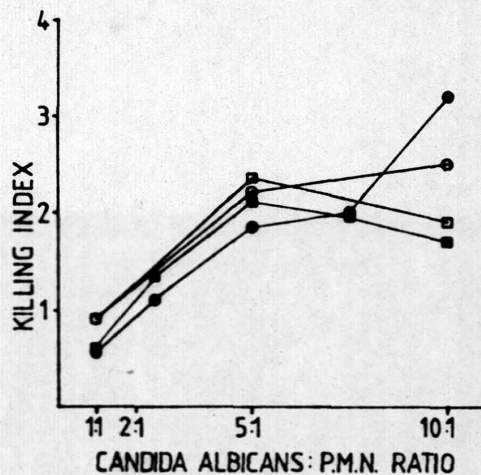


FIGURE 2.9. Effect of varying the C.albicans/PMN ratio on the killing index. Standard serum concn and incubation time. PMNs from four healthy donors.

Effect of varying the C.albicans/PMN ratio. The importance of determining the phagocytes' intrinsic killing capacity was discussed in the introduction (page 77). Figure 2.9. shows four experiments in which the organism/phagocyte ratio was altered. The ratio was altered by varying the number of PMNs whilst keeping the Candida numbers constant so that the humoral factor-Candida relationship remained the same. This ensures that the phagocytes' intrinsic ability to kill at different organism/phagocyte ratios is not due to availability of serum factors.

It was evident that up to a ratio of 5:1 there was a linear relationship between the number of Candida presented to the PMNs and the number killed by each cell. At ratios greater than 5:1 PMNs from different sources showed increased variability in killing despite consistently ingesting greater numbers of organisms at these ratios. Phagocytes from some individuals are able to kill in proportion to ingestion at high ratios. Figure 2.10. shows phagocytosis and killing at different ratios by PMNs from two different donors; in both experiments killing increased linearly with phagocytosis and at no ratio did the value of KI exceed that of PI. It was also noticed that the killing efficiency at low ratios was relatively low when compared to higher ratios.

It was concluded that the capacity of human PMNs to kill C.albicans may occasionally be overstretched at a ratio of 10:1 whereas low ratios do not induce maximal phagocytic killing. A ratio of 5:1 was chosen for the standard assay

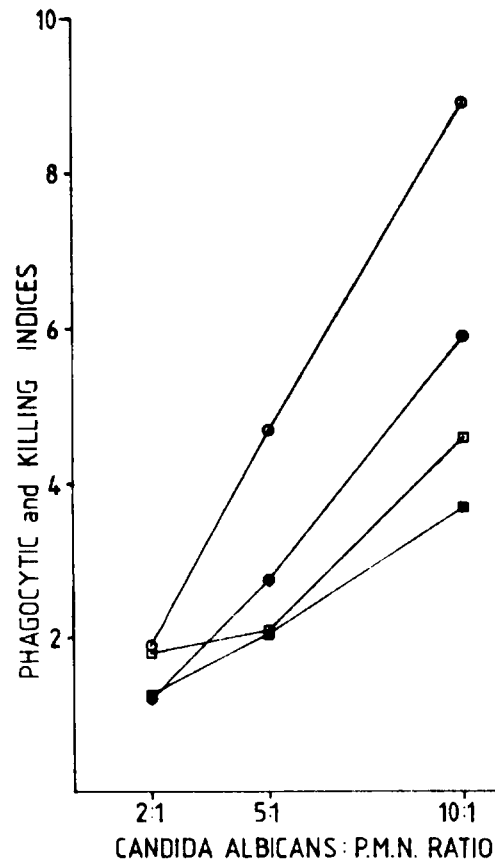


FIGURE 2.10. Phagocytosis and killing of C.albicans by PMNs at differing organism/ phagocyte ratios. Phagocytes from two healthy donors. Standard serum concn. and incubation time. OPEN symbols represent phagocytosis, CLOSED symbols represent killing.

since this appeared to provide a satisfactory challenge and a reasonably narrow normal range for PMNs from healthy donors.

Serum requirements. The intrinsic ability of phagocytes to ingest and kill must be measured under conditions where serum neither limits nor inhibits these functions. Figure 2.11. shows the results of a typical experiment in which phagocytosis and killing of C.albicans by normal PMNs was tested in normal AB serum, concentrations ranging from 0.31 to 20.0 per cent.

Phagocytosis always approached a maximum in 0.62-1.25% serum and remained on a plateau at concentrations between 2.5 and 20.0 per cent. On the other hand intracellular killing, being very low relative to phagocytosis in 0.62% serum, needed 5% serum to reach a maximum and then showed a consistent drop at concentrations between 10.0 and 20.0 per cent.

Figure 2.12. shows that the drop in intracellular killing at high serum concentrations was observed with PMNs from all donors tested.

Figure 2.13. shows phagocytosis and killing of C.albicans by the same PMNs tested in sera other than standard AB serum. It can be seen that these sera show the same characteristics as AB serum with the exception of serum 'B' which did not support killing at 1.25 or 2.5 per cent concentrations.

A serum concentration of 5% was therefore chosen as non-

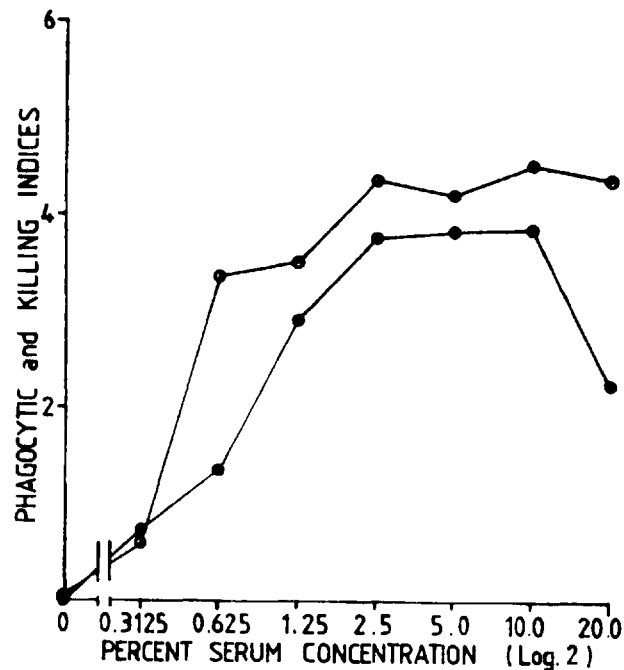


FIGURE 2.11. Effect of different serum (AB) concentrations on phagocytosis and killing of *C. albicans* by PMNs. Standard incubation time and Candida/PMN ratio. OPEN symbols represent phagocytosis, CLOSED symbols represent killing.

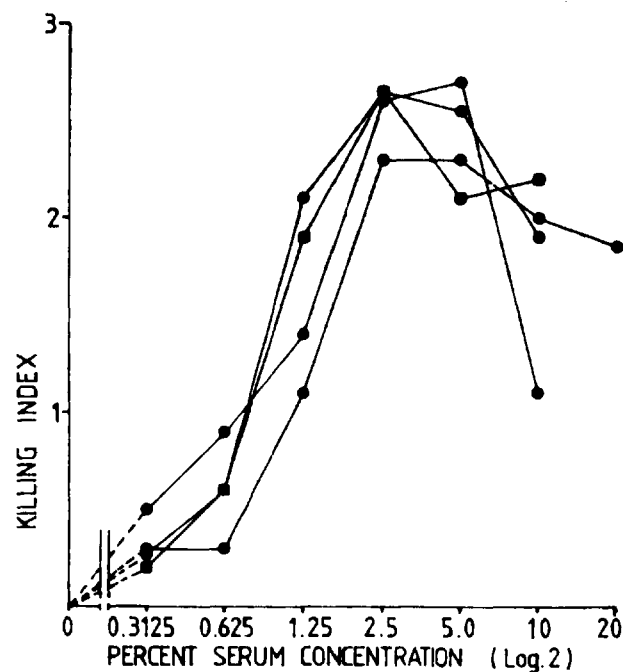


FIGURE 2.12. Effect of different serum (AB) concentrations on *C. albicans* killing using PMNs from different healthy donors. Standard organism/PMN ratio and incubation time.

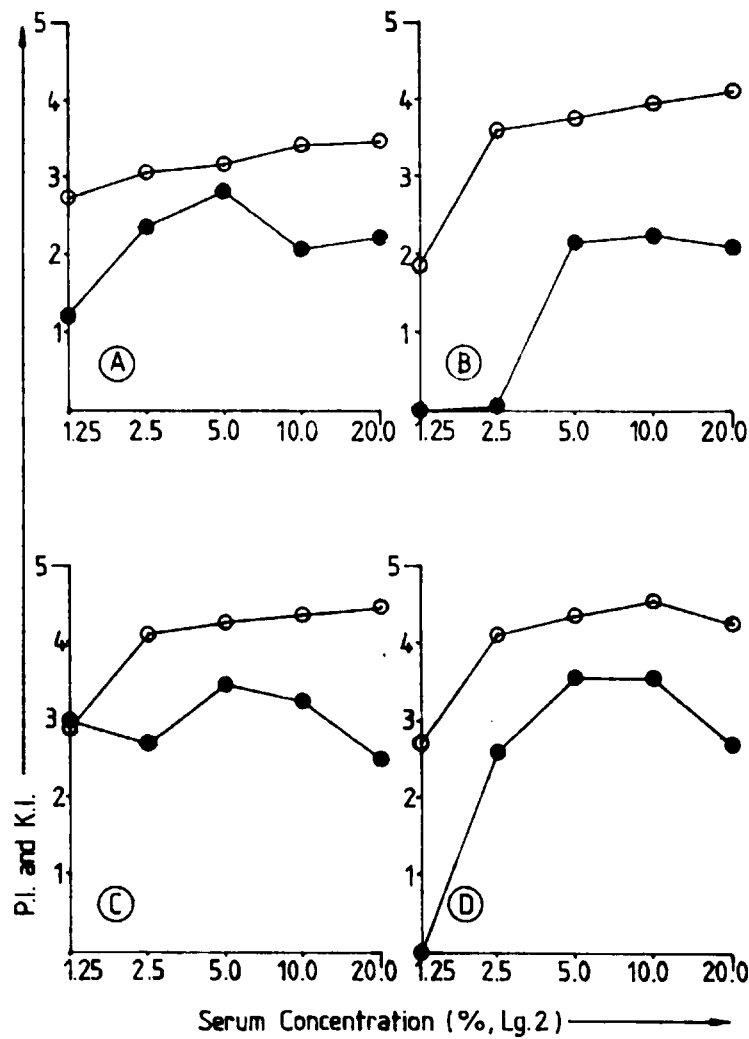


FIGURE 2.13. Effect of different serum concentrations on phagocytosis and killing of *C.albicans* by PMNs using sera from different donors. Standard organism/phagocyte ratio and incubation time. PMNs from the same source. OPEN symbols represent phagocytosis, CLOSED symbols represent killing.

All sera were obtained from healthy donors.

limiting and optimal in the combined assay for intrinsic phagocytosis and killing by human PMNs.

2-6. Comparison with other Candida killing assays.

Table 2.2. shows the results of six experiments in which the new radiometric assay was compared with conventional methods for determining phagocytic killing of C.albicans. All methods were performed using a Candida/PMN ratio of 5:1, a serum concentration of 5%, and an incubation time of 30 mins with mixing by end-over-end rotation at 37°C. The correlation between the different assays was considered satisfactory, but the radiometric assay gave the lowest replicate error.

TABLE 2.2. Comparison of the ³H-uridine incorporation assay with methylene blue dye-exclusion and colony counting as methods for assessing intracellular killing of Candida.

Subjects tested	Radiometric assay	Methylene* blue test	Colony* counting
1	2.75±0.30	nd	2.65±0.53
2	1.8±0.17	nd	1.0±0.17
3	2.28±0.14	nd	3.35±0.50
4	3.25±0.35	3.15±0.35	nd
5	2.4±0.10	2.85±0.57	nd
6	2.55±0.20	2.9±0.43	nd

* Results given as killing indices. nd = not done. PMNs from different sources were used. Standard AB serum was employed throughout.

The methylene blue dye-exclusion test was performed according to the method of Lehrer and Cline (1969b) with the aforementioned modifications regarding serum concentration, organism/phagocyte ratio, and incubation time. After the incubation phagocytes were lysed with sodium deoxycholate (final concn. = 0.625%) in the presence of DNAase (final concn. = 25 µg/ml) to prevent clumping. The released C.albicans were then exposed to methylene blue ($2 \times 10^{-4}M$) for five minutes before being concentrated by centrifugation at 400g for five minutes. The supernatant was removed leaving the organisms in a 100 µl volume which was then examined microscopically. C.albicans unable to exclude the dye were scored as dead. Control mixes without PMNs were examined in parallel and the organisms in these preparations had >95% viability.

Colony counting was carried out by plating sodium deoxycholate and DNAase treated mixtures onto 3% agar (1% pentone, 2% glucose) after appropriate dilution in medium. Killing was determined by calculating the reduction in colony forming units compared to controls in which no phagocytes were present.

The 3H -uridine incorporation assay was also compared with a ^{51}Cr release technique for measuring candidacidal activity of phagocytes (Yamamura, Boler, and Valdimarsson, 1976). The latter measures killing by virtue of phagocyte-mediated release of radiolabelled sodium chromate from C.albicans. Figure 2.14. shows percentage killing versus time for both types of assay conducted simultaneously using a Candida/PMN ratio of 1:1. It can be seen that killing evaluated by 3H -uridine incorporation reached a plateau at 20 minutes with approximately 50% organism death, whereas ^{51}Cr release showed a linear increase in killing up to 40 minutes when greater than 100% killing was apparently achieved. It was also noted that 17% of routine clinical tests over a period of 8 months gave similar results suggesting that the ^{51}Cr release method

was measuring digestion in addition to killing.

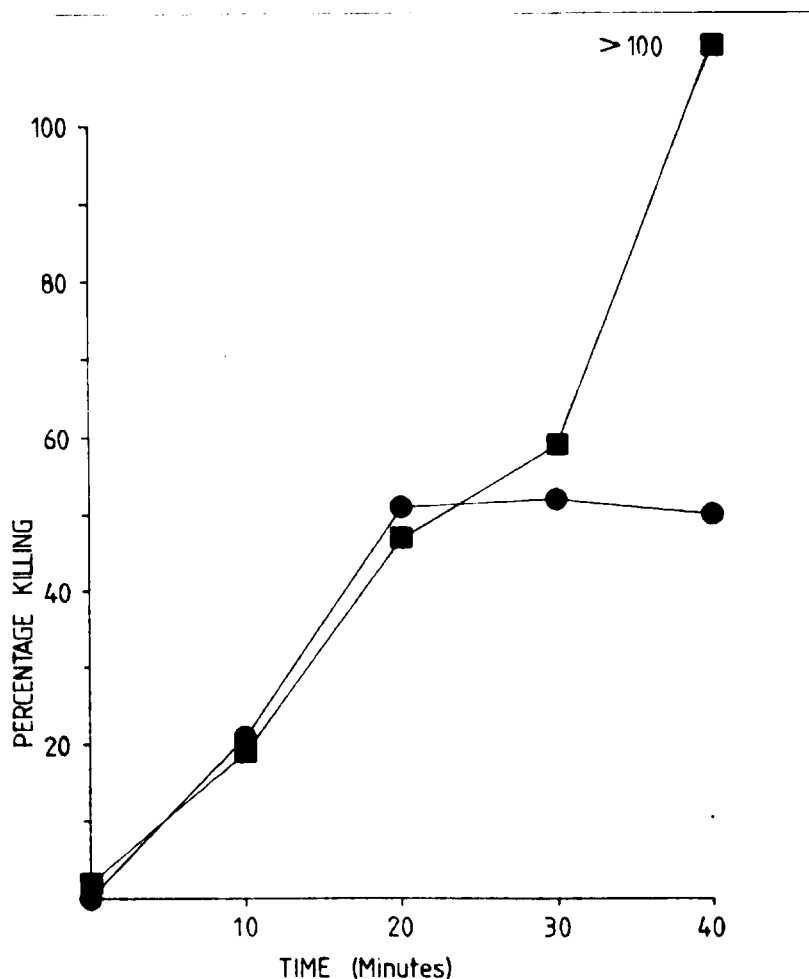


FIGURE 2.14. Comparison of ^3H -uridine incorporation with ^{51}Cr release for measuring intracellular killing of *C.albicans* by PMNs. ROUND symbols represent ^3H -uridine method, SQUARE symbols represent ^{51}Cr release method.

The method was carried out according to Yamamura et al (1976). The preparation of ^{51}Cr labelled *C.albicans* has already been described (page 86). The standard assay conditions for ^{51}Cr release were as follows: 5×10^5 *C.albicans* and 5×10^5 PMNs were mixed at 37°C with end-over-end rotation in 0.5ml medium containing 2.5% normal AB serum. Control tubes without PMNs were also set up. After incubation 0.2 ml DNAase and 0.1 ml sodium deoxycholate were added. The resulting mixtures were incubated for a further 15 mins at 37°C . The tubes were then centrifuged at 2500g for 10 mins, 0.2 ml supernatants collected and counted in a Packard γ -spectrometer. Results are derived from the following formula: Percentage killing = $100 [(X-S) \div (T-S)]$, where X = release in presence of PMNs, S = spontaneous release. Total releasable chromium (T) was determined by incubating

C.albicans with PMNs as above but followed by sodium deoxycholate and boiling for 30 mins.

2-7. Validation of the ^3H -uridine incorporation assay using PMNs from CGD patients.

The validity of the combined phagocytosis and killing assay was examined by using PMNs from patients suffering from chronic granulomatous disease. The results of two such experiments using CGD PMNs together with control cells tested at the same time are shown in Table 2.3. As expected CGD leucocytes ingested but failed to kill C.albicans.

TABLE 2.3. Ingestion and phagocytic killing by PMNs from patients with CGD.

Phagocytes	PI	KI	KE(%)
CGD (MC)	2.9	0	0
Control (1)	3.3	2.6	78
CGD (JV)	3.4	0.4	12
Control (2)	3.8	2.4	63

2-8. Normal ranges and assay stability.

Results for intrinsic phagocytosis and killing by PMNs from 20 healthy adults, all tested within 36 hours, are presented in Table 2.4. Replicate variability within an experiment never exceeded 11%. Initially approximately 50 per cent of all experiments had to be discarded because low values were

obtained for either phagocytosis or killing by PMNs from standard control subjects. These early problems were most apparent whilst the laboratory was being decorated, but they may also in part have been due to toxic medium or plastics. Latterly more than 85% of tests have been technically acceptable and Table 2.5. shows consecutive results from two of the most frequently used standard control subjects over twelve months.

TABLE 2.4. Intrinsic phagocytosis and killing of C.albicans using PMNs from 20 healthy individuals tested November 1978

	PI	KI	KE (%)
Mean	4.0	2.7	68
Mean 2tsd	3.1-4.9	2.0-3.4	40-96

Standard AB serum concn (5%), organism/PMN ratio (5:1), and incubation time.

TABLE 2.5. Consecutive results for intrinsic phagocytosis and killing of C.albicans using PMNs from two standard control subjects.

Date of testing	PI	KI	KE (%)	
CONTROL 1	6.10.78	2.15	2.15	100
	5.12.78	4.55	3.25	71
	10.1.79	4.05	1.80	44
	1.08.79	3.15	2.55	81
	26.9.79	3.95	3.35	85
	24.10.79	3.85	3.45	90

continued on page 105/

TABLE 2.5. continued from previous page.

	Date	PI	KI	KE (%)
CONTROL 2	29.11.78	4.65	3.40	73
	13.12.78	4.35	2.70	62
	27.06.79	3.45	2.65	77
	29.08.79	3.55	2.20	62
	17.10.79	4.35	3.75	86

Re-examination of the normal range for intrinsic phagocytosis and killing 16 months after construction of the original range showed no significant difference in PI between the two. However, the values of KI were significantly higher in the more recent data. The new normal ranges are given in Figure 2.15. which shows KI plotted against corresponding values of PI. Normal ranges for extrinsic phagocytic activity will be given with clinical data in Chapter 5.

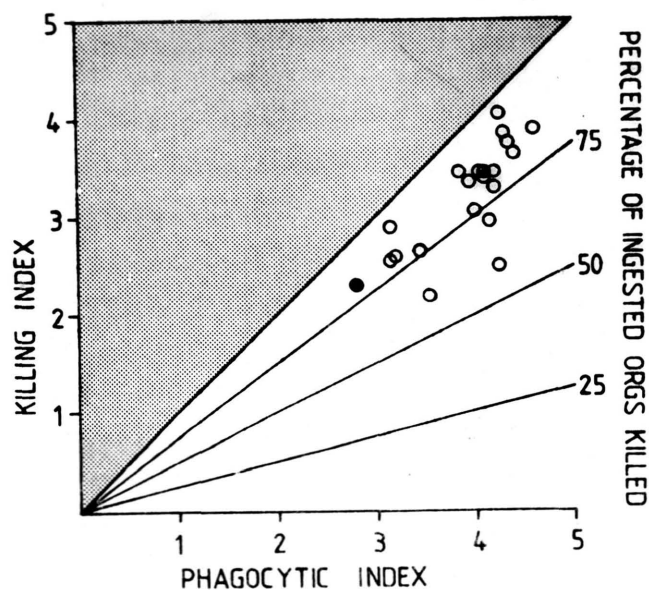


FIGURE 2.15. Normal range for intrinsic phagocytosis and killing of *C.albicans* by PMNs. Standard assay procedure. CLOSED symbol represents two points. Range, PI = 2.75-5.0. Range, KI = 1.95-4.3. Mean KE = 84. n = 22.

2-9. Standardisation of the assay to measure phagocytosis and killing of *C.parapsilosis* by PMNs.

Optimal assay conditions for the phagocytosis and killing of *C.parapsilosis* were investigated. This was in order that these organisms could be directly compared when examining phagocyte function in further studies.

Time course. The time course for phagocytosis and killing of *C.parapsilosis* is presented by a typical experiment in Figure 2.16. It can be seen that the kinetics of ingestion and killing were similar to that of previous studies with *C.albicans*. Phagocytosis was at a maximum within the first ten minutes of incubation whilst optimum killing was apparent at 30-40 mins. A standard incubation time of 40 minutes was therefore chosen to comply with that of the *C.albicans* assay.

Effect of varying the *C.parapsilosis*/PMN ratio.

The organism/phagocyte ratio was altered altered in the same way as described previously (page 95). Figure 2.17. shows that phagocytosis and killing increased in a linear manner up to a ratio of 5:1. However, at a ratio of 10:1, although there was greater ingestion, the ability of PMNs to kill was variable. Killing efficiency at ratios of 2:1 and below were not as great as a 5:1 ratio. In view of these findings a ratio of 5:1 was chosen for use in the standard assay. This decision was again in line with that of the assay for *Candida albicans*.

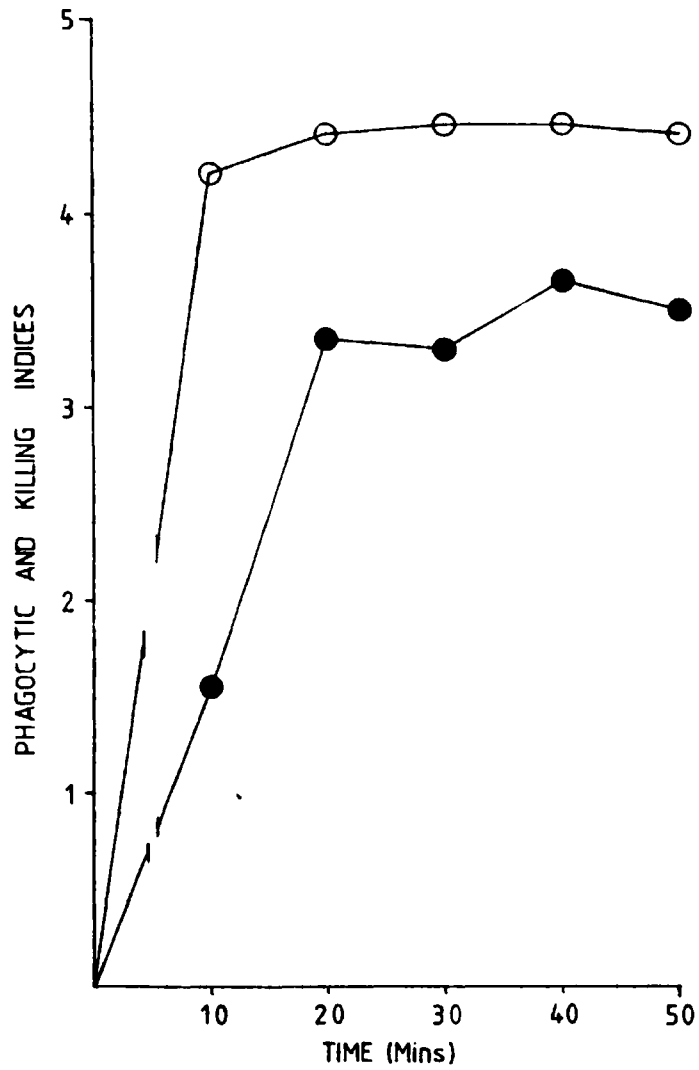


FIGURE 2.16. Phagocytosis and killing versus incubation time for C.parapsilosis and PMNs. Standard assay conditions. OPEN symbols represent phagocytosis, CLOSED symbols represent killing. Normal AB serum was used.

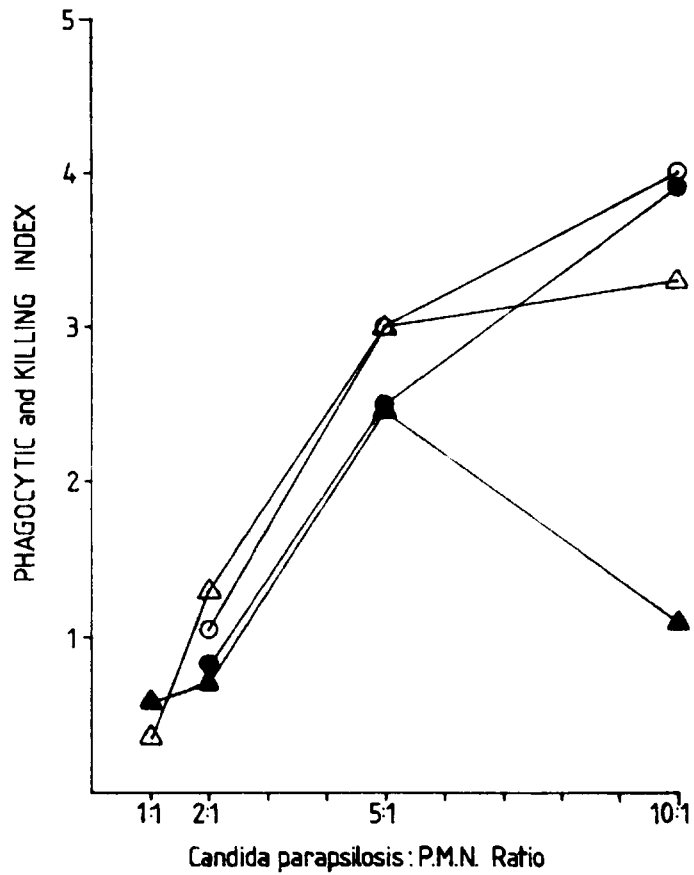


FIGURE 2.17. Effect of varying the Candida parapsilosis/PMN ratio on phagocytosis and killing. Standard AB serum concn and incubation time. OPEN symbols represent ingestion, CLOSED symbols represent killing. PMNs from two healthy individuals.

Serum requirements. Experiments in which the ability of normal PMNs to phagocytose and kill C.parapsilosis in differing serum concentrations are shown in Figure 2.18 a and b. In both sera phagocytosis increased linearly up to 2.5-5.0 per cent serum concentration, reminiscent of C.albicans engulfment under the same conditions. However, in serum concentrations above 5% there was a reduction in values for PI. This was most marked in non-AB normal serum (FIG. 2.18b). Phagocytic killing was low relative to ingestion at serum concentrations below 1.25-2.5 per cent. Optimum values for killing were reached at 2.5-5.0 per cent. At serum concentrations greater than 5.0% the drop in phagocytosis was mirrored by a fall in killing although the killing efficiency remained high.

It was evident that phagocytosis and killing of C.parapsilosis were optimal in 5.0% serum and therefore this concentration was chosen for use in the standard assay. The standard assay conditions for phagocytosis and killing of both C.albicans and C.parapsilosis were thus exactly the same in regard to serum concentration, organism/phagocyte ratio, and incubation time.

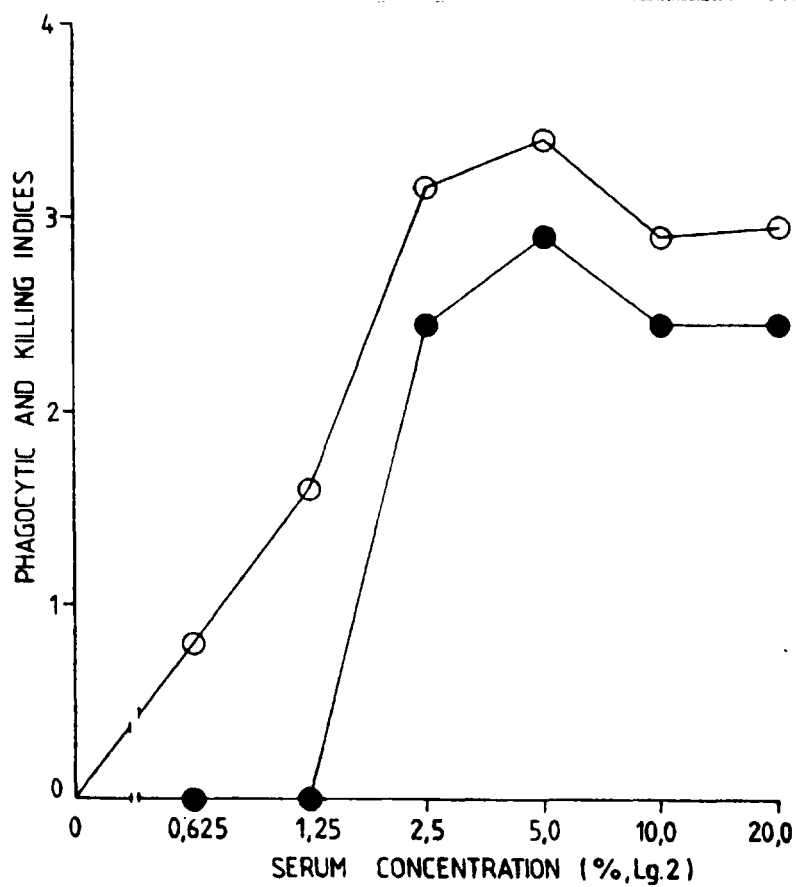


FIGURE 2.18a. Effect of serum concentration on the phagocytosis and killing of *C. parapsilosis* by PMNs.
 Normal AB serum was used under standard assay conditions regarding incubation time (40 mins) and organism/phagocyte ratio (5:1). PMNs from a healthy subject.

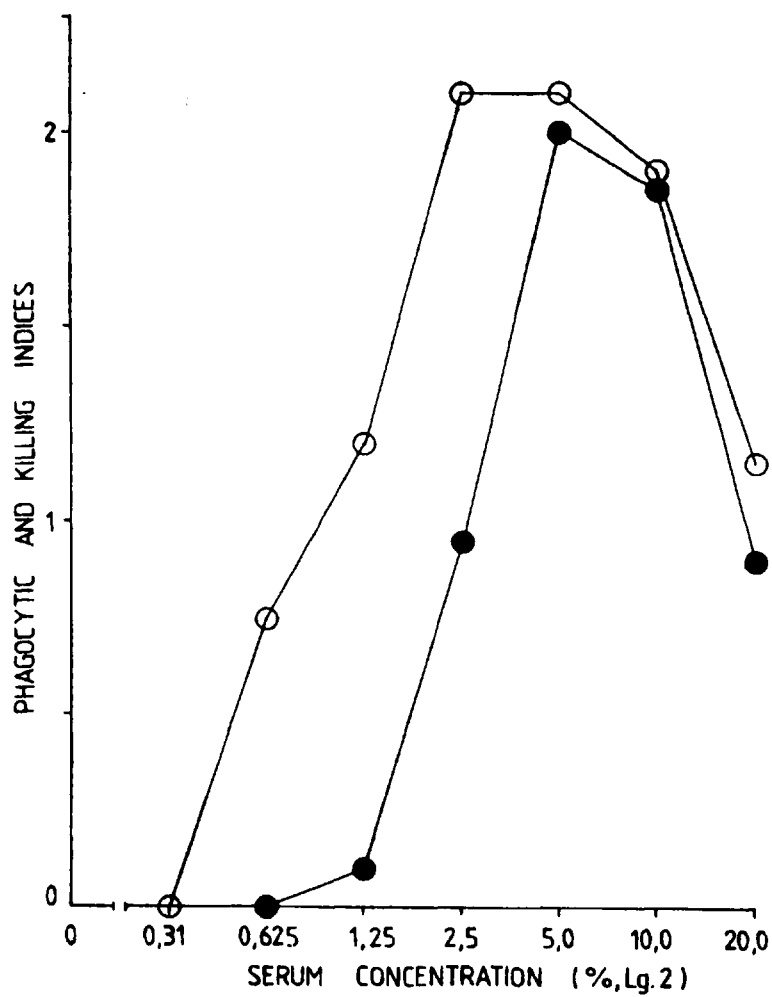


FIGURE 2.18b. Effect of serum concentration on the phagocytosis and killing of *C. parapsilosis* by PMNs.
Normal non-AB serum was used. Standard conditions as for FIG. 2.18a.

2-10. Modification of the assay to measure phagocytosis and killing of *C.albicans* by mononuclear phagocytes (MNPs).

The ability of MNPs (monocytes) to engulf and kill *C.albicans* was compared to PMNs. However, before analysing the time course, *Candida*/monocyte ratios, and serum requirements for monocyte associated phagocytic activity it was essential to rule out that 1) *Candida* organisms stimulate ^3H -uridine incorporation into mononuclear cells, and 2) mononuclear cells affect ^3H -uridine uptake by *Candida*.

Does the presence of *Candida* stimulate uridine uptake by mononuclear cells? Experiments designed to answer this question are presented in Table 2.6. It can be seen that organisms did not affect mononuclear cell uridine uptake.

TABLE 2.6. Effect of *Candida* on the incorporation of ^3H -uridine by blood mononuclear cells.

Mixture description	EXPT 1	EXPT 2
Monocytes + killed <u><i>Candida</i></u>	433±55	313±29
Monocytes alone	437±65	325±31
Killed <u><i>Candida</i></u> alone	21±4	18±5
Number of contaminating lymphocytes/well.	3.2×10^5	2.8×10^5

Uridine incorporation expressed as mean cpm±1 sd of twelve wells. Monocytes (4×10^4) and heat killed *C.albicans* (2×10^5) were placed in a microtitre well containing 0.2 μCi ^3H -uridine and 5% serum in a total volume of 200 μl . Controls containing either mononuclear cells or heat killed *Candida* alone were also

prepared. The resulting mixtures were incubated for two hours at 37°C before harvesting the cells and counting radioactivity in the standard manner.

Is the incorporation of ^3H -uridine by *Candida* affected by mononuclear cell products?

Figure 2.19. shows that there is reduced uridine uptake by *C.albicans* when incubated with supernatants from cultures containing monocytes for 90-120 minutes. Mononuclear cell-*Candida* supernatant and mononuclear cell supernatant did not significantly differ from one another in affecting ^3H -uridine incorporation by *C.albicans*. The possibility that mononuclear cells and *Candida* were competing for serum nutrients was not investigated. However, it was concluded that it was possible to use a radiometric assay of this type for measuring monocyte phagocytic activity provided the incubation time with radio-labelled uridine was restricted to 60 minutes.

2-11. Materials and Method.

Unless otherwise stated the assay procedure was as follows: quadruplicate mixtures of mononuclear cells (containing 4×10^4 monocytes) and *C.albicans* (2×10^5) were placed in 'U' bottom microtitre wells in a total volume of 100 μl and containing 2.5 per cent serum. Two control mixtures, also in quadruplicate, were prepared containing either mononuclear cells or *Candida* alone in the same quantities, volume and serum concentration as above. A second series of wells the same as the above were also set up in quadruplicates in the same microtitre plate (see Figure 2.20 for layout).

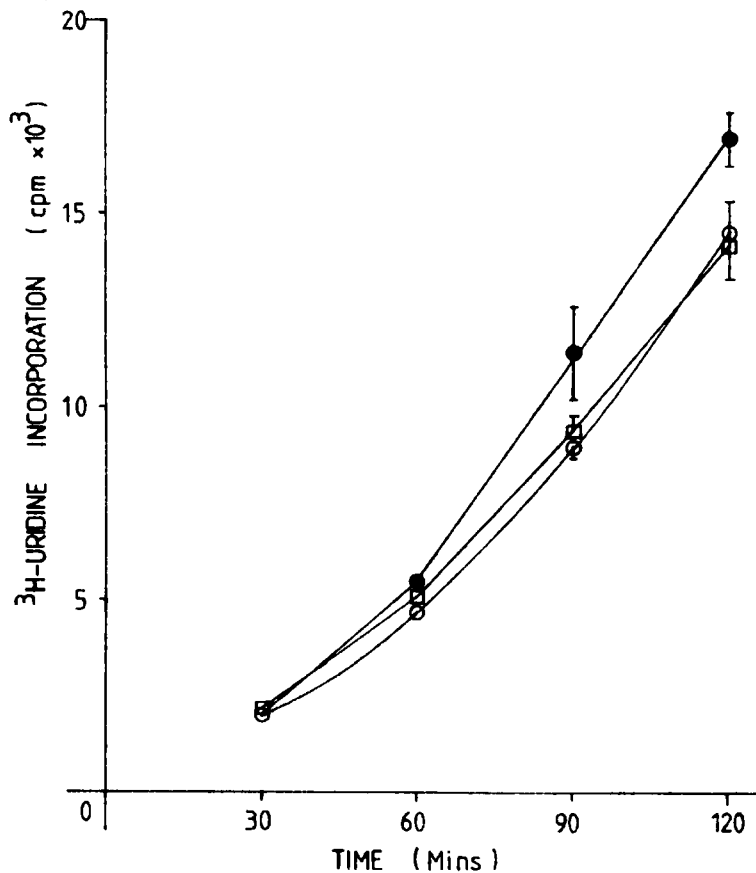


FIGURE 2.19. Effect of cultured blood mononuclear cell products on ^3H -uridine incorporation by *C.albicans*.

CLOSED ROUND symbols = Fresh medium. OPEN ROUND symbols = Mononuclear cell-*Candida* supernatant. OPEN SQUARE symbols = Mononuclear cell supernatant.

Mixtures containing 5×10^5 monocytes, 2.5×10^6 heat killed *C.albicans* and 5% serum in a total volume of 0.5 ml were incubated for two hours at 37°C . Controls with monocytes alone were also prepared. After incubation the mixtures were centrifuged at 2500g for 10 mins. The resulting supernatants (pH 7.2, 100 μl), or fresh medium (100 μl), and 2×10^5 live *C.albicans* (100 μl) were incubated together in microtitre wells for 40 mins at 37°C prior to adding 0.2 μCi of ^3H -uridine/well. *C.albicans* incubated in fresh medium also contained a final serum concentration of 2.5% thus making such preparations identical with the others. Incubation was allowed to continue for varying periods of time up to two hours. Time = 0 on the figure indicates time when radiolabel was added.

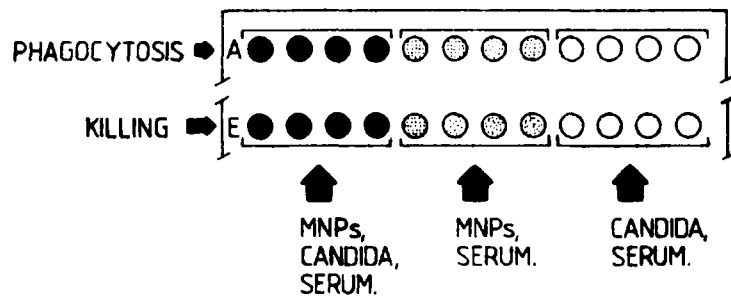


FIGURE 2.20. Schematic diagram showing layout of microtitre plate for measurement of phagocytosis and killing by MNPs.

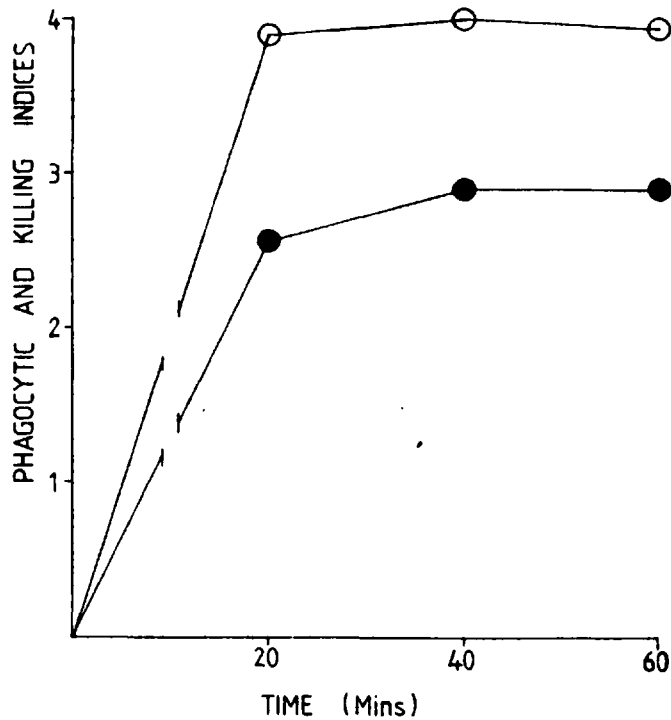


FIGURE 2.21. Phagocytosis and killing versus incubation time for MNPs and *C.albicans*. Standard assay conditions regarding organism/phagocyte ratio and serum concn. OPEN symbols represent phagocytosis, CLOSED symbols represent killing.

Ingestion was measured from the first series of wells and killing from the second series.

The microtitre plate was then incubated for 40 minutes at 37°C prior to adding 0.2 µCi of ³H-uridine to each well. 100 µl of medium was then added to wells destined to provide a measurement of phagocytosis whilst 50 µl sodium deoxycholate and 50 µl DNAase were added to the second series of wells. The plate was incubated for a further 60 minutes at 37°C before placing on ice for 15 minutes to detach any adherent cells. Finally, cells were harvested and radioactivity counted in the normal manner.

Presentation of data

The PI and KI for monocyte phagocytosis and killing of Candida albicans were calculated with a modified formula which took into account unstimulated uridine uptake by mononuclear cells:

$$\text{PI or KI} = \left[1 - \frac{\text{cpm } (\text{Candida} + \text{MNPs}) - (\text{MNPs alone})}{\text{cpm } \text{Candida} \text{ alone}} \right] \text{R}$$

The KE and OKE were calculated as described previously (page 90).

2-12. RESULTS

Time course. Figure 2.21 shows a typical experiment in which the assay was terminated at 20 minute intervals up to 60 mins. It is apparent that 40 minutes is a suitable length of time for incubation in the modified radiometric assay measuring phagocytic activity of monocytes.

Effect of varying the Candida/MNP ratio. Figure 2.22. shows two experiments using monocytes from healthy donors. Both phagocytosis and killing reached a plateau at a 5:1 ratio. At the higher ratio of 10:1 phagocytosis did not increase and the killing efficiency was comparable to a 5:1 ratio. The killing efficiency was lower at a ratio of 1:1 indicating poor induction of phagocytic killing. Phagocytosis and killing were both optimal at a 5:1 ratio, this ratio was therefore chosen for the standard assay.

Serum requirements. The effect of four different normal sera on monocyte phagocytosis and killing are shown in Figure 2.23. Phagocytosis and killing are both at a maximum in 1.25-2.5% serum. At serum concentrations greater than 2.5% sera B and D maintain a relatively high value for PI. On the other hand sera A and C show a substantial decrease in support of MNP phagocytosis at high serum concentrations. It appears that some sera inhibit ingestion at high concentrations. However, all sera fail to support monocyte killing at concentrations between 2.5 and 20.0 per cent. In view of these findings a serum concentration of 2.5% was chosen for the standard assay.

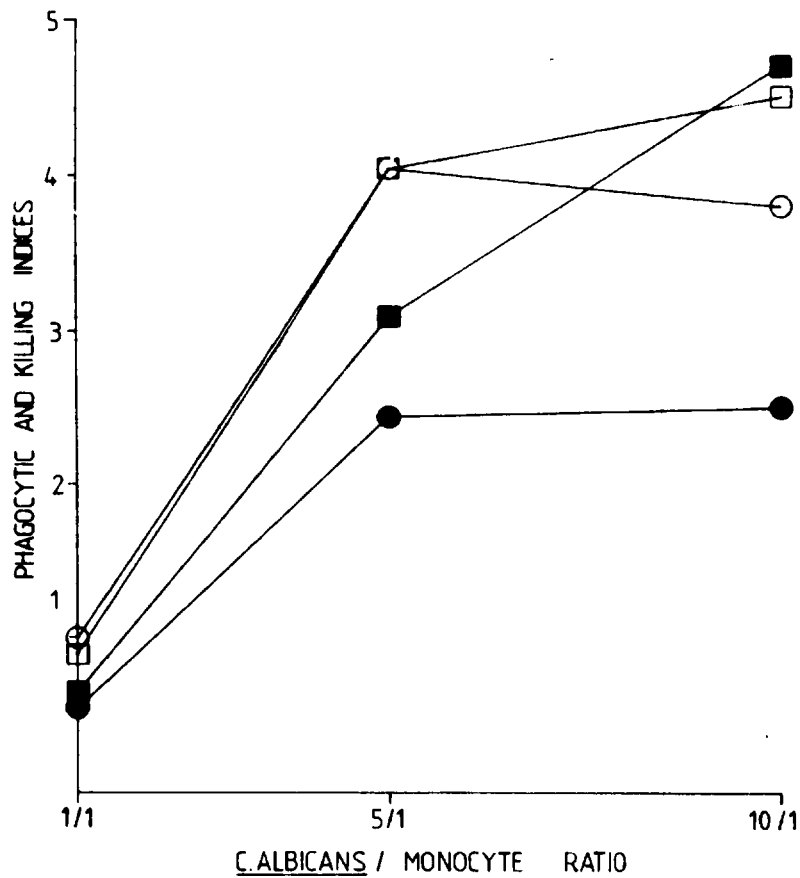


FIGURE 2.22. Effect of varying the *C. albicans*/MNP ratio on phagocytosis and killing.

OPEN symbols represent phagocytosis, CLOSED symbols represent killing. Standard assay conditions in regard to incubation time and AB serum concn. The organism/monocyte ratio was altered by varying phagocyte numbers.

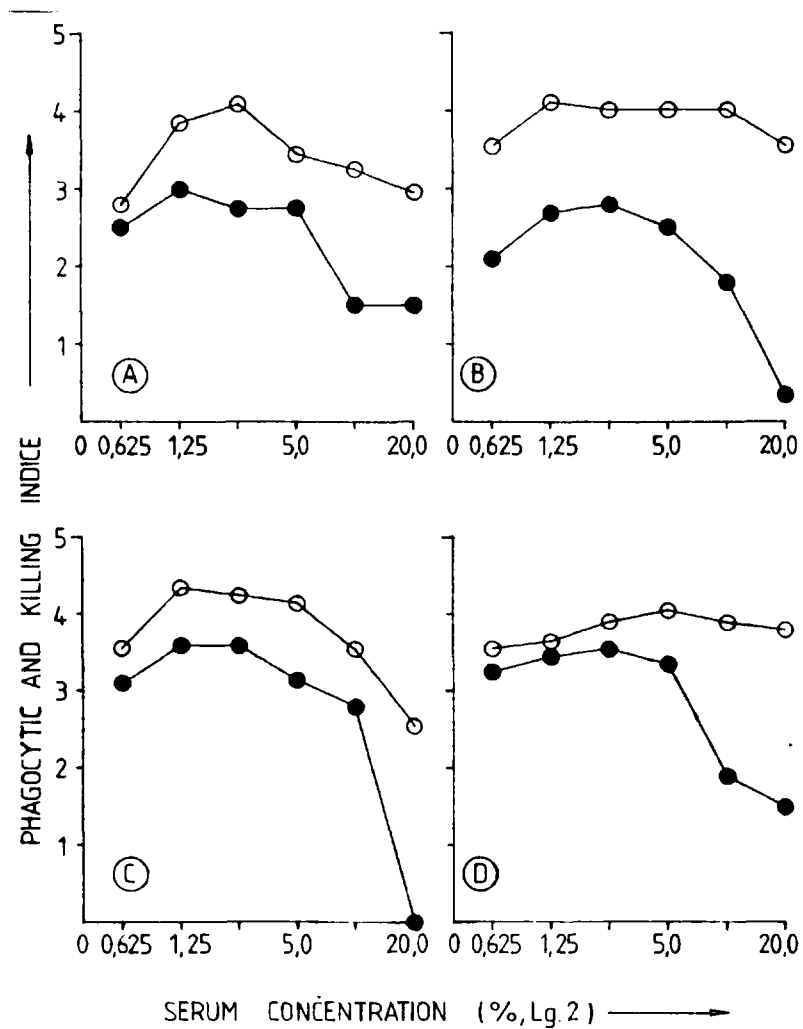


FIGURE 2.23. Effect of serum concentration on phagocytosis and killing of *C.albicans* by MNPs. OPEN symbols represent phagocytosis, CLOSED symbols represent killing. Standard assay conditions in regard to incubation time and organism/MNP ratio. All sera from healthy donors.

2-13. Discussion.

It is important to determine whether defective phagocytic killing is due to impaired ingestion or reduced phagocyte killing capacity. The combined assay described measures phagocytosis and killing independently from a single sample and has the additional advantages of objectivity and sensitivity. It is in part based on an earlier method for measuring phagocytosis (Yamamura et al., 1977), but also detects metabolic death by the absence of radiolabel incorporation by microorganisms. The original uridine incorporation assay has already been modified to measure phagocytosis of bacteria (Lam and Mathison, 1979) and it should be possible to adapt the method for measuring phagocytic killing of bacteria.

The data presented shows that assays for phagocytic function must be carefully standardised in terms of organism/phagocyte ratio, incubation time, and serum concentration in order to achieve acceptable sensitivity and analytical performance. Thus the ability of normal human phagocytes to kill fungal blastospores is not fully challenged at Candida/phagocyte ratios below 5:1 and a ratio of 10:1 may be necessary when agents are tested for their ability to enhance phagocytic candidacidal activity. At low organism/phagocyte ratios of 1:1 or 2:1 it would be expected that nearly all of the ingested organisms would be killed. However, this did not occur which suggests that there is insufficient activation of phagocytic killing when only one or two organisms are ingested per PMN or MNP. Presentation of large quantities of microbes to

phagocytes has been advocated by other workers (Clawson and Repine, 1976; Palmblad and Engstedt, 1979).

A serum concentration of 5.0-10.0 per cent is optimal for testing the intrinsic candidacidal capacity of human PMNs. At lower serum concentrations the killing is limited by humoral factors while higher concentrations consistently inhibit phagocytic killing of C.albicans without affecting its ingestion. In contrast, the low killing of Candida parapsilosis can be attributed to reduced ingestion. The different effects of high serum concentrations on phagocytosis and killing of these organisms demonstrates the analytical potential of this assay.

Similar phenomena in relation to serum concentration has been noted by Tofte and associates (1980) who found that relatively high serum concentrations (25%) and prolonged incubation for preopsonisation reduced the subsequent ingestion of certain bacteria. Prolonged preopsonisation was also found to impair the zymosan induced chemiluminescence of human PMNs (Williams, Hastings, Easmon, and Cole, 1980). Furthermore, the relationship between serum concentration and phagocytic activity is particularly salutary as many workers have used high serum concentrations as well as low organism/phagocyte ratios in their studies (Leijh, van Barselaar, and van Furth, 1977; Oseas and Lehrer, 1978; Wood and White, 1978).

Monocytes have different serum requirements for the ingestion and killing of C.albicans. First, they function best in serum

concentrations of 1.25-2.5% as opposed to 5.0-10.0%, and second, they are sensitive to high serum concentrations in two distinct ways. Relatively high serum concentrations can cause either a reduction in phagocytosis with attendant impaired killing, or, diminished killing with little effect on phagocytosis. Therefore the behaviour of monocytes to Candida albicans in high serum concentrations appeared to reflect the behaviour of PMNs to different organisms. It is unlikely that the disparate results with MNPs were the function of sera from different sources because the behaviour of normal PMNs towards C.albicans and C.parapsilosis with different sera was always consistent and depended on the organism. Since different MNPs were used it seems probable that they were responsible for the variation in phagocytic activity to C.albicans. Unfortunately, individual sera were not tested against several sources of MNPs. Although not proven here, the differential response of monocytes towards C.albicans could be related to their mode of storage as it has been reported that cooling can cause the loss of MNP Fc and C3 receptors (Kávai et al., 1980). Monocytes from cell-separator residues were subjected to 4°C storage prior to use as were freshly isolated MNPs on occasion.

A former killing assay which measures ^{51}Cr release from Candida albicans (Yamamura et al., 1976) occasionally gave values of killing greater than 100%, indicating that this technique might, in addition to killing, be measuring digestive release of ^{51}Cr from the organisms. Relevant to this was the observation that monocytes released ^{51}Cr from C.albicans in an 'organism age' related manner (Cooper, personal communication), suggesting

that older blastospores were more susceptible to killing and degradation. It was also noted that in experiments which did not demonstrate 100% phagocytic killing there was always a rapid release of label within the first 30 minutes followed by a much slower release (Yamamura et al., 1976; Author's observations). This is consistent with a rapid release of label associated with killing followed by a slower digestive release of label. Slow release of label could reflect the 'residual killing capacity' reported by other workers (Raeburn, van Zwet, Leijh, and van Furth, 1980). Interestingly, the ^3H -uridine incorporation technique showed rapid death followed by a plateau. More extensive studies are needed since the clinical potential of a digestion assay is very great particularly in relation to patients with chronic infections who may have impaired ability to digest and process antigen and may therefore be in a state of antigen overload.

CHAPTER 3

THE DEVELOPMENT AND TECHNICAL CONSIDERATIONS OF A MICRO-TEST
MEASURING IODINATION OF C.ALBICANS BY POLYMORPHONUCLEAR
LEUCOCYTES.

3-1. Introduction.

This chapter is devoted to the description of a micro-technique for assaying radioiodination of C.albicans by PMNs. Standard assay conditions for this test are examined and compared with those for the radiometric combined phagocytosis and killing assay.

Myeloperoxidase-halide- H_2O_2 mediated killing of microorganisms by phagocytes has been discussed in Chapter 1 (page 57) and therefore no further mention will be necessary.

3-2. Materials and Methods.

The standard procedure for micro-radioiodination of C.albicans was as follows unless otherwise stated. Quadruplicate 200 μ l volumes of medium containing 2×10^5 PMNs, 1×10^6 C.albicans, 10.0% serum and 0.2 μ Ci of 125 Iodine were placed in 'U' wells of a Sterilin M24AR microtitre plate. Control mixtures which did not contain PMNs or Candida were also set up in quadruplicate. A schematic diagram showing the layout of a typical microtitre plate is given in Figure 3.1. The microtitre plate was incubated for 90 minutes at $37^\circ C$ before collecting insoluble radioactivity onto glass fibre discs with a Skatron harvester. The discs were then dried and placed in 2ml flat-bottomed plastic tubes for counting in the γ -spectrometer.

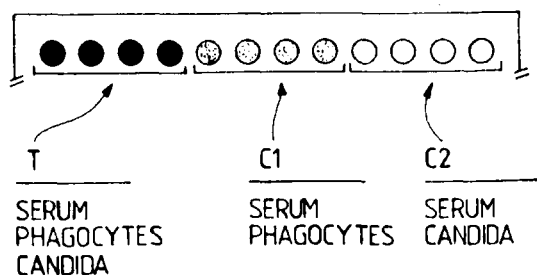


FIGURE 3.1. Schematic diagram showing layout of microtitre plate for microradioiodination of C.albicans by PMNs.

3-3. Presentation of data.

Results for iodination of C.albicans are expressed in raw counts per minute (cpm) and caculated by the following formula:

$$^{125}\text{I (cpm) fixed}/1 \times 10^6 \text{ blastospores} = T - (C1+C2)(\text{cpm})$$

where T = the cpm obtained from experimental wells, C1 represents cpm from mixtures without organisms, and C2 = cpm from mixtures without phagocytes.

Between 5 and 10 per cent of the original radioactivity was recoverable from insoluble material as a result of the interaction of Candida and phagocytes in optimal serum concentrations. Replicate error within an experiment was less than 8.0%. Because results are expressed in cpm values are corrected for ^{125}I decay when several experiments are shown together.

3-4. Technical comments.

a. Effect of different types of microtitre plate on the harvesting efficiency.

An initial problem of uneven counts was explained by the nature of the microtitre plate employed. Figure 3.2. shows that a substantial amount of radioactivity associated with discs was lost when experiments were conducted in Sterilin tissue culture grade (M24ART) plates. This was not so severe when ordinary (M24AR) plates were used. Moreover, replicate

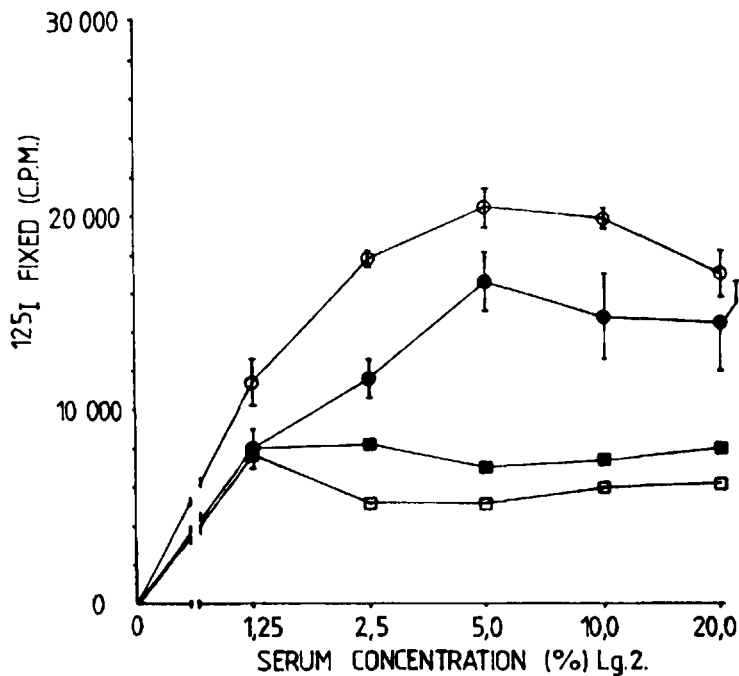


FIGURE 3.2. Effect of different types of microtitre plate on harvesting efficiency.

Two experiments using PMNs from healthy donors. Standard assay conditions in regard to organism/phagocyte ratio and incubation time.

ROUND symbols indicate radioactivity associated with discs, SQUARE symbols indicate radioactivity associated with wells. OPEN symbols represent experiments in Sterilin M24AR plates, CLOSED symbols represent experiments in Sterilin M24ART plates.

Radioactivity associated with microtitre wells was measured after cutting up microtitre plate with a bandsaw. Individual wells were placed in tubes and counted in γ -spectrometer.

error was less if non-tissue culture grade plates were used.

The proportion of radioactivity remaining in the wells of M24ART plates was greater if the Candida/PMN ratio was low (FIGURE 3.3.). When the effect of the Candida/PMN ratio was examined in M24AR plates it was found that negligible amounts

of radioactivity were lost even at low ratios.

As a result of these findings it was considered prudent to use M24AR plates in future experiments.

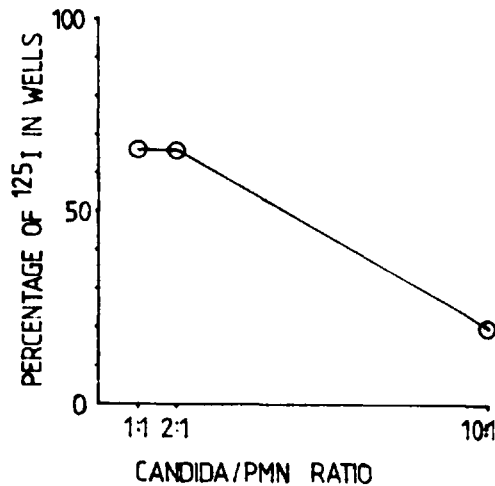


FIGURE 3.3. Effect of C.albicans/PMN ratio on harvesting efficiency when tissue culture grade plates are used.

Standard assay conditions regarding incubation time and serum concentration. Ratios altered by changing phagocyte numbers.

b. Harvesting procedure.

Trichloroacetic acid (TCA) is commonly used to precipitate protein bound radioactivity. The possibility that this could precipitate radioiodinated serum proteins was confirmed by the results shown in Figure 3.4. It can be seen that the amount of radioactivity collected in the presence of TCA was always greater than that collected by distilled water.

This was more pronounced at serum concentrations between 10.0 and 20.0 per cent. Although it is possible to remove this

source of error with appropriate controls it was considered preferable to use distilled water and avoid measuring the serum binding of iodide.

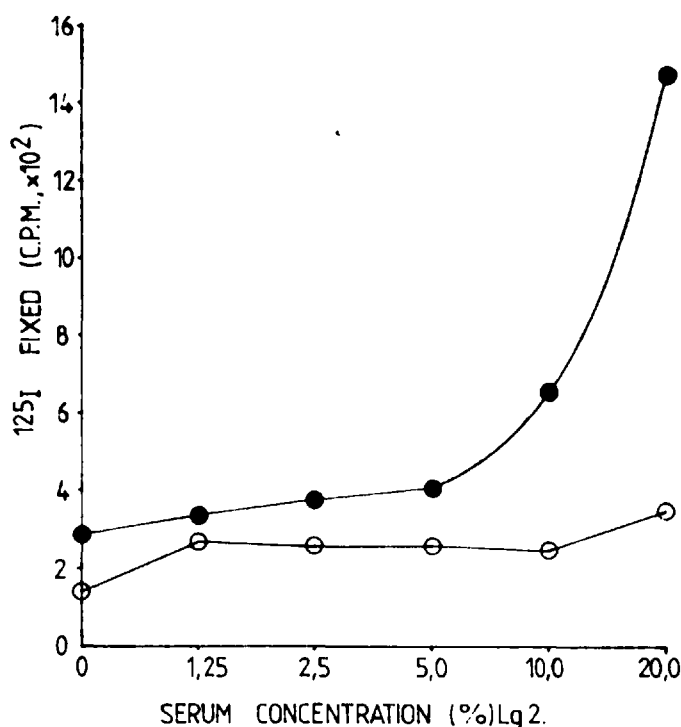


FIGURE 3.4. Comparison of trichloroacetic acid with distilled water for harvesting microradioiodination experiments.

200 μ l volumes of medium containing serum in concns ranging from 0-20.0% were placed in 'U' wells of a M24AR microtitre plate. Each serum concn was set up in sextuplet. Another microtitre plate was prepared in an identical manner. To each well was added 0.2 μ Ci 125 I and the plates incubated for 90 minutes. Radioactivity from one of the plates (CLOSED symbols) was collected with 10.0% TCA, whilst radioactivity from the other plate was collected with distilled water (OPEN symbols). Radiolabelled material was collected onto glass fibre discs and counted in the usual manner.

3-5. Standardisation of micro-iodination assay.

Time course. Iodination increased linearly up to 60 minutes (Figure 3.5.). However, maximal counts were usually obtained when the incubation time was 90 minutes. An incubation time of 90 minutes was therefore chosen for the standard assay.

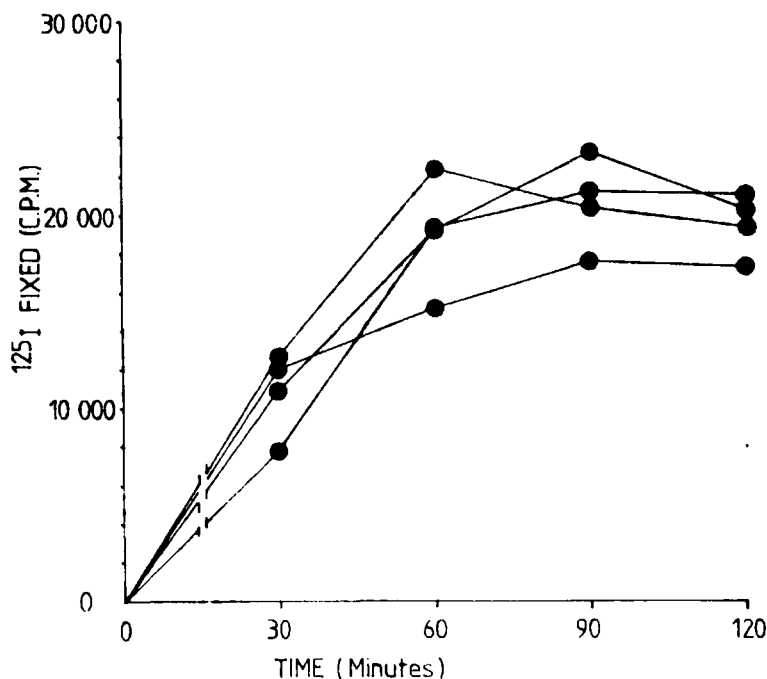


FIGURE 3.5. Iodination of C.albicans by PMNs versus incubation time. Standard assay conditions for serum concentration and organism/phagocyte ratio. PMNs and autologous sera from healthy donors.

The Candida/PMN ratio. Figure 3.6. shows that maximum iodination was achieved at a 5:1 ratio. There was less iodination at 10:1 ratios than at 1:1 Candida/PMN ratios. A ratio of 5:1 was therefore chosen for the standard assay.

Serum requirements. In normal serum iodination rose with increasing serum concentration until a peak was reached at 5.0-10.0 per cent serum. Iodination fell in higher serum concentrations (Figure 3.7.). Significantly lower iodination

occured when heat-inactivated serum was used (Fig.3.7.). A serum concentration of 10.0% was chosen for the standard assay.

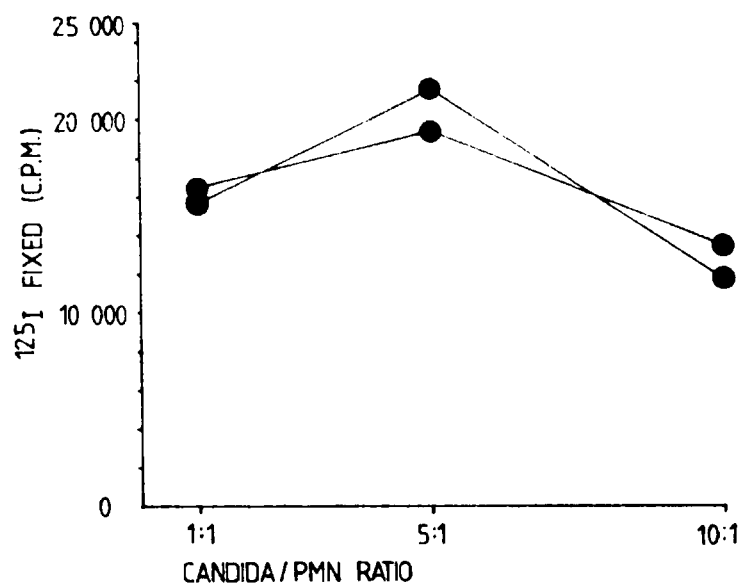


FIGURE 3.6. Effect of varying the organism/phagocyte ratio on iodination of *C.albicans* by PMNs. Ratio altered by changing phagocyte numbers. PMNs and sera from healthy donors. Standard assay conditions for serum concentration and incubation time.

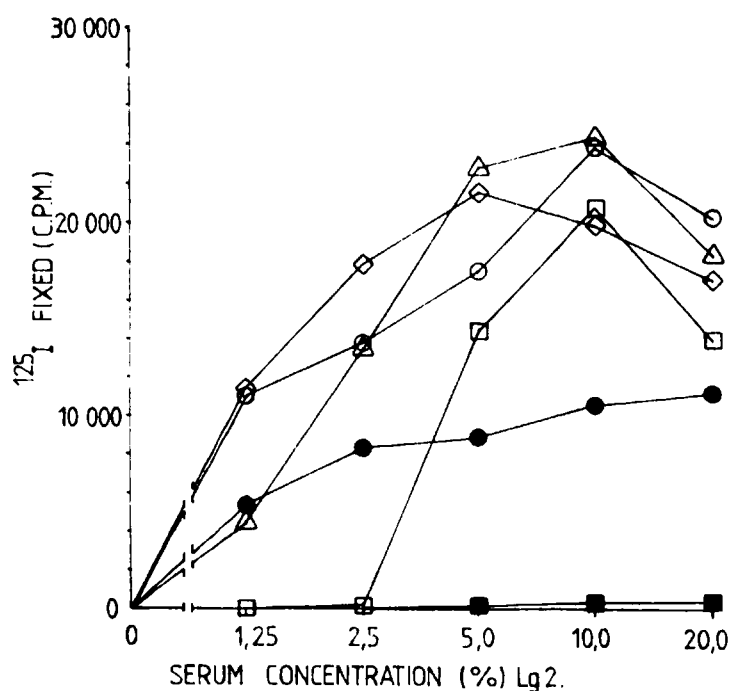


FIGURE 3.7. Effect of serum concentration on iodination of *C.albicans* by PMNs. OPEN symbols represent normal sera, CLOSED symbols represent sera which have been heat-inactivated (56°C/40 mins). PMNs and autologous sera from healthy donors. Standard assay conditions.

3-6. Discussion.

The microradioiodination method described is based on a similar technique devised by Hosking et al. (1978). It requires only small amounts of blood and is simple to perform.

The effect of organism/phagocyte ratios and serum requirements are in excellent agreement with the results obtained with the radiometric phagocytosis and killing assay described in the previous chapter. Of particular interest are the common optimal serum concentrations of 5-10% and the reduction in both Candida killing and iodination observed in higher serum concentrations. A strikingly similar dependence on serum concentration was demonstrated by Klebanoff and Clark (1977) in their improved assay for iodination of zymosan by PMNs. They also demonstrated that C3 or C4 deficient serum were incapable of supporting iodination of zymosan by PMNs. Since zymosan is opsonised via the alternative complement pathway it was argued that the low iodination in C3 deficient serum was due to poor ingestion. However, low iodination of C.albicans by PMNs was observed in serum concentrations which fully supported phagocytosis. It therefore seems likely that stimulation of iodination was independent of antibody mediated opsonisation. Moreover, in heat-inactivated serum (FIG.3.7.) reduced ingestion does not fully account for inefficient iodination. Therefore, it is possible that complement components are not only opsonic but also are involved in the processes which terminate in microbial iodination. This will be examined further in the next chapter.

Iodination at organism/phagocyte ratios of 1:1 was low when

compared to a 5:1 ratio. This finding supports the idea that killing processes are not fully induced when PMNs are challenged with low numbers of Candida. High organism/phagocyte ratios were also relatively inefficient at promoting iodination which suggests that killing processes may be saturated or antagonised by phagocytic ingestion of large quantities of microorganisms.

Care should be exercised when selecting commercially available products for laboratory use. The results for iodination in two types of microtitre plate confirm this and emphasise that the more expensive materials are not always the best for the job. Finally, investigation of simple procedures can avoid unnecessary work. This is apparent from the observation that distilled water was preferable to 10.0% TCA as a harvesting fluid in the micro-iodination technique.

CHAPTER 4

THE BASIS OF EXTRINSIC PHAGOCYtic KILLING: THE INFLUENCE OF
C3 ON INTRACELLULAR KILLING BY PMNs AND MNPs.

4-1 Introduction

The salient feature of extrinsic phagocytic activity in relation to C.albicans and PMNs was the disparity between phagocytosis and killing at low and high serum concentrations. Since it had already been shown that C3 is required for phagocytic killing of C.albicans (Yamamura and Valdimarsson, 1977) it seemed likely that the observed differences between opsonisation and the humoral support of killing reflected differences in the effect of specific antibody and C3 on the phagocytic process. Therefore, at concentrations of intact serum below 5.0% enough opsonising antibody is present to promote ingestion whereas killing is probably limited by the availability of activated C3. Similarly, the drop in phagocytic killing in serum concentrations above 10.0% could be due to relatively rapid inactivation of particle-bound C3b by the combined action of β_1H and C3bINA (Whaley and Ruddy, 1976; Pangburn, Schreiber, and Muller-Eberhard, 1977). A process which may be less effective at lower serum concentrations because of insufficient amounts of inactivators.

This chapter illustrates various experimental approaches which sought to confirm the view that C3 is required for phagocytic killing of Candida by PMNs. Additionally, an examination of the role of C3 in monocyte killing of Candida albicans will be presented.

4-2. Materials and Methods.

Standard assay procedures for phagocytosis and killing and micro-iodination were followed unless otherwise stated. Descriptions of other methods are placed with the relevant results.

RESULTS

4-3. The effect of heat-inactivated serum on phagocytosis and killing.

A significant reduction in microbial iodination by PMNs occurred when heat-inactivated (56°C/40 mins) serum was used in place of fresh serum (Figure 3.7.). This could have been due to poor opsonisation and subsequent phagocytosis, or, alternatively, normal levels of phagocytosis but impaired killing. The ability of two normal but heat-inactivated sera to support phagocytosis and killing of C.albicans by PMNs is shown in Figure 4.1. Compared with intact serum there was reduced ingestion in the presence of heated serum, although maximal phagocytosis was achieved at the same serum concentration (1.25%). However, the failure of heated serum to support intracellular killing was much more striking as killing was virtually absent at concentrations below 5.0% and increased only marginally up to 20.0%.

Heat-inactivated serum had a similar effect on the phagocytosis and killing of C.parapsilosis by PMNs (Figure 4.2.), but the reduction in killing was more severe in that death of this

organism was detectable only in 20.0% serum.

Monocytes, in contrast to PMNs, did not appear to have their function blunted by heat-inactivated serum, instead maintaining good levels of phagocytosis and killing at heated serum concentrations of between 1.25% and 10.0% (Figure 4.3.). It was also noticed that in high concentrations of heated serum there was more killing than in corresponding concentrations of normal fresh serum.

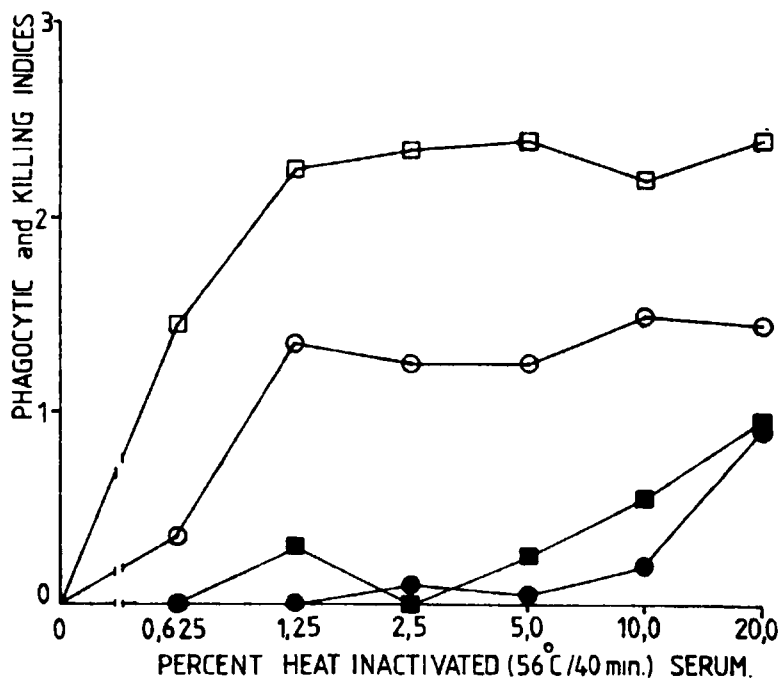


FIGURE 4.1. Two experiments showing the effect of heat-inactivated serum on phagocytosis and killing of *C. albicans* by PMNs.

Standard assay procedure. Sera from two healthy donors.

OPEN symbols represent phagocytosis, CLOSED symbols represent killing.

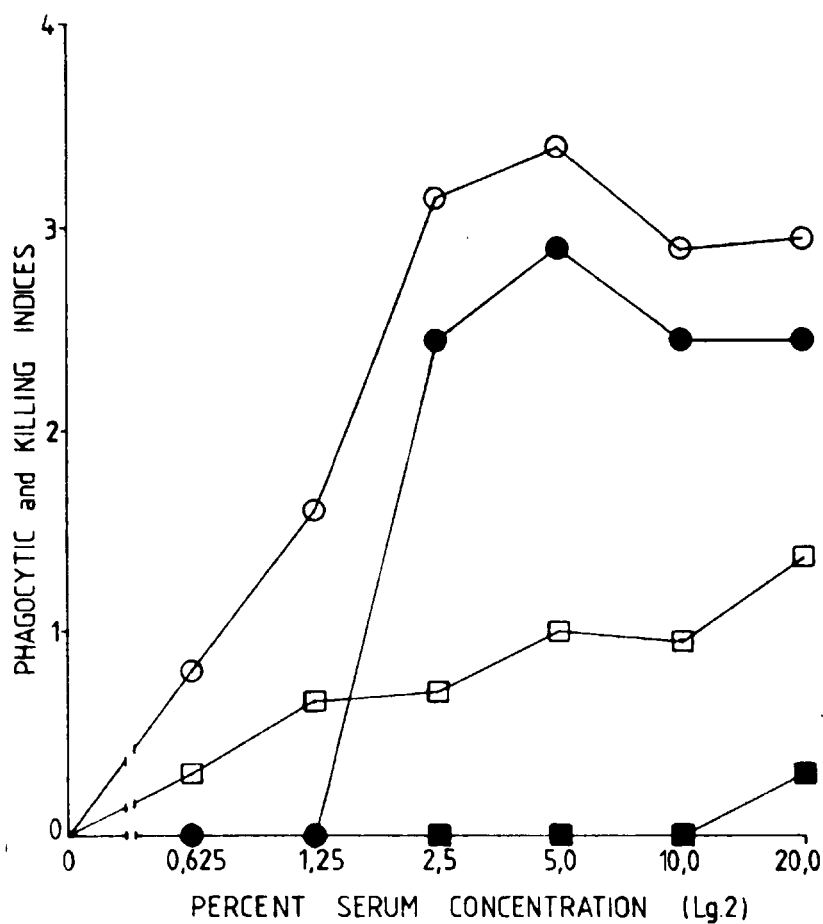


FIGURE 4.2. Effect of heat-inactivated serum on phagocytosis and killing of *C. parapsilosis* by PMNs.

ROUND symbols represent normal AB serum, SQUARE symbols represent heat-inactivated (56°C/40 mins) AB serum. OPEN symbols represent phagocytosis, CLOSED symbols represent killing. Standard assay conditions.

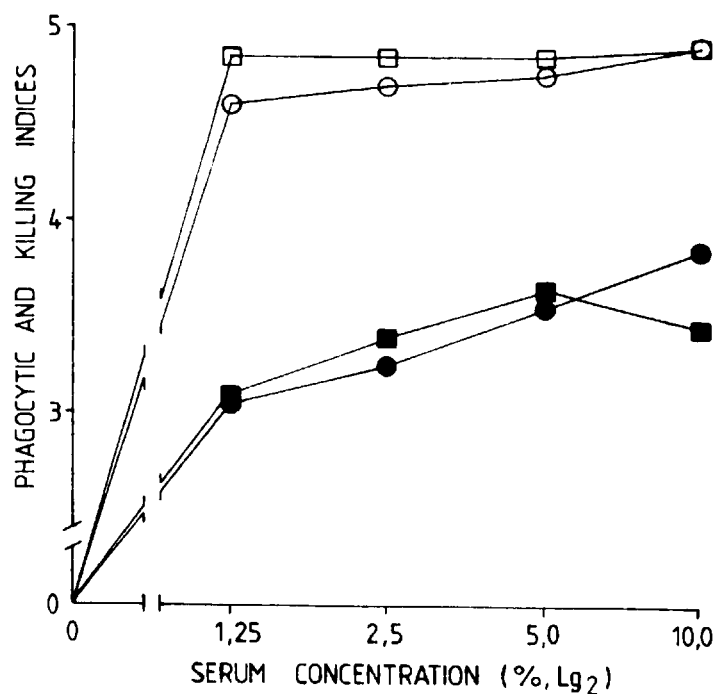


FIGURE 4.3. Effect of heat-inactivated serum on phagocytosis and killing of *C.albicans* by MNPs. Standard assay conditions.

SQUARE symbols represent normal, fresh serum. ROUND symbols represent serum which has been heat-inactivated (56°C/40 mins). Serum was obtained from same healthy donor.

OPEN symbols = phagocytosis, CLOSED symbols = killing.

4-4. Effect of heat-labile serum components on PMN oxygen consumption.

An increase in oxygen consumption is the cardinal feature of the metabolic changes in PMNs associated with microbial killing (see Chapter 1, Section B).

Table 4.1 shows that there was a significant reduction in oxygen consumption by PMNs subjected to C.albicans opsonised in heat-inactivated serum when compared with normal fresh serum. It can be seen that failure to provoke oxygen uptake in the presence of heated serum was not due to poor ingestion (Table 4.2.). Also, adequate oxygen consumption in the presence of serum-treated, washed organisms proves that the blastospores were coated with a serum factor, or factors, which promoted O₂ uptake by PMNs and that soluble substances were not involved.

Heated serum with high antibody titres to C.albicans failed to stimulate oxygen consumption or phagocytic killing by PMNs. This implies that antibody does not participate in the metabolic stimulation of PMNs which leads to the death of C.albicans.

There was no lag between addition of Candida and uptake of oxygen by PMNs in any of the sera tested which indicated that organisms were optimally opsonised (Nakamura et al., 1981). This was also confirmed microscopically.

TABLE 4.1. The influence of heat-labile serum components on oxygen consumption by PMNs stimulated with *C.albicans*.

RUN	Basal Rate	Normal human serum (NHS)	Normal human serum (56°C/40 mins)	NHS High titre ab.	Normal human serum. Washed <u>Candida</u>
1	0	58.80	2.20	11.10	60.20
2	0	73.50	2.40	nd	71.30
3	0	73.70	0	3.0	73.40

Results expressed as $\text{nM O}_2 / 2 \times 10^7$ PMNs/minute.

Oxygen consumption was measured using a Clark-type oxygen electrode in a water-jacketed vessel at 37°C. Mixing was accomplished by an integral magnetic stirrer. Opsonised Candida were added to PMN suspensions to give a final organism/phagocyte ratio of 5:1. The volume of the mixtures was 1.2 ml. Candida blastospores were opsonised in appropriate volumes of 5.0% serum at 37°C/15 mins before concentrating by centrifugation.

The human serum with high antibody titres to C.albicans was the gift of Dr DWR Mackenzie, LSHTM, London WC1E 7HT. This serum had an agglutinating titre of 1/32 according to the method of Warnock et al., 1979. It was heat-inactivated to avoid heat labile serum component-mediated stimulation of O_2 consumption or phagocytosis and killing.

Opsonised washed organisms were prepared as above but were washed three times in medium after serum treatment.

TABLE 4.2. Phagocytosis and killing of *C.albicans* by PMNs using the same sera employed in the experiments presented in Table 4.1.

Serum (5.0%)	PI	KI	KE (%)
NHS	4.40	3.0	68
NHS (56°C/40 mins)	2.30	0.55	24
NHS (high ab. titre)	3.50	0.50	14

4-5. Effect of C3 on intracellular killing by PMNs.

The previous results are consistent with a complement dependent candidacidal activity of PMNs. Conversely, it would appear that monocytes are unaffected by the absence of heat-labile serum factors (Figure 4.3.). To examine the nature of heat-labile serum dependent killing various complement deficient sera were examined for their ability to support phagocytosis and killing of C.albicans by PMNs.

Table 4.3. shows that although C3 is not essential for phagocytosis of C.albicans it is required for intracellular killing of this organism by PMNs at low serum concentrations in vitro. There was no such requirement for early classical pathway activity nor was activation of C5-C9 needed. In another experiment it was shown that purified C3 could replace the extrinsic phagocytic activity of C3 deficient serum (Table 4.4.).

TABLE 4.3. Effect of various complement deficiencies on the phagocytic killing of C.albicans by PMNs in vitro.

Serum (5.0%)	PI	KI	KE (%)
C2-deficient human serum	3.45	2.75	80
C3-deficient human serum	3.55	0	0
C5-deficient mouse serum	3.20	3.15	98
Heat-inactivated (56°C/40 mins) human serum	3.60	0	0
Normal human serum	4.65	3.10	67

TABLE 4.4. Restoration by purified C3 of intracellular killing in C3 deficient human serum.

Serum (5.0%)	PI	KI	KE (%)
Normal human serum	3.85	2.50	65
C3 deficient human serum	2.65	0.40	15
C3 deficient serum + purified C3 (40.0 μ g/ml)	3.0	1.65	55

4-6. Effect of C3 on iodination by PMNs.

Table 4.5. illustrates three experiments which confirm that there is a concomitant reduction in iodination of C.albicans by PMNs in C3 deficient serum. Also demonstrated is the dependence for C3 in PMN killing of C.parapsilosis, again accompanied by poor microbial iodination. The reduction in iodination which took place in C3 deficient serum could not be fully accounted for by diminished ingestion.

4-7. Monocytes kill C.albicans in C3 deficient serum.

The ability of monocytes to phagocytose and kill C.albicans in heat-inactivated serum is explained by their independence of humoral C3 (Table 4.6.). These results however do not rule out the possibility that monocytes utilise their own resources of this molecule when it is not available in serum (Brade et al., 1977; Whaley, 1980).

TABLE 4.5. Phagocytosis, killing, and iodination of C.albicans and C.parapsilosis by PMNs in C3 deficient human serum.

	<u>C.albicans</u>			<u>C.parapsilosis</u>		
	EXPT No.			EXPT No.		
	1	2	3	1	2	3
C3 DEFICIENT SERUM (5%) NORMAL SERUM (5%)	PI	2.60	2.75	2.50	2.30	2.75
	KI	1.80	2.05	2.10	2.35	2.80
	KE (%)	69	75	84	100	100
	Iodination (cpm)	19750	18300	17750	22400	20400
	PI	1.70	2.95	2.70	1.75	3.65
	KI	0	0.45	0.70	0	0.35
	KE (%)	0	15	26	0	9.5
	Iodination (cpm)	8000	4500	6800	11000	7600

TABLE 4.6. Phagocytosis and killing of C.albicans by MNPs in C3 deficient human serum.

Serum	PI	KI	KE (%)
Normal human serum (5.0%)	3.05	2.0	65
Normal human serum (2.5%)	3.25	3.05	94
C3 deficient human serum (5.0%)	3.30	3.15	95
C3 deficient human serum (2.5%)	3.10	3.30	100

4-8. Effect of trypan blue and heparin on phagocytosis and killing by PMNs.

The dye trypan blue has been shown to inhibit attachment of PMNs to C3 coated sepharose beads and uptake of complement opsonised Staph.aureus (Guckian, Christensen, and Fine,1978). This activity was restricted to the C3 receptor and did not affect the Fc receptor. Moreover, it was shown that C3 receptor bearing cells other than PMNs could be similarly antagonised (Harper, Fine, Guckian, and Cavallo,1981).

The presence of trypan blue in the phagocytosis and killing assay should therefore be reflected by it's action on the PMN C3 receptor. Figure 4.4. demonstrates that trypan blue inhibits Candida killing by PMNs in a concentration dependent manner. The results are presented as values of KE since there was also a deleterious effect on ingestion.

Heparin modulates the alternative pathway C3 convertase by preventing it's formation (Weiler et al.,1978) or causing breakdown by enhancement of β 1H and C3INA activity (Kazatchkine et al.,1979b). It also has inhibitory activity with respect to the classical pathway (Raepple, Hill, and Loos,1976; Loos, Volanakis, and Stroud,1976a and b). It's anticomplementary activity is demonstrated by the absence of C3 breakdown in the presence of C.albicans and heparin but not C.albicans alone (Figure 4.5.). The addition of heparin to normal serum in the phagocytosis and killing assay should therefore make the serum behave as if it was C3 deficient.

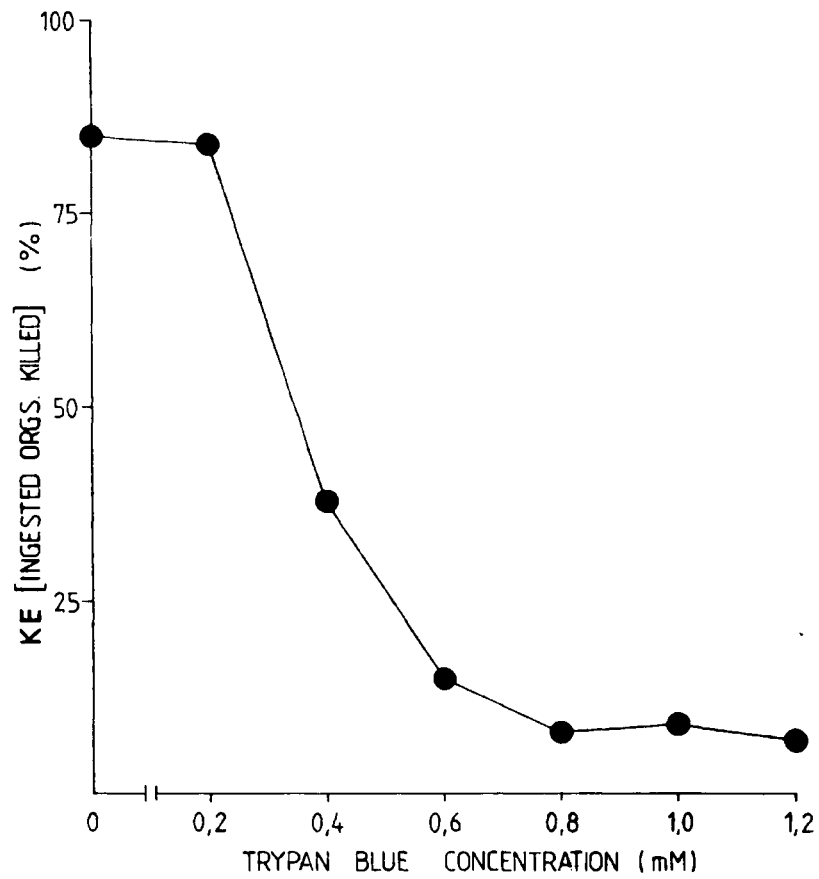


FIGURE 4.4. Effect of trypan blue on phagocytic killing of C.albicans by PMNs.

Standard assay conditions.

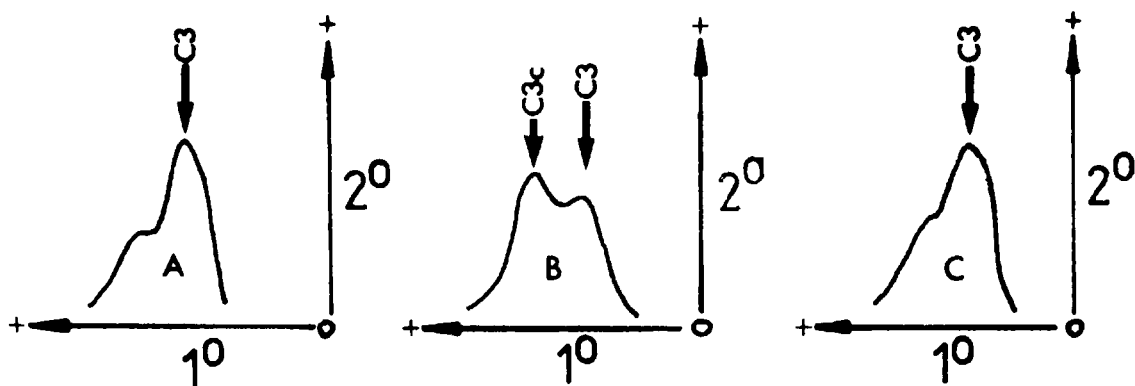


FIGURE 4.5. Effect of heparin on complement activation.

Tracings of two dimensional immunoelectrophoresis patterns showing the absence of C3 conversion by C.albicans in the presence of 200 U heparin (tracing A). Tracing B shows a normal pattern of C3 breakdown in the presence of Candida. Tracing C is the control which shows that C3 remains unconverted if Candida is absent.

Conversion was allowed to proceed for 20 mins/37°C in 1.0 ml volumes of medium containing 20.0% normal serum with 2×10^6 blastospores and 200 U/ml heparin. Control mixtures without heparin or without C.albicans were also prepared. The reaction was stopped by the addition of EDTA (final concn. 1 mM). Samples were electrophoresed in the first dimension (1°) in 1.20% agarose containing 20 mM barbitone buffer pH 8.6 for 60 mins 10V/cm. The gels were then cut and electrophoresed in the second dimension (2°) against 1.2% agarose 20 mM barbitone buffer containing antibody to human C3 (Seward)(20 µg/ml gel) for 2 hours 6-8V/cm.

The plates were then dried and stained with Coomassie Brilliant Blue (0.5% w/v in 10% glacial acetic acid-45% ethanol soln.). Destaining was carried out in acid-ethanol soln. without dye.

Figure 4.6. shows that heparin has a dose dependent effect on phagocytic killing of C.albicans by PMNs.

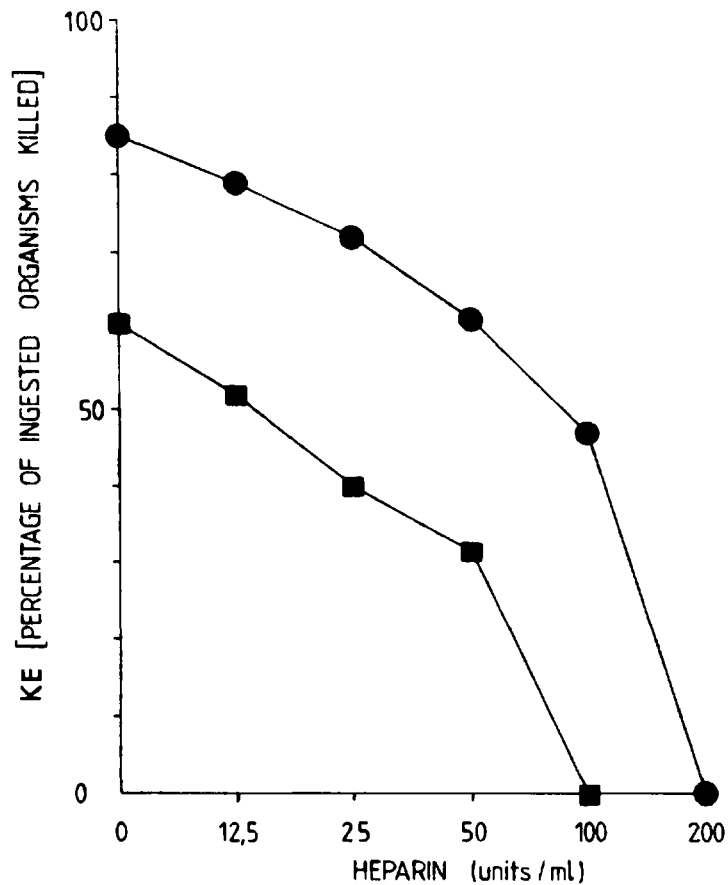


FIGURE 4.6. Effect of heparin on phagocytic killing of Candida albicans by PMNs. Standard assay conditions.

The failure of Saccharomyces cerevisiae to be opsonised by C3 in the presence of heparin is shown in Figure 4.7. which, as a comparison, includes phagocytosis and killing of Candida albicans at the same heparin concentration. The normal opsonic activity of serum for S.cerevisiae is similar to that of C.albicans (Figure 4.8.). Also included in the latter figure is the serum of a healthy individual who exhibits an opsonin defect at serum concentrations lower than 20.0%.

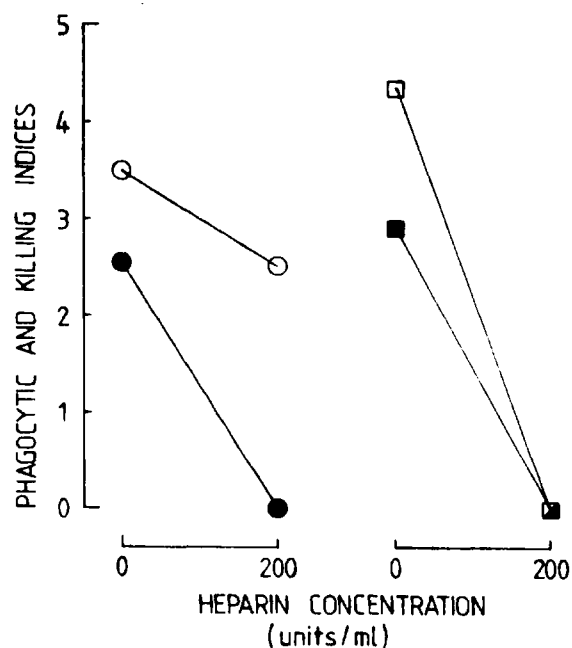


FIGURE 4.7. Effect of heparin on the opsonisation of Saccharomyces cerevisiae. Standard assay conditions. OPEN symbols represent phagocytosis, CLOSED symbols represent killing. ROUND symbols = Candida albicans, SQUARE symbols = S.cerevisiae.

The action of heparin on monocyte phagocytosis and killing of C.albicans is shown in Table 4.7. It can be seen that heparin does not affect the candidacidal capacity of MNPs in either normal or heat-inactivated serum.

TABLE 4.7. Phagocytosis and killing of C.albicans by MNPs in the presence of heparin.

Serum (2.50%)	PI	KI	KE (%)
Normal human serum (NHS)	4.40	3.10	70
NHS + heparin (200 U/ml)	3.50	3.35	96
Human serum (56°C/40 mins)	3.50	3.0	86
Human serum (56°C/40 mins) + heparin (200 U/ml)	3.70	3.05	82

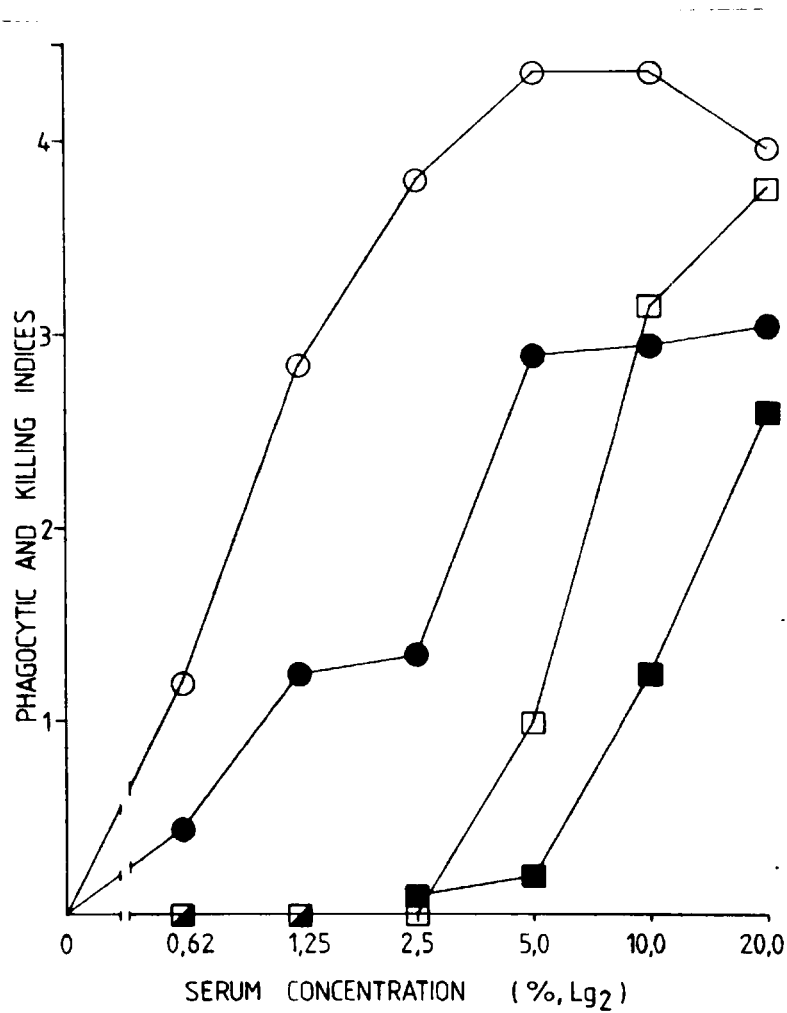


FIGURE 4.8. Effect of serum concentration on phagocytosis and killing of *S.cerevisiae* by PMNs.

OPEN symbols represent phagocytosis, CLOSED symbols represent killing. ROUND symbols represent serum from a healthy donor, SQUARE symbols represent serum from a healthy donor with an antibody-independent opsonin defect.

Standard assay conditions as for phagocytosis and killing of *C.albicans* by PMNs.

4-9. Do CGD PMNs lack the C3 receptor?

All of the previous results are consistent with a requirement for C3 in the phagocytic killing of C.albicans by PMNs. The question then arises as to whether CGD PMNs lack a C3b receptor which can transmit the stimulatory signal given by C3 coated particles. To test this idea PMNs from an individual with CGD were analysed for their ability to ingest S.cerevisiae in serum concentrations which do not provide antibody for opsonisation (Yamamura and Valdimarsson, 1978). The results, which are shown in Table 4.8., indicate that this individual does possess C3 receptors since Saccharomyces has been ingested as well as C.albicans or C.parapsilosis.

TABLE 4.8. Phagocytosis and killing of C.albicans, C.parapsilosis, and S.cerevisiae by CGD PMNs.

CGD PMNs (J.V.)

ORGANISM	PI	KI	KE (%)
<u>C.albicans</u>	3.30	0.35	11
<u>C.parapsilosis</u>	2.65	0.50	19
<u>S.cerevisiae</u>	3.0	0.65	22

CONTROL PMNs

<u>C.albicans</u>	3.75	2.50	67
<u>C.parapsilosis</u>	2.95	2.70	91
<u>S.cerevisiae</u>	3.90	2.85	73

4-10. Discussion.

Antibody and complement act in concert to promote ingestion of particles by PMNs (Mantovani, 1975; Scribner and Fahrney, 1976). In this aspect of phagocytic activity it is believed that the C3b receptor is primarily involved with recognition and attachment of particles whilst the Fc receptor is responsible for ingestion. Division of labour between immunoglobulin and complement is also extended to metabolic stimulation of PMNs since C3 coated sepharose beads can cause superoxide anion production in the absence of IgG (Goldstein, Roos, Kaplan, and Weissmann, 1975; Goldstein, Kaplan, Radin, and Frosch, 1976; Tedesco, Trani, Soranzo, and Patriarca, 1975). However, IgG, if present on the bead surface, can increase the superoxide response to C3 as well as initiate degranulation. It was also shown in these studies that $F(ab')_2$ anti C3 abolishes superoxide anion generation by PMNs in response to sepharose-C3 whereas $F(ab')_2$ anti-IgG reduced but did not abolish the oxidative response to sepharose-C3-IgG. Stimulation is unrelated to ingestion in this system since the sepharose beads were far larger than the PMNs. However, it is not known if immunoglobulin by itself can stimulate superoxide production since beads treated with heat-inactivated serum or normal serum in the presence of EDTA did not attach to PMNs.

There have been reports that aggregated IgG, when attached to non-phagocytosable surfaces, can induce PMNs to increase their oxidative metabolism (Henson and Oades, 1975; Johnston and

Lehmeyer, 1976; Kiyotaki, Shimizu, Watanabe, and Yamamura, 1978). All of the latter studies have the disadvantages that it is difficult to ascertain 1) the effect of immunoglobulin or C3 density on the particle surface, 2) receptor accessibility to immunoglobulin or C3 coated onto particles, and 3) the particle surface itself when in apposition to the phagocyte membrane with immunoglobulin or C3.

The results presented in this chapter give further credence to the dual nature of extrinsic phagocytic activity. In regard to this, the term 'procidin' has been proposed by Valdimarsson and Yamamura (1979) for humoral substances which render micro-organisms susceptible to the action of intracellular killing mechanisms. Such activity should be clearly distinguished from opsonisation even if a single substance displays both. Specific antibodies have only opsonic activity and do not act as procidins for PMN killing of either C.albicans or C.parapsilosis. On the other hand C3 has both opsonin and procidin activity towards both of these organisms.

Assigning a dual role for C3 in opsonisation and procidination of S.cerevisiae is not feasible because immunoglobulin, at the serum concentrations used in the assay, does not figure significantly in the PMN phagocytic response to this organism. The absence of C3 necessarily results in the abolition of ingestion and therefore determining the direct importance of C3 in phagocytic killing of S.cerevisiae is impossible.

In contrast, the presence of Ig and absence of C3 in engulfment and killing of C.albicans or C.parapsilosis has a twofold effect.

First, there was a reduction in phagocytosis indicating opsonin activity for C3. Second, there was total abrogation of intracellular killing confirming absolute dependence on C3 for its function as a procoagulant.

Several studies have demonstrated that the oxidative system of PMNs is associated with the plasma membrane. Experimental approaches to this subject have included morphometric work (Nathan, Baehner, and Weaver, 1969; Briggs, Drath, Karnovsky, and Karnovsky, 1975; Briggs, Karnovsky, and Karnovsky, 1977), cell fractionation (Segal and Peters, 1977; Segal and Jones, 1979a), and use of surface acting agents (Goldstein et al., 1977; Takanaka and O'Brien, 1975). Further evidence for an ectoenzyme system can be derived from phagocyte-parasite interactions (Mackenzie et al., 1981) and lectins (Romeo, Zabucchi, and Rossi, 1973).

It therefore seems likely that C3 coated onto the microbe surface initiates oxidative metabolism by interacting with the PMN C3b receptor. This is supported by the observation that there was insignificant oxygen consumption by PMNs when stimulated by C.albicans in the presence of heated serum. What is more it was found that trypan blue, which interferes with C3 receptors, also inhibited phagocytic killing. Heparin, which prevents C3 conversion, had a similar effect.

The ability of some organisms to provoke oxygen consumption when coated only with antibody might be related to the points raised earlier concerning ligand surface density or accessibility. Another possibility is that these organisms possess surface molecules which mimic C3b (Fine et al., 1980).

The biological advantage attending a microorganism with surface C3b-like molecules is difficult to envisage unless these structures interfere with true complement activation or, alternatively, are used to gain entry into a cell for the purpose of establishing a parasitic existence (Jack and Ward, 1980). Conversely, the power of other organisms to escape intracellular destruction could be related to a failure of the humoral immune system. A possible example is virulent Legionella pneumophila which evades intracellular destruction and has very low complement fixing activity (Horwitz and Silverstein, 1981).

Monocytes appear to be totally independent of serum derived C3 for phagocytic killing of C.albicans. Therefore, they either use endogenous sources of this molecule or employ different methods to initiate killing processes. It was interesting to note that they were incapable of killing efficiently in high concentrations of normal but not heat inactivated serum. A possible interpretation is that monocyte derived C3 is subject to inactivation in high concentrations of fresh serum but not serum which has been heated.

Other workers have found that heat labile serum factors raise the microbicidal capacity of monocytes (Leijh et al., 1979). This activity was largely dependent on the ability of the test organism to produce catalase (Leijh, van Zwet, and van Furth, 1980). It was found that catalase negative streptococci were killed in the absence of serum much more effectively than catalase positive streptococci or E.coli. Moreover,

the presence of serum increased the ability of monocytes to kill catalase positive microbes but did not enhance their capacity to kill catalase negative organisms. This was because efficient killing of catalase negative organisms was already taking place in serum free conditions. Taken together these results imply that catalase negative organisms commit suicide by providing the phagocyte with H_2O_2 which can partake in peroxide dependent killing. On the other hand, catalase positive organisms do not release H_2O_2 and intracellular killing therefore relies upon stimulation of oxidative MNP metabolism by heat labile serum factors.

Although the findings of Leijh and his associates do not agree with the results presented here contrary lines of evidence suggest that serum is not involved in monocyte killing of C.albicans (Diamond and Haudenschield, 1981). In the latter study a radiometric technique to measure the metabolic activity of hyphae was used to examine MNP-mediated damage to hyphae and pseudohyphae. It was found that monocytes could damage C.albicans in the absence of serum unlike PMNs which did require serum for optimal hyphal damage (Diamond, Krzesicki, and Jao, 1978). Similarly, macrophage cytotoxicity towards tumour cells has been shown to be independent of serum (Keller, 1979). These discrepancies of monocyte/macrophage function are probably related to the wide spectrum of MNP differentiation or the experimental approaches adopted by different workers.

Trypan blue has been reported to delay rejection of skin grafts

(Brent and Medawar,1961;Kripke, Norbury, Gruys, and Hibbs, 1977) and syngeneic tumours (Kripke et al.,1977). It also suppresses cell mediated cytotoxicity towards tumour cells and appears to prevent antibody binding to target cells (Scornik, Ruiz, and Hoffman,1979).

The results presented here suggest that the immunosuppressive properties of trypan blue are related to antagonism of the C3 receptor. Although trypan blue activity was restricted to analysis in the PMN phagocytosis and killing assay, this conclusion is consistent with other reports which infer that mononuclear cell cytotoxicity is related to endogenously produced complement components (Sundsmo and Müller-Eberhard, 1979; Simone and Henkart,1980).

Heparin, in addition to anticomplement and anticoagulant activity, has been noted to interfere with chemotactic factors in vivo (Berghem et al.,1978) and PMN cationic protein function in vitro (Clark and Higginbotham,1970). With respect to the last study there is no direct evidence for the antimicrobial action of human PMN cationic proteins in vivo. The significance of in vitro microbicidal activity is clouded because the antibacterial properties of these proteins are independent of alleged enzymic function (Lehrer, Mitchell, and Hake,1973; Odeberg and Olsson,1975). Nevertheless, cationic proteins have been alluded to in connection with oxygen-independent (Lehrer and Cline,1969a;Lehrer, Hanifin, and Cline,1969; Mandell,1974) and oxygen-dependent killing mechanisms (Segal et al.,1981). The present studies do not discount the possibility that heparin interferes with cationic proteins in vivo.

However, the overwhelming effect on PMN candidacidal function concomitant with absence of significant complement fixation are together strongly suggestive of the two being closely linked.

An examination of PMNs from a patient suffering from CGD for the presence of C3 receptors was prompted by the data shown here and two sets of observations. First, the findings of Segal and others that the spectrum of a membrane bound b-type cytochrome was missing in PMNs from individuals suffering from X-linked CGD (Segal and Jones, 1978; Segal, Jones, Webster, and Allison, 1978; Segal and Jones, 1979b). Second, the report that the Kell phenotype Ko was strongly associated with CGD (Giblett et al., 1971; Marsh, Uretsky, and Douglas, 1975; Marsh, Øyen, and Nichols, 1976). The Ko phenotype is the result of an absent gene product called Kx which is normally present on PMNs, monocytes, and red cells. The Ko phenotype is found in boys with the X-linked form of the disease but not in the autosomal form of girls. The exciting notion that the C3 receptor, cytochrome b, and Kell antigen were all one and the same was further reinforced by the observation of C3 receptors on erythrocytes (Fine et al., 1980).

The results presented here do not confirm that theory. Nevertheless, since this particular patient lacked cytochrome b activity it is still possible that in normal cells the cytochrome is antigenically represented as Kx and that the C3 receptor is a separate entity. An alternative interpretation is that cytochrome is present in the membrane but is unable to produce a characteristic spectrum in redox conditions due

to a missing prosthetic group. If this is correct then it may be inferred that the cytochrome is synonymous with a receptor which is still capable of binding C3 coated particles but with no effect in certain forms of CGD. In any event it would be worth investigating a larger group of patients.

A second mechanism, independent of oxygen, for phagocytic killing of C.parapsilosis (Lehrer,1972; Lehrer,1975; Lehrer, Ladra, and Hake,1975) was not confirmed in the radiometric phagocytosis and killing assay because CGD PMNs were unable to kill this organism efficiently. It must be remembered that the techniques adopted by Lehrer and his associates were not optimal in terms of serum concentration or organism/phagocyte ratio. Moreover, since the Giemsa staining method measures digestion of ribonucleoprotein (Lehrer,1970) it is probable that lysosomal enzyme degradation is reflected in the results. If the radiometric assay was used with an extended incubation time it might give results similar to those of Lehrer. A method specifically for measuring phagocytic digestion may also be useful in this regard.

CHAPTER 5CLINICAL APPLICATION OF THE PHAGOCYTOSIS AND KILLING ASSAY.

5-1. Introduction.

The majority of patients with abnormal infections and suspected immunodeficiency do not have a classifiable immune deficiency disorder. Nevertheless, it is likely that many of these individuals have some primary immunological irregularity. However, their conditions remain undefined because the available laboratory tests do not detect their disorder or, alternatively, are insensitive or fail to mirror in vivo states. Increased susceptibility to infection may also result from an accumulation of borderline defects affecting two or more components of the immune system. Unfortunately, it is not yet possible to analyse, in a meaningful way, the overall effect of several defects on total bodily defence.

This chapter is partially devoted to preliminary clinical application of the combined phagocytosis and killing assay. The effect of various antibiotics on in vitro phagocytic activity is also presented.

It should be pointed out that routine tests of immunological function were not performed by the author. These tests were carried out by members of the Clinical Immunology Dept., SMHMS, London W2 with the exception of isohaemagglutinins and immunoglobulins which were determined by staff of the Blood Transfusion Dept., SMHMS, and Chemical Pathology, SMH, London W9.

5-2. Materials and methods.

Intrinsic phagocytic activity was examined using non-limiting conditions in regard to organism/phagocyte ratio (5:1) and serum concentration (5.0%). Patients' cells were tested in both control and autologous plasma or serum. Internal control mixtures with healthy donor phagocytes in standard control plasma or serum were also prepared.

The procidin activity of patients' serum was tested against healthy control cells using a C.albicans/PMN ratio of 5:1 and serum concentrations of 2.5% and 5.0%. Internal control mixtures containing healthy donor phagocytes and sera from standard control donors were also prepared. The lower serum concentration (2.5%) was used because phagocytic activity is linearly related to serum concentrations below 5.0% (see page 77). The normal ranges for extrinsic phagocytosis and killing are given in Table 5.1.

TABLE 5.1. Normal range for extrinsic phagocytosis and killing of C.albicans by PMNs.

Serum concentration	PI	KI	KE (%)
5.0%	3.10-4.90	2.20-3.90	76
2.5%	2.80-4.60	2.0-3.20	70

n=32. Standard organism/phagocyte ratio of 5:1.

Antibody independent opsonisation of S.cerevisiae was measured with the radiometric assay devised by Yamamura and Valdimarsson (1978). An organism/phagocyte ratio of 10:1 and serum concentrations of 1.25 and 2.50 per cent were used in the standard assay. Patient's serum was tested against healthy donor PMNs. Internal control mixtures contained serum from standard control subjects.

Other studies included T lymphocyte enumeration by E-rosettes; B lymphocyte enumeration by membrane fluorescence for surface immunoglobulins; in vitro lymphocyte transformation with PHA, PWM, Con A, PPD, Herpes antigen; skin tests for delayed hypersensitivity; IgE levels; serum immunoglobulins; isohaemagglutinins; viral antibodies; complement profile (C1q, C4, C3, and CH₅₀); PMN chemotaxis; PMN reduction of NBT.

Some patients had all of the above tests whilst others had selected tests of immune status.

390 patients were referred to the Consultant Clinical Immunologist, SMHMS, because they had a history of infections which were unusually frequent, persistent, severe, or complicated. The majority were referred by dermatologists, paediatricians, chest physicians, or allergists. When feasible patients with abnormal test results were investigated again in remission and significant defects only recorded if present when the individual was free of overt disease.

The effect of antibiotics on normal phagocytic activity was analysed using a 5:1 C.albicans/PMN ratio and normal serum concentration of 5.0%. The antibiotics were added at the start of incubation or preincubated with phagocytes for 60 minutes prior to addition of serum and blastospores.

Most of the antibiotics were obtained as commercially available injectable preparations. Gramicidin base was the gift of E.R.Squibb and Sons Ltd., erythromycin was obtained as a base from Abbot Laboratories, and ketoconazole base was the gift of Janssen Pharmaceuticals. Care was taken to avoid any toxic preservatives or pH changes in the assay.

5-3. Clinical Results.

Figure 5.1. shows the percentage of defects found in the 390 patients suffering from abnormal infections. The majority of defects were associated with reduced phagocytic activity (44%), only 7.0% had abnormal lymphocyte function and 3.0% a classifiable immune defect (Table 5.2.). Approximately fifty per cent of the patients had no discernible defect.

TABLE 5.2. Classifiable immune deficiency diseases observed in patients with abnormal infections.

DISEASE	Number of patients
Hypogammaglobulinaemia	5
Wiskott-Aldrich syndrome	3
Ataxia telangiectasia	1
Chronic granulomatous disease	2
Lazy leucocyte syndrome	<u>1</u>
	12

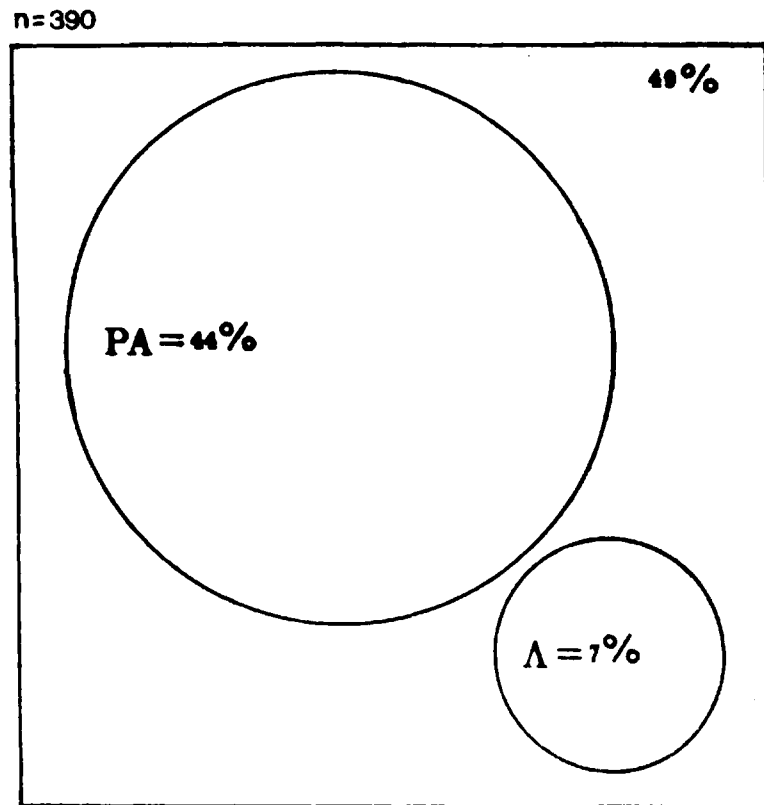


FIGURE 5.1. Venn-Euler diagram showing distribution of defects found in patients suffering from abnormal infections.
PA = phagocytic activity defects, Λ = defects of lymphocyte function. $n = 390$.

Table 5.3. demonstrates the prevalence of various disorders of phagocytic activity as defined by the radiometric tests. Twenty-two per cent of the patients tested had low intrinsic killing. No defects of intrinsic ingestion were discovered. However, abnormal humoral phagocytic activity was relatively common since 31% of the patients tested had low procidin activity and 42% reduced antibody-independent opsonisation. Nearly 25% of the patients tested for antibody-independent opsonisation and procidins had both defects (Figure 5.2.), whilst only 11% had abnormalities of both intrinsic killing and antibody-independent opsonisation. There was also a high association between procidin and intrinsic killing defects; 20% of those tested had both defects (Figure 5.2.).

TABLE 5.3. Various defects of phagocytic activity in patients with abnormal infections.

Defect of phagocytic activity	Number positive/ total number tested	(%)
Intrinsic ingestion	0/112	0
Intrinsic killing	25/112	22
Procidins	18/58	31
Antibody-independent opsonins	129/310	42

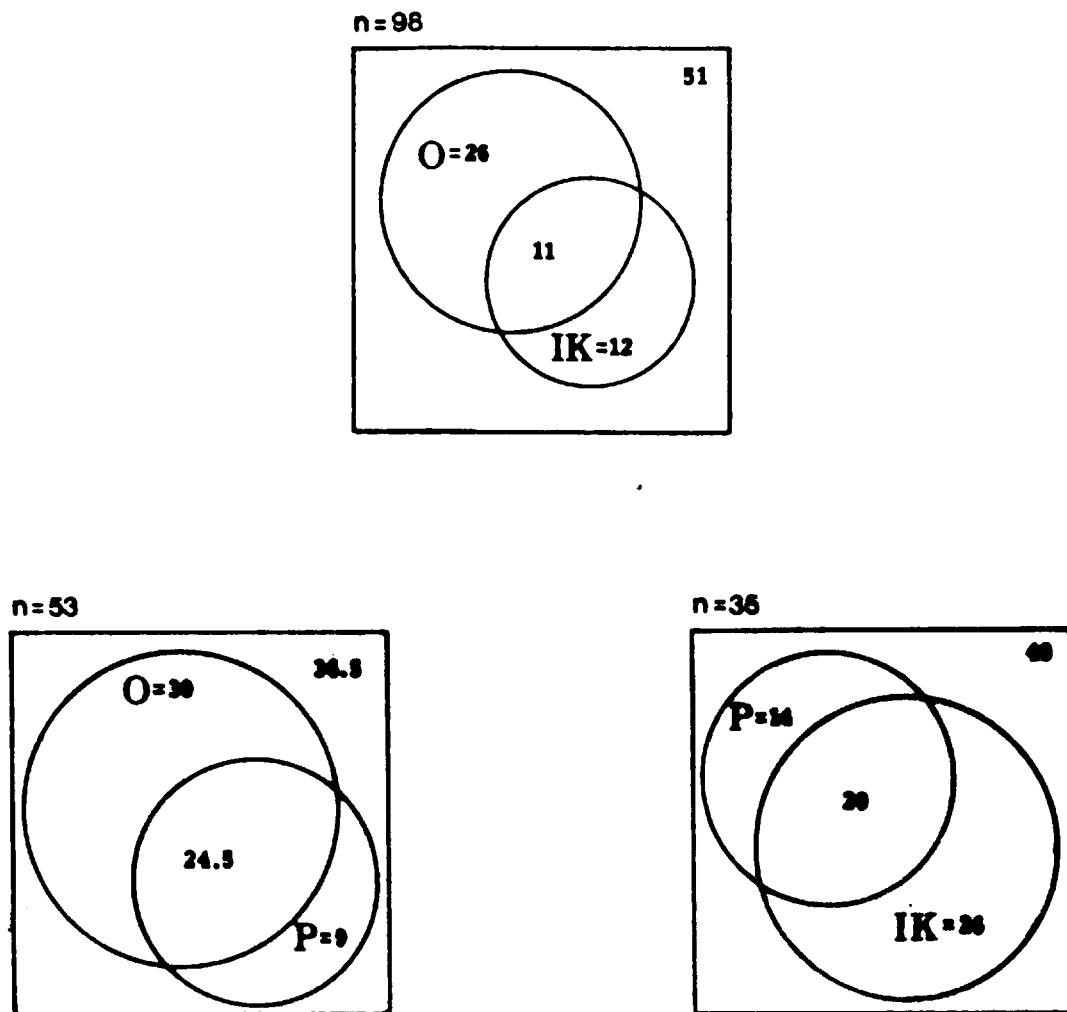


FIGURE 5.2. Venn-Euler diagrams showing various defects of phagocytic activity in patients suffering from abnormal infections. Results expressed as a percentage.
 O = antibody-independent opsonins, P = procidins, IK = intrinsic killing. See text for details.

5-4. Effect of antibiotics on phagocyte function.

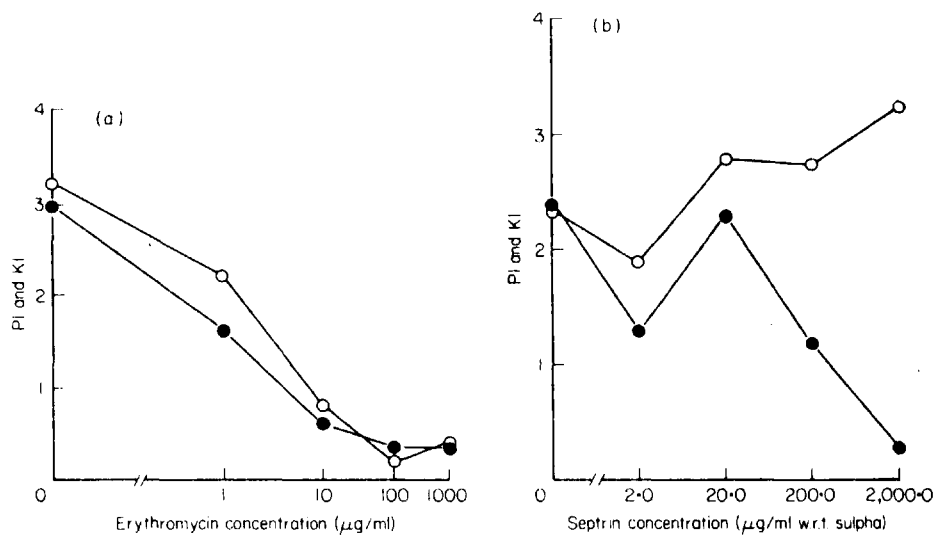
The analytical potential of the radiometric phagocytosis and killing assay is particularly well demonstrated by the results presented in Figure 5.3. Erythromycin showed a marked inhibition of phagocytosis and consequent reduction in killing at 10 µg/ml. Septrin however did not inhibit phagocytosis of C.albicans at concentrations which completely abolished phagocytic killing of this organism. Septrin-induced microbial survival was associated with low PMN mediated iodination of Candida (inset Fig.5.3.). The antifungal drug ketoconazole was able to improve phagocytic killing of C.albicans if incubated with fungal blastospores for 2½ hours prior to addition of phagocytes (Table 5.4.).

The antibiotics which had no deleterious effect upon PMN phagocytic activity included penicillin, gramicidin, gentamicin, tetracycline, and metronidazole. Figure 5.4. shows the effect of penicillin or gramicidin on phagocytosis and killing of C.albicans by PMNs.

TABLE 5.4. The effect of ketoconazole on phagocytosis and killing of C.albicans blastospores which have been pre-incubated in 5.0% serum.

Ketoconazole (final concn. µg/ml)	PI	KI	KE (%)
0.10	2.35	2.35	100
0.01	2.35	1.35	57
0.001	1.85	1.40	76
-	2.20	1.65	75

Blastospores were preincubated for 2.5 hours.



	PI	KI	Iodination (cpm)	NBT reduction (% +ve cells)
SEPTRIN (2000 $\mu\text{g/ml}$ wrt sulpha.)	4.15	0.30	1780	90
CONTROL	3.0	2.0	20500	88

FIGURE 5.3. Experiments showing the effect of erythromycin or co-trimoxazole (Septrin) on phagocytosis and killing of *C. albicans* by PMNs.

Standard assay procedure. OPEN symbols represent phagocytosis, CLOSED symbols represent killing. Control mixtures (without PMNs) contained the same amount of antibiotic as test mixtures (with PMNs). INSET shows the effect of Septrin on iodination of Candida by PMNs as well as nitroblue tetrazolium reduction.

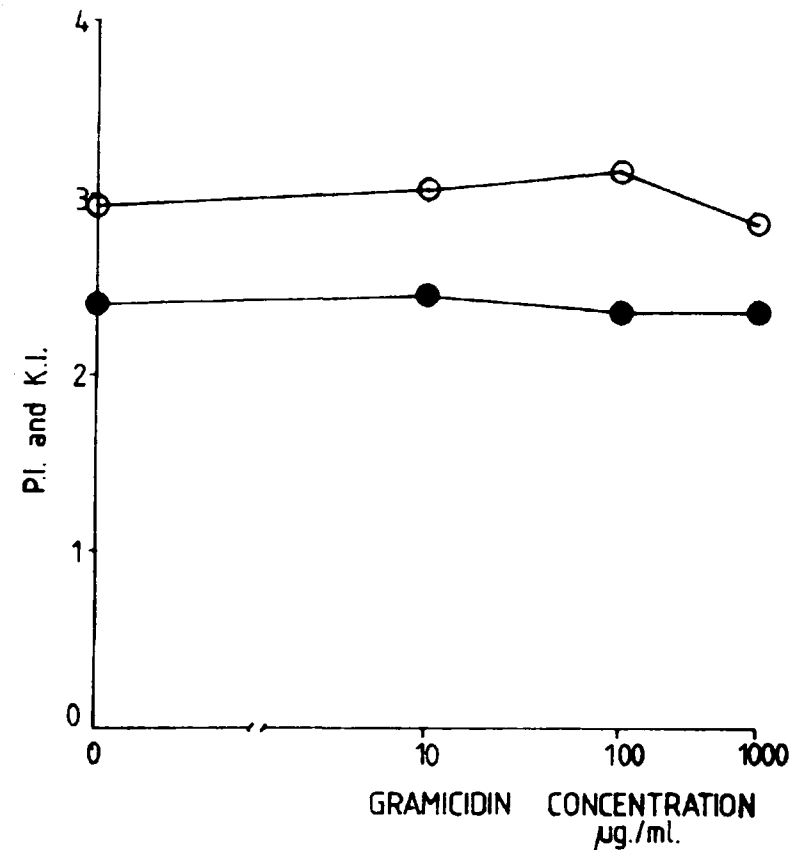
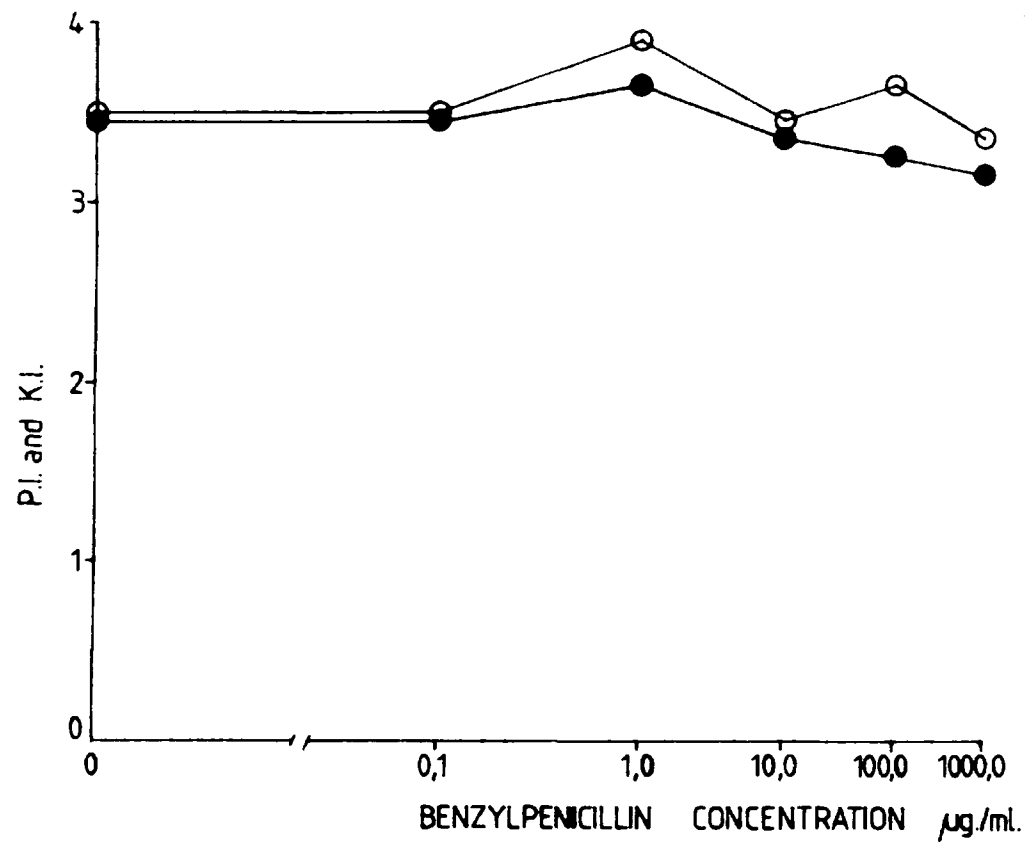


FIGURE 5.4. Experiments showing the effect of benzyl penicillin or gramicidin on phagocytosis and killing of *C. albicans* by PMNs. Standard assay procedure. OPEN symbols represent phagocytosis, CLOSED symbols represent killing. Control mixtures (without PMNs) contained the same amount of antibiotic as test mixtures (with PMNs).

5-5. Discussion.

Results from the clinical studies highlight the preponderance of defects of non-specific immunity over those of specific immunity. However, approximately half the patients examined in this series had no abnormality uncovered by these tests. As expected there was a relatively high association between low antibody-independent opsonisation of S.cerevisiae and procidination of C.albicans since both depend upon C3 activation. It is noteworthy that none of these patients had abnormal complement activity as defined by other criteria. There was also a high number of patients with defects of both intrinsic killing and procidination. Relatively few patients had both intrinsic killing defects and low antibody-independent opsonisation indicating that such abnormalities are not causatively related. Although an effort was made to test patients' phagocytic activity when they were free of infection, it is impossible at this early stage to determine the number of individuals with primary defects. Nonetheless, it is believed that assessment of phagocytic activity is clinically valuable regardless of whether primary or secondary abnormalities are being detected. Corrective measures, even when directed at a secondary defect, may break a vicious circle which is a common feature of persistent infections.

Antibiotics are administered with the aim of directly antagonising microbial growth with little thought to their effect, be it harmful or helpful, on the immune system. The in vitro effect on phagocytic activity of Septrin and Erythromycin, on

the one hand, and ketoconazole on the other, demonstrate both sides of the coin. Septrin (co-trimoxazole) is a combination of trimethoprim and sulphamethoxazole. Sulpha drugs have already been found to inhibit phagocytic killing (Stendahl et al., 1978; Lehrer, 1971). Dimethyl sulphoxide also inhibits phagocytic killing and it has been suggested that this is due to $\text{OH}^\bullet$ scavenging (Repine et al., 1981). Since all sulpha drugs with inhibitory activity possess a sulphoxide group it seems likely that an oxygen atom with a delocalised electron cloud is responsible for radical scavenging. PMNs were still capable of reducing NBT in the presence of Septrin although iodination of C.albicans was significantly diminished. This indicates that production of superoxide is unaffected whilst the $\text{MPO-halide-H}_2\text{O}_2$ system is disturbed.

Ketoconazole only showed a stabilising effect on phagocytic activity when preincubated with blastospores. Since this drug was found to inhibit germ tube formation in this and other studies it is possible that it prevents full expression of microbial virulence prior to phagocyte encounter. Similar work has shown that preincubation of antibiotics with microbes at sub-minimum inhibitory concentrations can enhance phagocytosis (Gammel et al., 1981) or killing (Root et al., 1981). It is therefore suggested that in some circumstances antibiotics make microorganisms prone to host immune mechanisms rather than directly antagonising their growth. It is possible that ketoconazole acts as a synthetic procidin in vivo either alone or in concert with a humoral component.

CHAPTER 6GENERAL DISCUSSION.

The radiometric combined assay for measurement of phagocytosis and killing makes it possible to determine whether defective phagocytic killing is due to impaired ingestion or reduced phagocyte microbicidal capacity. It can also be used to detect both cellular and humoral abnormalities of phagocytic activity in an objective and sensitive manner. The technique is in part based on an earlier method for measuring phagocytosis (Yamamura et al., 1977), but in addition quantitates intracellular killing by the failure of microorganisms to incorporate a radiolabel.

The combined assay has been calibrated for phagocytosis and killing by polymorphonuclear and mononuclear phagocytes in relation to fungal blastospores. However, different species of microorganism differ in their susceptibility to ingestion and killing. Furthermore, some microbes can decrease phagocytic activity towards unrelated species in mixed infections (Ingham et al., 1977). It would therefore be of benefit to assess phagocytic activity in relation to a panel of microbes using a simple assay. The original uridine incorporation method for evaluating phagocytosis has already been modified to measure the ingestion of Staph.aureus (Lam and Mathison, 1979) and it should be possible to adapt the combined assay principle for measuring phagocytic killing of bacteria. Indeed, further modifications have allowed such measurements to be made in relation to mycobacteria (GAW Rook, personal communication).

Simultaneous evaluation of phagocytosis and killing invests the assay with good analytical potential because the efficiency

of phagocyte microbicidal activity can be readily quantitated. This property of the assay is particularly useful when examining the effect of various drugs or serum components on phagocytic activity.

Wright and Douglas (1903) postulated that serum contained substances which acted directly on phagocytes to enhance their function. They coined the term 'stimulins' for such factors but were unable to prove their existence and concluded that 'the question as to whether the blood fluids contain, in addition to opsonins, also an element which directly stimulates the phagocytes, remains for the present unresolved'. The importance of humoral support in phagocytic killing was noted in the 1960's and early 1970's (Li and Mudd, 1965; Shayegani and Mudd, 1966; Miya and Marcus, 1961; Solberg and Hellum, 1973) but surprisingly little attention was given to this work. However, it was later shown that heat-labile serum factors in the form of C3 could directly enhance phagocytic killing (Valdimarsson and Yamamura, 1977; Leijh et al., 1979; Leijh, van Zwet, and van Furth, 1980). The specific term 'procidin' has been proposed for humoral substances which render microorganisms susceptible to the action of intracellular killing mechanisms (Yamamura and Valdimarsson, 1979). The term first used by Wright and Douglas, 'stimulin' could be used as a collective term for procidins, chemoattractants, and lymphokines since all act directly on phagocytes.

Using the new combined assay it was confirmed that C3 is not necessary for phagocytosis of C.albicans but is required for phagocytic killing of this organism by PMNs in vitro. It was

also demonstrated that C3 has procidin activity for phagocytic killing of C.parapsilosis by PMNs. In contrast, it was not possible to show that monocytes needed extrinsic C3 for intracellular killing of fungal blastospores.

In the absence of C3 PMNs were incapable of efficient blastospore iodination and, moreover, heat-inactivated serum failed to promote PMN oxygen consumption. Despite promoting ingestion, immunoglobulin directed against blastospores did not encourage efficient killing or oxygen consumption. Taken together these findings indicate that C3b coated microbes preferentially stimulate PMN oxidative metabolism via C3 receptors thereby increasing H_2O_2 production for participation in MPO-mediated halogenation of ingested organisms. On the other hand Ig triggers ingestion via Fc receptors but plays no part in the procidination of Candida.

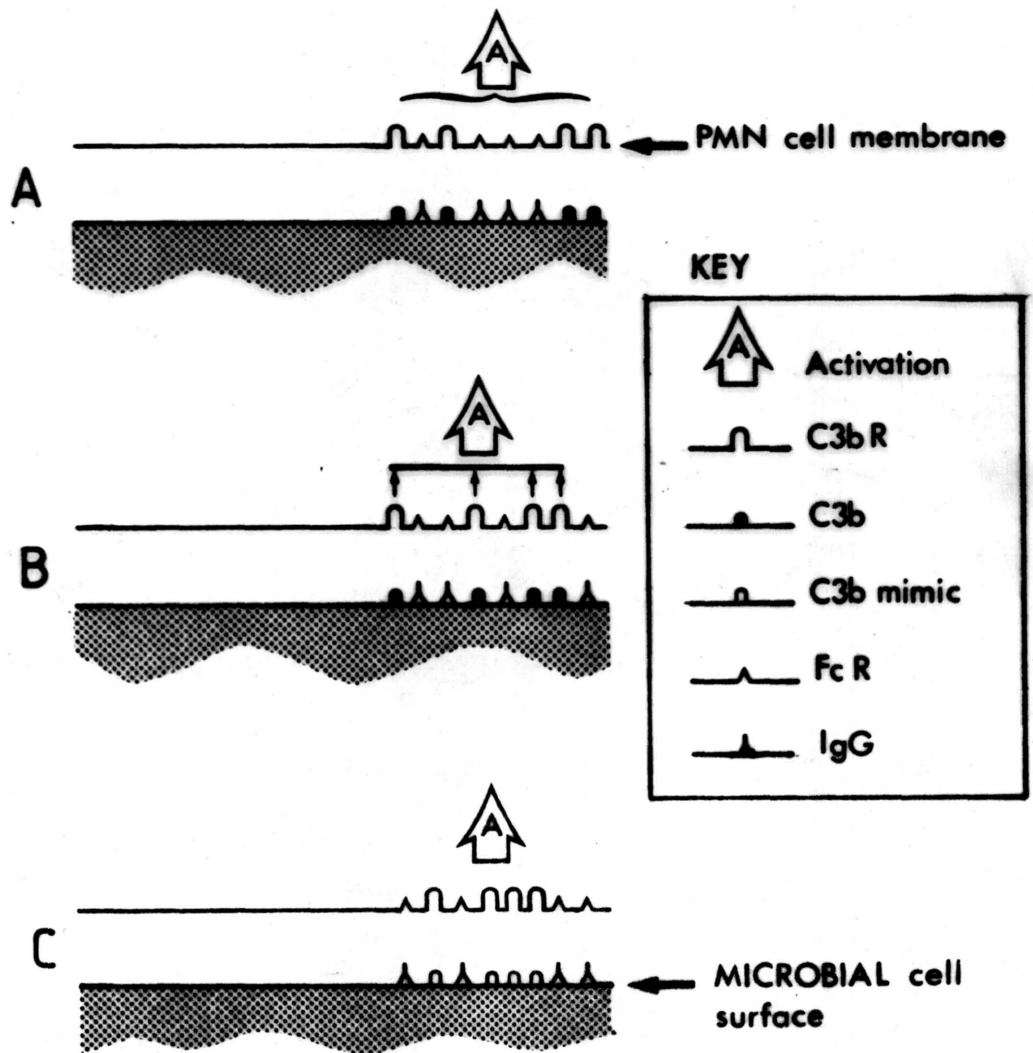
Other lines of evidence support the contention that C3 rather than IgG is responsible for the stimulation of PMN oxidative metabolism (Goldstein et al.,1976; Goldstein et al.,1975; Tedesco et al.,1975). The concept that antibody and complement have diverse functions is further supported by studies of their relative contributions to phagocytosis (Scribner and Fahrney, 1976) and also the development of B memory cells (Klaus and Humphrey,1977). However, immunoglobulin coated bacteria or latex particles can provoke oxygen consumption in the absence of complement which suggests that antibody is stimulatory if presented to the phagocyte surface in sufficient density. Alternatively, antibody may work in concert with microbially derived products which substitute for C3. See Figure 6.1. for

explanatory diagrams. In regard to these theoretical proposals it has been reported that microbes possess molecules which mimic bound C3 (Jack and Ward, 1980; Fine et al., 1980). It is also apparent that antigen density, and hence IgG density, is important to phagocytosis and cytolysis of chicken erythrocytes by human monocytes and K cells (Öhlander, Larsson, and Perlmann, 1981). Conversely, there is evidence that large quantities of antibody on Candida are detrimental to phagocytic killing (Laforce et al., 1975).

Further work is therefore required to analyse the effect of ligand density, be it IgG or C3b, on the activation of phagocytic killing processes. A study of microbe surfaces for the presence of C3-like molecules and the influence such molecules may have on phagocytic function would also be of value since it may define a relationship between virulence and evasion of phagocytic killing.

Although C3 is clearly involved in oxygen dependent phagocytic killing by PMNs it is possible that C3, or other complement components, may also cooperate with lysosomal enzymes (Li and Mudd, 1966), and this interaction may result in killing without digestion (van Houte et al., 1977). Alternatively, complement may facilitate digestion by lysosomal enzymes e.g. lysozyme. Microbes which evade, or are not subject to, oxygen dependent killing may thus find themselves digested alive. The microbicidal action of digestive granular enzymes is incidental to phagocytic killing if the organisms have already been killed by oxygen dependent mechanisms. Therefore serum factors which prepare organisms for digestion should be called 'prodigestins'

FIGURE 6.1. Schematic diagram showing various models of phagocyte stimulation.



A) Synergistic or cumulative activation of phagocyte metabolism by both C3b and Ig. B) Selective activation by microbially bound C3b. C) Activation of metabolism by molecules of microbial origin which resemble C3b. See text for details.

rather than procidins.

The design of the radiometric phagocytosis and killing assay, which relies upon active incorporation of a radiolabel by viable microbes rather than the passive release of label by dead ones, makes it unlikely that this method is measuring microbial degradation by granular contents. However, it remains to be established that organisms which escape primary killing systems can succumb to a death by digestion. Methods measuring phagocyte digestive capacity, using live but suitably prelabelled blastospores, should clarify these points and provide a novel and useful approach to clinical evaluation of phagocytic function, namely separate assessment of ingestion, killing, and digestion.

The observation that relatively high serum concentrations can inhibit phagocytic killing of Candida is reminiscent of reduced Schistosomula killing by eosinophils and neutrophils when high concentrations of complement are present (Anwar, McKean, Smithers, and Kay, 1980). Inactivation of C3b by β_1H and C3INA is the probable cause of the aforementioned phenomena but should be confirmed since this may be of pathogenetic importance in infection. Moreover, it should be noted that the concentration of protein in lymph and interstitial fluid although variable in different tissues corresponds to approximately 5-10 per cent serum. Human phagocytes may therefore show optimal microbicidal activity in the extravascular milieu during subclinical stages of infection and early inflammatory changes. Further studies to identify and quantitate the proteins found in extracellular

fluid are needed to clarify these speculations.

The mechanism by which particle associated C3b induces phagocytic killing is unknown. Ligand binding could induce conformational alterations in the C3 receptor or, alternatively, an intact microbially bound C3/C5 convertase may be responsible for cleavage of a specific PMN membrane protein. This problem could perhaps be answered by either inactivating the convertase, but leaving it stereochemically intact, or employing enzyme inhibitors.

PMNs from an individual suffering from X-linked CGD and who lacked the b-type cytochrome activity described by Segal and associates did possess a C3 receptor. This suggests that the cytochrome is functionally preceded by activation of the C3 receptor (Figure 6.2.). Supporting evidence for this concept is derived from studies on Epstein-Barr virus infected myeloid cells (Newburger et al.,1980). It was found that complement opsonised bacteria or zymosan were ingested by this cell line but were incapable of promoting O_2^- production. Soluble stimuli (phorbol myristate acetate) or unopsonised latex particles however did induce the respiratory burst. This indicates that stimulation via C3 receptors can be bypassed and since this cell line does not have Fc receptors it was not mediated by immunoglobulin or immunoglobulin-like molecules. Results from other workers also show that there are multiple means of starting the respiratory burst (McPhail, Henson, and Johnston,1981).

Cytochromes of the b-type are encoded in mitochondrial DNA (mt DNA)(see von Jagow and Sebald,1980; Whittaker and Danks,1978).

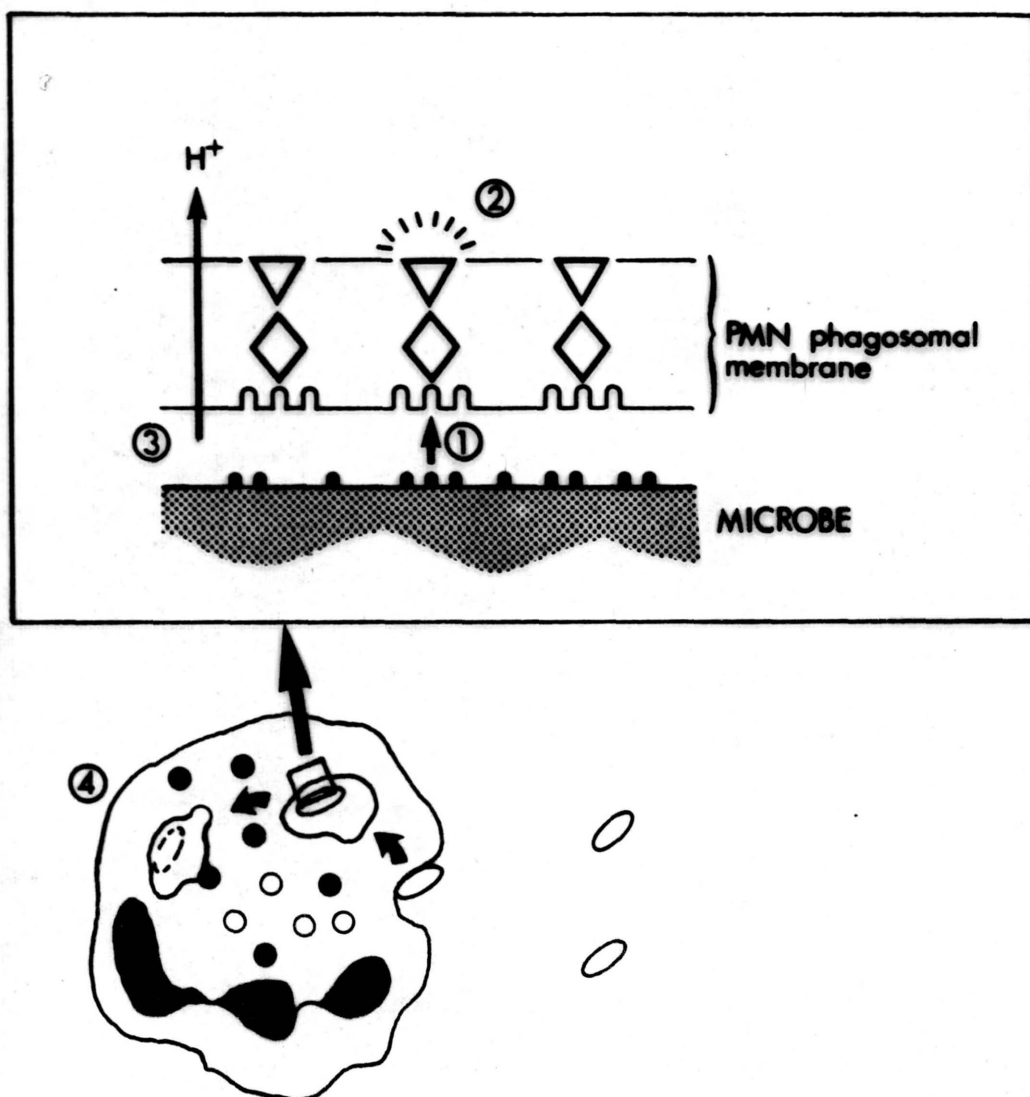


FIGURE 6.2. Unifying model for phagocytic killing by PMNs. Interaction of microbially bound C3b with clustered C3b receptors on the PMN surface (1)(Fearon, Kaneko, and Thompson, 1981) activates a multicomponent membrane situated oxidase system (2)(Babior et al., 1981) which produces toxic oxygen products and a transient rise in vacuolar pH (3)(Segal et al., 1981). The rise in pH favours the microbicidal activity of cationic proteins in azurophil granules. The MPO- H_2O_2 -~~HALOX~~ ~~halogen~~ system imposes a further threat to microbial survival. Finally, the microbe is subjected to degradation by hydrolytic enzymes in an acid environment (4).

Therefore, it would be interesting to discover if cytochrome b_{-245} is similarly encoded because human mtDNA appears to have some redundancy (Anderson et al.,1981). If mRNA for this cytochrome is transcribed from mtDNA then, at first sight, the X-linked transmission of CGD could be explained by cytoplasmic inheritance as virtually all mitochondria are derived from the egg and not the spermatozoon (Billet,1979). However, this theory is flawed because one would expect all the progeny of an affected mother to inherit the disease. Since this does not happen the putative transcription of mRNA for cytochrome b_{-245} from mtDNA must be subject to X-chromosome control.

Approximately 31% of 58 patients with suspected immunodeficiency were found to have subnormal procidin values while 22% of 112 patients tested had defective phagocytic killing. No intrinsic abnormalities of ingestion were discovered indicating that this function is highly conserved or lethal when defective. It should be pointed out that although an effort was made to test these patients when they were free of infection, it is not possible at this stage to tell how many of these defects were of a secondary nature. Nevertheless, it is believed that a careful study of phagocytic function is clinically important regardless of whether primary or secondary abnormalities are being detected. It is evident that complications may result from inefficient phagocytic activity (Levinsky et al.,1981), therefore corrective measures, even when directed at a secondary defect, may break a vicious circle which is a feature of most persistent infections.

MATERIALS, EQUIPMENT and SUPPLIERS

ABBREVIATIONS

REFERENCES

MATERIALS, EQUIPMENT and SUPPLIERS.

Acridine orange/ethidium bromide	Sigma Chemical Co.
Agar	Oxoid
Agarose	BDH
Barbituric acid/Na.barbitone	BDH
Centrifuges	MSE/Fisons
Cytocentrifuge	Shandon
Dextran 110 (6% w/v 0.9% NaCl)	Fisons
DNAase (DN100)	Sigma Chemical Co.
Electrophoresis equipment	Shandon,
Ficoll-Hypaque (Lymphoprep)	Nyegaard & Co., A/S Oslo
Fluorescence microscope	Leitz SM-Lux
Glass plates	Ilford Ltd
Glucose	BDH
Goat anti-human whole C3 antiserum	Seward
Heparin (1000 IU/ml, preservative free)	Paines and Byrne Ltd.
Kenacid Blue R	BDH
May-Grunwald Giemsa stain	Hopkin and Williams/Gurr
Microsyringe with dispenser	Hamilton
Microtitre plates	Sterilin/Linbro
Peptone	Difco
Phase contrast microscope	Wild
Plastic tubes	Raven, Luckam, Sterilin
PPO	BDH
Radiochemicals	Radiochemical Centre, Amersham.
Rotator	Jencons
RPMI 1640 (20 mM Hepes)	Flow Laboratories
Skatron automatic multiple channel cell harvester	Flow Laboratories
Sodium deoxycholate	BDH
Spectrometers:	
β -counter	Tri-carb scintillation spectrometer Model B- 2450, Packard
γ -counter	Auto-gamma scintillation spectrometer 5266, Packard.

Toluene (scintillation grade)

BDH

Trypan Blue

Sigma Chemical Co.

ABBREVIATIONS

C3b R	C3b receptor
Ci	Curie
CFU	Colony forming unit
Con A	Concanavalin A
concn	concentration
condtns	conditions
CPD	Citrate-phosphate-dextrose
cpm	counts per minute
DNAase	Deoxyribonuclease
EDTA	Ethylene diamine tetra-acetic acid
Fc R	Receptor for the Fc portion of Ig.
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
Ig	Immunoglobulin
KE	Killing efficiency
KI	Killing Index
LSHTM	London School of Hygiene and Tropical Medicine
MNP	Mononuclear phagocyte
MPO	Myeloperoxidase
mRNA	messenger Ribonucleic acid
mtDNA	mitochondrial Deoxyribonucleic acid
n	Number of subjects in sample
nd	Not done
OKE	Overall Killing Efficiency
PA	Phagocytic activity
PBS	Phosphate buffered saline
PHA	Phytohaemagglutinin
PI	Phagocytic Index
PMN	Polymorphonuclear phagocyte
PPD	Purified protein derivative of mycobacteria
PPO	Diphenyloxazole
PWM	Pokeweed mitogen
r	correlation coefficient
sd	Standard deviation
SMH/SMHMS	St. Mary's Hospital/SMH Medical School
TCA	Trichloroacetic acid

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