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LEUKOCYTE FUNCTION IN MYELOPROLIFERATIVE DISORDERS

BY

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"Drugs can only repress symptoms: they cannot eradicate disease. The true remedy for all diseases is Nature's remedy. Nature and Science are at one, Sir Patrick, believe me; though you were taught differently. 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 of all diseases, and that is to stimulate the phagocytes. Stimulate the phagocytes. Drugs are a delusion."

G. B. Shaw (1906) The Doctor's Dilemma. Act 1.

ABSTRACT

Myeloproliferative disorders are characterised by abnormal proliferation of the myeloid tissues in the bone marrow and in metaplastic sites including the liver and spleen. When this work started in 1973, very little was known about the functional and enzymic abnormalities of the leukocytes in these disorders. During the last three years I have been able to set up quantitative and qualitative procedures for the estimation of leukocytes primary granular enzymes, such as peroxidase, acid phosphatase and β-glucuronidase as well as secondary granular enzymes, such as alkaline phosphatase. Also, I have been able to set up a quantitative method for the estimation of the neutrophil function which included phagocytosis and killing of Candida guillermondii as well as degranulation and H_2O_2 production during phagocytosis.

Fifty healthy subjects, 21 males and 29 females, ranging between 17 and 74 years of age, were investigated. These were compared to 13 Chronic Granulocytic Leukaemia, 12 Myeloid Metaplasia and 7 Polycythaemia Rubra Vera. The principle abnormalities that have been demonstrated in the Chronic Granulocytic Leukaemia neutrophils were the significant reduction in the fungicidal capacity, the impairment of degranulation and the significant reduction in H_2O_2 production during phagocytosis. Myeloid metaplasia neutrophils had a significant reduction in the fungicidal capacity and a low peroxidase level which correlated

significantly with the corresponding fungicidal ability. Polycythaemia Rubra Vera neutrophils, also, had a significant reduction in the fungicidal capacity.

Quantitative and comparative studies on the cytochemistry and function of the leukocytes from these myeloproliferative disorders was valuable in distinguishing the different disorders and throwing some light on the different mechanisms which appear to be responsible for their functional impairment.

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CHAPTER ONE

CHAPTER ONE

INTRODUCTION

1. Symbols and Abbreviations

Patients have been designated a number and this number was replaced by a symbol during graphic representations.

Patient No.	Symbol	Patient No.	Symbol
1 =	○	8 =	▲
2 =	□	9 =	▼
3 =	△	10 =	◆
4 =	▽	11 =	◬
5 =	◇	12 =	⊙
6 =	●	13 =	⊡
7 =	■		

s	second
'	minute
min	minute
d	day
nm	nanometer (10^{-9})
mm^3	millimeter cube
cm^3	centimeter cube
mg	milligramme
l	litre
C^0	degree centigrade
%	percentage
/	per
S.E.	standard error
N.S.	not significant
p	significance probability
CGL	Chronic Granulocytic Leukaemia

CGD	Chronic Granulomatous Disease
MM	Myeloid Metaplasia
PRV	Polycythaemia Rubra Vera
SI	Severe Infection
PMN	Polymorphonuclear neutrophil
TLC	Total leukocyte count
H_2O_2	Hydrogen peroxide
NAD (H_2)	Nicotinamide adenine dinucleotide (reduced)
NADP (H_2)	Nicotinamide adenine dinucleotide phosphate (reduced)
EDTA	Ethylenediaminetetra-acetic acid
O.D.	Optical density
M	Molar
mM	Millimolar

2. Reasons for doing the work

The Myeloproliferative disorders are characterised by abnormal proliferation of myeloid tissues, that is erythrocytes, granulocytes and megakaryocytes, in the bone marrow and in metaplastic sites including the liver and spleen. One member of this group of disorders is Chronic Granulocytic Leukaemia (CGL) which can be distinguished by the presence of the Philadelphia Chromosome. When this work started in 1973, a certain amount was known about the functional and enzymic abnormalities of the CGL granulocytes. These abnormalities included defective phagocytosis of bacteria as well as very low leukocyte alkaline phosphatase content. A relationship between the lack of leukocyte alkaline phosphatase and the ability to phagocytose was suggested. Very little was known about the ability of these cells to kill microorganisms.

There was hardly any information on the granulocyte function or the levels of the granular enzymes of the other myeloproliferative disorders. Some patients with Myeloid Metaplasia show a high peripheral granulocyte count and used to be diagnosed as suffering from Chronic Granulocytic Leukaemia. However, the cells lack a Philadelphia chromosome and usually show a high leukocyte alkaline phosphatase score. Polycythaemia Rubra Vera, a third member of the group, often has raised granulocyte counts and may show a high leukocyte alkaline phosphatase score. It was not known whether there was any correlation between the level of the granular enzymes of the granulocytes of these disorders and their

ability to phagocytose and kill microorganisms. Quantitative and comparative studies on the cytochemistry and function of the leukocytes from these myeloproliferative disorders was valuable in distinguishing the different disorders, throwing some light on the maturity of their leukocytes and revealing the different mechanisms which appear to be responsible for their functional impairment.

3. History of the Granulocytes

In 1749, J. E. de Senac described the "globules blancs du pus" although it was the Englishman Hewson, who in 1773 gave the first detailed account of the white cells in the blood. He considered that the "colourless corpuscles" were derived from the lymphatic glands and thymus, entered the circulation through the thoracic duct and reached the spleen where they became transformed into red corpuscles. This idea was accepted for a century until the 1880's when the origin of the granular leukocytes in the bone marrow was established.

The function of the white cells in inflammation was first studied by Addison in 1843. He described different types of white cells and pointed out the granules. He stated that the leukocytes formed pus although he believed that epithelial cells were also derived from them. Addison further described the increased number of white cells in Scarlet Fever and in the blood taken from the neighbourhood of a boil.

In 1846, Waller described the active nature of leukocyte migration.

Haeckel (1862) was the first to demonstrate that leukocytes were capable of taking up foreign bodies into their own cytoplasm.

Schultze (1865) was able to observe white blood cells kept at body temperature and he depicted a "finely granular cell" in the amaeboid form. This cell had ingested fine droplets of milk which he had mixed with the human blood cells.

Migration of leukocytes from vessels was confirmed by Schultze in 1866.

Between 1870 and 1880 Ehrlich, using special staining, was able to recognise the five species that form the basis of the present day classification of leukocytes. Leber (1888) first described and demonstrated chemotaxis of leukocytes. Nearly a century ago, Metchnikoff's classical studies, starting with starfish larvae and wood splinters and continuing with investigations on the phagocytic functions of granular leukocytes, led to his cellular theory of immunity. He deduced that phagocytic cells protected higher creatures from assault by the ubiquitous micro-organisms in the internal and external environment and championed the cause of phagocytosis as the basis of survival against pyogenic infections. Metchnikoff's crusade (1905) provoked opposition from the proponents of humoral mechanisms of host protection, but from this controversy arose the realization that both humoral factors and phagocytosis co-operate (Wright et al., 1904). In his later writing,

Metchnikoff stated that excessive phagocytosis was responsible for aging and thus first suggested neutrophil-induced tissue damage. He pursued a quixotic search for a serum to prevent old age.

The description of opsonins by Wright & Douglas (1903) related phagocytosis to the rapidly expanding field of immunology, a relationship dramatised by G. B. Shaw in "The Doctor's Dilemma". Since Hewson's descriptions and speculations about hematopoiesis some 200 years ago, we have been able to recognize, characterize and quantitate the various cellular elements of the white blood cell system. We have come to understand many of the functions they perform and how they are carried out, but we have yet to characterize clearly all of the processes involved in the maturation and production of this cellular system. It is likely that some day our present knowledge will be reviewed in dusty historical libraries with perhaps the same air of superior knowledge with which we view the above work today.

4. Production and development of the granulocytes

The bone marrow is responsible for production of the neutrophilic polymorphonuclear leukocytes (PMN). The most striking feature about the kinetics of granulopoiesis is the enormous turnover of 126×10^9 neutrophils in a 70kg man per day, which lies behind the steady-state neutrophil concentration in the blood (Cartwright et al.,1964). The duration of neutrophil survival in the circulation, a half-time of six to seven hours, is much shorter than the maturation time of these cells

in the marrow, which is measured in days. During this prolonged preparation time, the neutrophil acquires the machinery to enable it to perform the functions of phagocytosis.

5. Stages of maturation of the polymorphonuclear neutrophil leukocyte

a) The myeloblast: This cell is a relatively undifferentiated or embryonic cell with a large oval nucleus and a cytoplasm lacking granules but containing abundant ribosomes, mitochondria and a small, rudimentary Golgi complex. The myeloblast is the origin of all myeloid or granular cells. The cell varies in size but normally its mean diameter is approximately 16μm. The usual variation is given as 11 to 18μm. The cell is normally spherical. Warm stage preparations do not show any evidence of motility; tissue cultures, however, may reveal slight movement. Myeloblasts are found, normally, only in the marrow and comprise about 2% of the total cells found. They are not found in normal peripheral blood.

b) The promyelocyte: This cell is also spherical and is smaller than the myeloblast measuring between 11 and 16μm. The nucleus is reduced in size but still maintains its reticular appearance. The promyelocyte is a stage of intense secretory activity associated with the formation of azurophilic or primary granules. These granules are formed by condensation of secretory material along the proximal or concave face of the Golgi complex. They are uniformly

dense, rounded or elongated in shape, about 600-800nm in diameter and contain Myeloperoxidase, acid phosphatase, β-Glucuronidase, cationic proteins and lysozyme. A warm-stage film shows that promyelocytes are motile in supravital conditions and may show some amaeboid movement. In tissue culture, motility is a characteristic of the promyelocyte. The cell is found only in the marrow, and normally comprises between 0.5 and 5% of the total cells observed. It is not found in normal peripheral blood.

c) The Myelocyte: This cell, when seen in normal marrow, is slightly smaller than its two predecessors and varies from 11 to 16μm. The nucleus is reduced in size and occupies less space in the cytoplasm. It shows a reticular pattern with little or no chromatin and no nucleoli. The myelocyte is also a stage of intense secretory activity and is associated with the formation of specific or secondary granules. These granules are formed by condensation of secretory material along the distal or convex Golgi face. They are smaller and less dense than azurophilic granules. They are variable in shape and measure between 300 and 500nm. They contain alkaline phosphatase, lactoferrin and lysozyme. The myelocyte is motile by warm-stage methods and in vital stained preparations it shows amaeboid movement. In tissue cultures it also can be recognized by its characteristic movement. The myelocyte is normally found in the marrow only. The neutrophil myelocyte predominates and forms 2 to 8% of the total cells. On the other hand, myelocytes may be found in small numbers in peripheral blood as a result of

stimulus to bone marrow.

d) The Metamyelocyte and Band cell: These are non-secretory, transitional stages during which there is a progressive decrease in the cell size, 11 to 15μm associated with changes in the shape of the nucleus, which now takes on a "dumb-bell" shape. The nucleus still retains its reticular appearance. There is also a gradual reduction in the numbers of most cytoplasmic organelles. The metamyelocyte is normally found in the marrow. The neutrophil metamyelocyte forms 10 to 25% of the total cells. Induced activity of the marrow may cause a temporary appearance in the peripheral blood.

e) The Polymorphonuclear neutrophil leukocyte (PMN): This is the final product of the maturation process. It is the most common of all nucleated cells found in the marrow and in the peripheral blood. The PMN is about 10-12μm in diameter which is considerably smaller than any of its immediate predecessors but almost twice the size of the normal erythrocyte. The appearance of the nucleus gives the name "polymorphonuclear" to the cell. The nucleus develops several lobes, usually three or four, joined by thin strands of chromatin. The cytoplasm contains primarily glycogen and granules, of which 75-90% are specific granules and only 10-25% are azurophilic granules. PMN leukocytes are present in the peripheral blood and in the marrow, and counts range from 2,500 to 7,500 mm^{-3} (40 to 75% of the total white cells) in the peripheral blood.

6. The Phagocytic activities of the PMN

Phagocytosis, the process whereby single cells internalize objects that they encounter, is a matter of survival for many unicellular organisms, which use it to gain nourishment. In the 1880's, Metchnikoff had deduced that phagocytic cells protected higher creatures from assault by microorganisms. For phagocytes to perform their protective role in the host, they must be produced and mobilized in a state of functional preparedness. Although various types of cells can be induced on occasion to engulf particles, the 'professional' phagocytes in defence against microbes are the polymorphonuclear neutrophilic leukocytes, the blood monocytes and the tissues macrophages.

For the ingestion (phagocytosis) of microorganisms, a series of distinct events occur within polymorphonuclear neutrophils (PMN's) which leads ultimately to the destruction of the invading organisms. This sequence includes: chemotaxis, recognition, ingestion, degranulation, peroxide formation, killing and the digestion of the ingested organisms.

a) Chemotaxis

Chemotaxis is the vectorial motile response of phagocytes to inflammation.

(i) Humoral aspects of Chemotaxis: The interactions between microorganisms and host tissue leads to the generation of chemotactic factors by several mechanisms (Sorkin et al., 1970). Certain bacteria release substances that, without further alteration, have the capacity to attract phagocytes. However, the principle mechanism by which microbes generate chemotactic activity for phagocytes is by

activating serum complement in a number of different ways. Antibody reacts with the surface of a microbe, and the antigen-antibody complex sequentially activates the haemolytic complement components C1, C4 and C2. The antigen-antibody-C142 complex attacks C3 and C5 in the serum to yield, among other fragments, C3a and C5a which are low-molecular-weight peptides with chemotactic activity (Sorkin et al., 1970). In the absence of specific antibody or in response to antibody of certain subclass specificity, microorganisms can generate C5a and C3a from C5 and C3 in serum by activating the incompletely defined alternate complement pathway or properdin system (Sandberg et al., 1972). Non-specific proteases released from bacteria or damaged tissue can attack C3 and C5 directly to yield C3a and C5a (Ward et al., 1973). Ingestion of particulate matter by neutrophils causes them to release a factor that has the capacity to attract other neutrophils in the absence of serum (Zigmond et al., 1973). Lymphocytes responding to antigens elaborate lymphokines, which, among other properties, have chemotactic activity (Altman et al., 1973; David et al., 1966).

(ii) Cellular aspects of Chemotaxis: Migrating polymorphonuclear leukocytes have an area of cytoplasm at the outer cell margin that is glossy or hyaline in appearance and is devoid of organelles such as granules or nucleus. This "hyaline ectoplasm" is particularly prominent in the direction of cell movement, where it forms a broad, thin veil (lamellipodium) and gives the appearance of being relatively stiff whereas the internal contents of the cell appear more fluid (Zigmond et al., 1973;McCutcheon, 1946).

At the opposite pole of the cell, there is a knob-like hyaline tail. The internal cytoplasm streams towards the direction of cell movement and the organelles within it exhibit rapid vibrational activity just behind the lamellipodium.

Ultrastructurally, the hyaline ectoplasm of neutrophils is composed of glycogen particles and of fine filaments, 6nm in diameter (Dale et al., 1971). These microfilaments have been identified as actin polymers (Pollard et al., 1974). Myosins with structural and enzymatic similarities to muscle myosin and with ATP hydrolyzing activity have been purified from PMN, strengthening the intriguing notion that movement of phagocytes may have molecular features similar to muscle contraction (Tatsumi et al., 1973; Stossell et al., 1972). The intracellular concentration of calcium could influence movement of actin filaments by myosin ATPase in phagocytes.

Another cytoplasmic structure that may be important for the mobility of phagocytes is the microtubule. They are hollow fibres with a diameter of 24nm. Their role in cellular motility is as yet unclear.

Chemotaxis and migration of PMN are inhibited by agents that interfere with ATP production and occur optimally in the presence of divalent cations (Bryant et al., 1966; Gallin et al., 1973). Substances that increase intracellular levels of cyclic-AMP in phagocytes inhibit motility whereas agents that raise levels of cyclic-GMP have been reported to enhance it. This reciprocal relation of these cyclic nucleotides may modulate cellular mobility, possibly by influencing the

stability of microtubules (Estensen et al., 1973). However, it has not been shown that changes in intracellular levels of these cyclic nucleotides occur in response to initiation of chemotaxis.

b) Recognition

Having arrived at the inflamed or invaded site, phagocytes must recognize what to attack. They demonstrate remarkable selectivity. The ingestibility of particles can be influenced by variation in their net surface charge or their hydrophobic properties (Stossel et al., 1972). Many microorganisms, particularly encapsulated ones, resist ingestion by phagocytes, a characteristic that contributes to their pathogenicity. Also particles that have interacted with fresh serum or are coated with sufficient amount of specific antibody are ingested by PMN with great avidity. This coating of particles with serum proteins that accelerate endocytosis is called opsonization (Greek - to prepare for eating).

(i) Humoral aspects of recognition: When present in sufficiently high concentrations, antibody directed against the surface of particles opsonizes these particles for ingestion by PMN. Only antibodies of the IgG class are opsonically active (Huber et al., 1968). Since the immunoglobulins of the IgG class are resistant to heating, the heat-stable opsonic power of serum is due to the presence of antibody. Normal serum has little heat-stable opsonic capacity, whereas serum activity of this nature from hyperimmunized subjects may be considerable (Quie, 1972; Young et al., 1972). On the other hand, all normal sera have

opsonic activity that is destroyed by heating and which can be attributed entirely to complement proteins. A quantity of antibody, insufficient to opsonize on its own, may react with bacteria and activate sequentially the haemolytic complement proteins C1, C4 and C2. Bacteria can also activate the properdin system. The C142 complex or the properdin system acts on C3 to promote the deposition of an opsonically active fragment of C3 on the surface of the microbe (Gigli et al., 1968; Stossel et al., 1973). This fragment, a peptide with a subunit molecular weight of 70,000, is extremely resistant to attack by physical agents and is attached to the particle surface by a firm hydrophobic bond (Stossel et al., 1974). C5, which is structurally similar to C3 but present in serum at 3% the concentration, may contribute somewhat to the opsonic power of fresh serum (Smith et al., 1969).

(ii) Cellular aspects of recognition: Opsonization of particles changes the ingestion by phagocytes from a slow or negligible basal rate to a rapid one. The rigorous specificities for the expression of opsonic activity suggest that the activation of ingestion by opsonized particles involves the engagement of receptor molecules on the surface of the phagocytes by the particle-fixed opsonins. There is also some indication that there are differences between the behaviour of C3 and that of IgG receptors of neutrophils (Holland et al., 1972; Rabinovitch et al., 1973; Stossel, 1972).

It must be emphasized that cell-particle interaction and adherence is not invariably associated with endocytosis and,

although cell-particle adherence must precede ingestion, the concept of an "attachment stage of phagocytosis" as defined by morphology must be used with caution (Rabinovitch, 1968).

c) Ingestion

The normal consequence of recognition is the initiation of engulfment by the phagocyte.

(i) Mechanisms of ingestion: Upon contact with the particle, the hyaline ectoplasm extends to form pseudopodia and, if migration toward the particle preceded contact, the lamellipodium simply surrounds the particle. The movement of the pseudopodia is a spreading one and is similar to the spreading of phagocytes on surfaces. The pseudopodia fuse at the distal side of the particle, which thereby becomes encased within a phagocytic vesicle or phagosome, the lining of this structure being inverted plasma membrane. The vesicle buds off from the cell periphery and moves away from the hyaline ectoplasm.

Ingestion of particles is an active energy-dependent event. Engulfment activates ATP generating processes, specifically glycolysis and glycogenolysis in neutrophils (Karnovsky, 1962) and is inhibited by metabolic poisons. These facts are consistent with the idea that an energy-utilizing mechanism, such as myosin-actin interaction, drives ingestion as well as migration. The anatomy of engulfment supports this concept. The network of microfilaments that comprises the lamellipodium of migrating phagocytes is prominent in the pseudopods that embrace the particle during ingestion (Reaven et al., 1973). Cytochalasin B,

which is thought to alter microfilament function, inhibits endocytosis as well as migration (Allison et al., 1971; Malawista et al., 1971). Colchicine, which disrupts microtubules, can also impair ingestion (Stossel et al., 1972). Although it is not clear how microtubules are involved in ingestion, they do penetrate into the cell periphery in regions of the cell in contact with particles being internalized (Reaven et al., 1973). In addition to the events which are occurring within the cell in order to propel the membrane about the particle to be ingested, the membrane itself must deform and fuse to complete formation of the phagosome. Ingestion accelerates the rate at which phagocytes acylate lysophosphatides added to the medium bathing the cells, and this is interesting because lysocompounds can induce the fusion of cell membranes (Elsbach, 1972).

During the uptake of large numbers of particles, phagocytes internalize a substantial quantity of plasma membrane, an action that is possible because of redundancy of the cell surface (Bessis, 1973).

(ii) Translation of recognition into ingestion: The manner in which "recognized" particles elicit ingestion is unknown. One clue is the fact that divalent cations (Mg^{++} and Ca^{++}) affect ingestion as they do migration and spreading. These ions must be in the extracellular medium for the phagocytes to ingest many types of particles (Stossel et al., 1972; Stossel, 1973). After opsonization with C3, particles can be taken up in the presence of much lower divalent cation concentrations (Stossel et al., 1973; Stossel, 1973). Drugs that increase intracellular levels of cyclic AMP in phagocytes

inhibit the rate of ingestion (Stossel et al., 1972; Bourne et al., 1971). However, uptake of particles does not alter intracellular levels of cyclic AMP and it has not yet been possible to assign a role of cyclic AMP as a mediator of the ingestion process or of its associated metabolic changes (Stossel et al., 1970). Cell-particle contact, the metabolic integrity of the cell and the ingestion mechanism itself are sensitive to the extracellular medium. Hypertonicity markedly inhibits ingestion (Sabarra et al., 1963). Inhibitors of glycolysis, surface-active agents such as ethanol, or hydrocortisone in high doses, drugs that bind to sulphydryl groups and chelators of divalent cations all impair engulfment (Karnovisky, 1962; Stossel et al., 1972; Wurster et al., 1971).

d) Degranulation

As the phagosome forms during engulfment, cytoplasmic organelles fuse with its membrane. In polymorphonuclear leukocytes, these organelles are the primary and secondary granules, which undergo violent movements in proximity to the phagosome, fuse with the vacuole and disappear from the cytoplasm (hence "degranulation") (Hirsch et al., 1960). The contents of these granules can be observed ultrastructurally in phagosomes as dense amorphous material (Steigbigel et al., 1974) and have been identified as granule-associated enzymes both histochemically and biochemically (Horn et al., 1964; Stossel et al., 1971). Thus, degranulation is a mechanism whereby these enzymes are delivered to their operational sites without subjecting the cytoplasms of the phagocyte to their potentially injurious

effects. Degranulation occurs in concert with ingestion, suggesting that the triggering mechanism for both is either similar or the same (Stossel et al., 1971; Stossel et al., 1972). The primary and secondary granules of neutrophils degranulate at different rates during ingestion (Stossel et al., 1971) and histochemical evidence suggests that secondary granules fuse with the phagosome first, followed by the primary granules (Bainton, 1973). The sequential degranulation and the segregation of enzymes into different classes of granules is very important. Probably the most important consequence of sequential degranulation is that the content of the phagocytic vacuole changes with time, thus permitting the contents of the specific granules to function independently. By observing early changes in the colour of ingested indicator-stained yeast, it has been established that intravacuolar acidity drops from about pH 7.0 to pH 6.5 in 3-4 minutes, and to pH 4 in 7-8 minutes (Jensen et al., 1971; Jensen et al., 1973). Therefore, during the first 3 minutes after phagocytosis, the time when it acquires the contents of specific granules, the pH of the phagocytic vacuole is nearly neutral. These granules are known to contain alkaline phosphatase, lactoferrin and lysozyme (Cohn et al., 1960) and all these substances have pH optima in the neutral or alkaline range (Jensen et al., 1973). Only later (after 3 minutes), when the pH is rapidly dropping to 4, are primary granule contents, most of which have pH optima in the acid range, discharged. These granules contain peroxidase, lysosomal enzymes, lysozyme and acid mucosubstance (Hardin et al., 1971). Peroxidase, a potent

bactericidal and virucidal enzyme, has a pH optimum of 4.5 (Jensen et al., 1973), while the lysosomal enzymes are largely inactive above pH 4.5. Hence, the sequence of granule discharge roughly parallels pH changes, providing conditions which favour sequential granule interaction (Bainton, 1973). Another possible consequence of this sequentical discharge is that secondary granule contents may be digested or altered and thus activated on entry of the primary enzymes (Bainton, 1973).

(i) Mechanisms of degranulation:

The mechanism(s) by which specific granules selectively fuse with the newly formed vacuoles before azurophil fusion is unknown. Although it is possible that the initial alkaline phosphatase reactivity may be due to the fact that specific granules outnumber azurophils in each cell by about 3:1, this explanation seems untenable because, if the two types of granules did empty simultaneously, the contents of the phagocytic vacuole would be constant and this is clearly not the case at the early intervals. Perhaps specific granules, in view of their smaller size, move more rapidly than azurophils, increasing the chance of collision with the vacuole. This possibility, however, remains untested. Some new information concerning the differing nature of the membranes of the two granule types is available. Nachman et al. (1972) analyzed membranes from both kinds of granules separated by zonal differential centrifugation and demonstrated distinct variations in their cholesterol - phospholipid ratios and protein components. Moreover, Elsbach and his co-workers (1968) have reported that phagocytizing PMN synthesize new

membrane lipid and that almost all of this lipid can be found within the membrane of the newly formed phagocytic vacuoles (Elsbach et al., 1972). In isolated granule fractions, it is observed that the two types of granules respond differently to osmotic changes. Specific granules do not rupture or change shape in hypotonic buffer or even in distilled water, whereas azurophils become markedly shrunken and distorted and rupture easily in distilled water. Considered together, these studies prove that the membranes of each granule type have distinctive properties. It is not yet clear what property, or properties, of the membrane may account for the initial selective fusion of specific granules and phagosome membranes (Bainton, 1973). In addition to discharge into phagocytic vacuoles, granular enzymes may be released outside the cell (Goldstein et al, 1973; Woodwin, 1962; Henson, 1971). This extracellular degranulation can result from leakages of granule contents out of incompletely fused phagosomes, from the phagocyte's attempts to ingest a very large particle that it is incapable of enveloping, from disruption of the cell or by active secretion after exposure of the cell to certain drugs and proteins.

As in migration, chemotaxis and ingestion, the microtubules and microfilaments of the cell cytoplasm appear to be involved in the mechanism of degranulation. In the resting cell, the hyaline ectoplasm acts as a barrier between the cytoplasmic granules and the plasma membrane. As the phagosome forms, the microfilaments, normally closely apposed to the surface membrane of the cell,

dissipate from the membrane, which is now part of the phagosome, allowing the granules to approach it. Cytochalasin B, which is believed to disrupt the active filaments in phagocytes, enhances extracellular degranulation of the phagocytes exposed to particles, suggesting that it removes the barrier to granule fusion with the plasma membrane (Surier et al., 1973; Skosey, 1973). The extracellular degranulation is impaired by agents that increase intracellular cyclic AMP levels and by substances that can interact with microtubules (Zurier et al., 1974). On the other hand, compounds that may increase intracellular cyclic GMP concentrations further enhance extracellular degranulation (Zurier et al., 1974; Ignarro, 1974). The findings have been interpreted to mean that assembly of microtubules is crucial for degranulation and that the assembly might be regulated by cyclic nucleotides (Zurier et al., 1974). Since these substances tend to have similar effects on migration and ingestion, a close relation of the mechanism by which they occur to that of degranulation is possible. The actual events that are responsible for the fusion of granule and phagosomal membranes during normal degranulation are still unknown.

7. The Biochemistry of Phagocytosis

Ingestion of particles is an active energy-dependent event. In the guinea pig, PMN QO_2 increases two-to-three fold and this increase is almost entirely insensitive to cyanide. The glucose consumption and lactate production

increases by about 25% and the amount of glucose metabolized via the hexose monophosphate shunt (HMPS) increases from about 1% in resting cells to 10% in phagocytosing cells. H_2O_2 is produced in the respiratory burst. The incorporation of labelled glucose and acetate carbon, and of phosphate and lysolecithin, into lipids is stimulated, tentatively suggesting that membrane synthesis or turnover is affected by ingestion of particles (Karnovsky et al., 1972).

a) Carbohydrate metabolism

It is well established now that PMN's depend very heavily on glycolysis for their energy supply (Cohn et al., 1960; Sbarra et al., 1959). The citric acid cycle (Krebs cycle) is less important in these cells (Karnovsky, 1962) and indeed this might be expected from the examination of their ultrastructure, which reveals rather few mitrochondria in mature PMN (Low et al., 1958). Nevertheless, these cells do exhibit a modest Pasteur effect (depression of glycolysis under aerobic conditions) and a Crabtree effect (depression of respiration by glucose) (Oren et al., 1963; Sbarra et al., 1959). Phagocytosis does result in increased glycolysis, upon which the ingestion of particles has been shown to be dependent (Martin et al., 1958). The critical fact is that phagocytosis appears to be equally efficient under aerobic or anaerobic conditions (Sbarra et al., 1959). However, the curious observation was made that respiration is greatly stimulated during phagocytosis (Karnovsky, 1962; Sbarra et al., 1959) and that this is linked to an enhanced passage of glucose carbon through the

hexose-monophosphate shunt (Karnovsky, 1962; Stähelin et al., 1957; Karnovsky, 1968). This linkage has been the subject of several speculations since it could not be altered by metabolic inhibitors of mitochondrial function such as cyanides. Furthermore, phagocytosis is accompanied by the formation of H_2O_2 which was infered from the important finding of Lyler et al. (1961) that formate oxidation in PMN was stimulated during phagocytosis (Karnovsky, 1968; Nathan et al., 1970).

b) Lipid metabolism

Lipid biosynthesis, as indicated by the incorporation of ^{14}C-acetate, occurs in granulocytes (Elsbach, 1959; Sbarra et al., 1960; Karnovsky, 1968). Two-thirds of the radioactivity is incorporated in the neutral lipids and the remainder into phospho-lipids (Marks et al., 1960). ^{32}P was rapidly incorporated into lecithin, phosphatidyl ethanolamine, phosphatidyl serine, inositol phosphatide and sphingomyelin (Firkin et al., 1961; Karnovsky et al., 1961; Sbarra et al., 1960; Karnovsky, 1968). A striking stimulation of the incorporation of lysolecithin in lecithin occurs during phagocytosis.

c) Peroxide formation and detoxification

(i) NADPH/NADH oxidase: The search for the enzymatic basis of the respiratory burst and increased peroxide production which occur during phagocytosis has been carried out in many laboratories. The characteristics of the enzyme system must include insensitivity to cyanide and hydrogen peroxide must be the product. Several potential leukocyte enzymes, including NADH oxidase, NADPH oxidase and amino acid oxidase have been investigated. Cagen et al. (1964) demon-

strated NADH oxidase activity in leukocytes obtained from the peritoneal cavity of guinea pigs. These investigations showed that the enzyme catalyzed the reaction between NADH and oxygen to yield H_2O_2. It had a pH optimum of 5, a Km for NADH of 1×10^{-3}M, contained FAD and was insensitive to 1mM KCN. The activity of this enzyme was sufficient to explain the respiratory burst of guinea pig leukocytes and was confined to the soluble subcellular fraction in alkaline KCl homogenates. Zatti et al., (1966) have found a granule-associated NADPH oxidase in guinea pig leukocytes which also produces hydrogen peroxide. However Cagan et al. (1964) found that most of the NADPH oxidase activity was sensitive to 1mM cyanide. Later on, Cline et al., (1969) have pointed out that human and guinea pig leukocytes contain D-amino acid oxidase activity. They postulated that hydrogen peroxide might be formed as a result of the interaction of the D-amino acids of bacterial cell walls with this enzyme. However, evidence gathered in a model human disorder of PMN respiration, Chronic Granulomatous Disease (CGD), indicates that amino acid oxidase, which is normal in the CGD cells, plays little if any physiological role.

The contribution of H_2O_2 to the bactericidal function of PMN has recently become much clearer. This will be discussed further later. (See page 42).

Baehner et al. (1970) studied the human NADH oxidase and found results similar to those detected in guinea pig PMN, except that the Km was 5×10^{-4}M rather than 10^{-3}M and V_{max} was half that of the guinea pig. Baehner et al. (1968)

also found that the enzyme is deficient in the leukocytes of patients with CGD in whose PMN the respiratory response to phagocytosis is markedly reduced or absent.

It must be emphasized that in certain laboratories NADPH rather than NADH is thought to be the primary hydrogen donor for a cyanide-insensitive NADPH oxidase. Two lines of clinical evidence concerning a possible primary role for NADPH in H_2O_2 production by PMN have emerged.

First, Cooper et al. (1970) have described a patient with profound susceptibility to staphylococcal infection and totally absent granulocyte G-6-PD activity. This patient's PMN did not produce H_2O_2 and did not consume additional oxygen during phagocytosis. NADH oxidase and NADPH oxidase activities were normal. Secondly, it has been noted by Baehner et al. (1971) that the leukocytes of some caucasians with very low G-6-PD activity are somewhat less efficient in bactericidal capacity than are normal cells. These findings lend support to an important role for NADPH in H_2O_2 production by human PMN, but the findings do not necessarily imply that an NADPH oxidase is active. Evans et al. (1966) have reported pyridine nucleotide transhydrogenase activity in human PMN. It is possible that the NADH, which serves as substrate for NADH oxidase, is provided from NADPH via a transhydrogenase reaction in human cells where:

$$NADPH + H^+ + NAD \rightarrow NADH + NADP + H^+$$

If this reaction is active in PMN, a requirement for the glutathione system in the hexose monophosphate shunt (HMPS)

response to phagocytosis would not be present. Maintenance of a normal HMPS response during phagocytosis without glutathione reductase activity would lend further credence to the importance of NADPH either as a direct or indirect donor of hydrogen to oxygen via an NADPH oxidase or a transhydrogenase reaction.

(ii) Superoxide anion as an intermediate in H_2O_2 production: Univalent reduction of oxygen can result in the formation of the hydroperoxy radical ($HO_2^{\cdot}$) and its conjugate base, the superoxide anion ($O_2^{\cdot-}$). At neutral pH, the equilibrium of the reaction $HO_2^{\cdot} \rightleftharpoons H^+ + O_2^{\cdot-}$ lies to the right. The superoxide anion can be oxidized to oxygen by acting as a reducing agent as in the case of the reduction of ferricytochrome C. Or it could be reduced to H_2O_2 by acting as an oxidizing agent as in the case of epinephrine oxidation. Superoxide dismutase, an enzyme which has been detected in the cytosol of human neutrophils (Dechatelet et al., 1971) can catalyze the dismutation of two molecules of superoxide anion with the formation of oxygen and H_2O_2.

The reduction of ferricytochrome C and the inhibition of this reaction in the intact PMN by superoxide dismutase suggests the formation of the superoxide anion.

Phagocytosing leukocytes of normal subjects demonstrate increased formation of $O_2^{\cdot-}$, while those of patients with Chronic Granulomatous disease (CGD) do not (Curnutte et al., 1974). This has been taken to suggest that superoxide anion can act as an intermediate in H_2O_2 formation.

(iii) Detoxification of peroxides: The production of oxygen metabolites by phagocytosing leukocytes leads to their accumulation in the phagosome. But these metabolites can be as toxic to the leukocyte itself as to the microorganism. Mason et al. (1972) have found that H_2O_2 and hydroxyl radicals can lead to the peroxidation of membrane lipids in the phagosome. Also H_2O_2 can easily cross biological membranes and enter the cytoplasm. Superoxide anions have been detected in the extra cellular medium surrounding resting neutrophils and the rate of accumulation was increased during phagocytosis (Mandell, 1970). It is not clear whether this superoxide is synthesized at the external surface of the plasma membrane, leaked out of incompletely fused phagosomes or diffused through the cytosol and the plasma membrane from an intracellular source. Reed (1969) and Khandwala et al. (1973) have found that there are at least two systems in the cytosol of neutrophils which are capable of detoxifying H_2O_2. These are catalase and reduced glutathione. Catalase breaks down H_2O_2 that has entered the cytoplasm to oxygen and water while reduced glutathione breaks it down to water. Reduced glutathione is regenerated by means of the hexose monophosphate shunt (Reed, 1969; Khandwala et al., 1973). Large increases in the hexose monophosphate shunt activity during phagocytosis has been reported (Karnovsky, 1962). Glucose-6-phosphate dehydrogenase, an enzyme which is required for the hexose monophosphate shunt activity, appears to be involved in both the production and detoxification of H_2O_2. About 3% of the H_2O_2 produced during

phagocytosis may contribute towards formate oxidation in the presence of catalase (Baehner, 1970). The myeloperoxidase-halide antimicrobial system does utilise some of the H_2O_2 within the phagocytic vacuole (Klebanoff, 1968; Klebanoff et al., 1966). Direct catalytic conversion and the glutathione system disposes of considerable amounts of the H_2O_2 generated during phagocytosis. Sbarra et al. (1972) have found that H_2O_2 can also escape in large amounts to the extracellular medium.

8. Microbicidal systems in polymorphonuclear neutrophils

Neutrophils are equipped with a comprehensive range of antimicrobial activities enabling them to perform their functions under varied conditions. These activities can be divided into first the oxygen independent system which operates under anaerobic conditions and comprises acid, lysozyme, lactoferrin or granular cationic proteins. Secondly there is the oxygen dependent system which requires an aerobic environment and which works in conjunction with oxygen metabolites i.e. hydrogen peroxide (H_2O_2), superoxide anion ($\bar{O}_2^{\cdot}$), hydroxyl radicals ($OH^{\cdot}$) or singlet oxygen ($\underline{\bar{O}}$). This second oxygen dependent mechanism can be further sub-divided into a system which is independent of myeloperoxidase and another system dependent upon myeloperoxidase and for which a halide is needed.

This classification of the microbicidal systems of PMN and the description of their actions is based on the excellent and comprehensive recent review by Klebanoff (1975).

I Antimicrobial systems not needing oxygen

The fact that neutrophil antimicrobial activity is not abolished under anaerobic conditions suggests the presence of an oxygen-independent antimicrobial system. The presence inside the neutrophil of substances such as acid, lysozyme, lactoferrin and cationic proteins would also contribute to an oxygen-independent antimicrobial system.

a) Acid

It has long been known that the pH of the phagosome is lower than the surrounding cytoplasm (Metchnikoff, 1905). Jensen et al (1973) have confirmed such acidity which could be as low as pH 3.0. The increase in lactic acid production during phagocytosis has been associated with the drop in phagosomal pH (Kakinuma, 1970; Lehrer et al., 1969). The nature of the acid as well as the hydrogen ion concentration are important in producing an effective toxicity against a given organism (Dubos, 1953).

b) Lysozyme

Lysozyme is present in both the primary and secondary granules of neutrophils (Baggiolini et al., 1969; Cohn et al., 1960a). It is a basic protein, isoelectric point pH 10.5-11, with a molecular weight of about 14,500. Lysozyme hydrolyzes microbial cell and is more likely to be microbiolytic rather than microbicidal.

c) Lactoferrin

Lactoferrin is present in the specific granules of neutrophils (Baggiolini et al., 1970; Leffell et al., 1972;

Masson et al., 1969). It is an iron-binding protein capable of interfering with microbial growth by competing for essential iron (Bullen et al., 1972; Kirkpatric et al., 1971; Masson et al., 1966; Oram et al., 1968). Lactoferrin is more likely to serve a microbiostatic rather than microbiocidal function.

d) Granular Cationic Proteins

Cationic, heat-stable, acid resistant substances with antimicrobial properties have been isolated from neutrophils (Cohn et al., 1960a). Phagocytin was described by Hirsch (1956) and leukin by Skarnes et al. (1956). These agents have been detected inside the phagosome during phagocytosis (Spitznagel et al., 1963) and believed to interfere with microbial viability by binding to acidic groups on the surface of the organism.

II Antimicrobial systems needing oxygen

An atmosphere of nitrogen will reduce the antimicrobial activity of the leukocyte exposed to it (Holmes et al., 1968; Klebanoff et al., 1972; Lehrer et al., 1969; Mandell, 1974; McRipley, 1967; Selvaraj et al., 1966), but does not affect phagocytosis (Sbarra et al., 1959).

a) MPO-independent antimicrobial systems

Antimicrobial activity is not completely abolished in myeloperoxidase deficient neutrophils. Only the efficiency of such leukocytes is decreased, and death of ingested organisms can take five times as long to achieve the same degree of killing as that produced by normal cells (Lehrer et al., 1969a). The MPO-independent antimicrobial

systems of PMN may include H_2O_2, superoxide anion, hydroxyl radicals, or singlet oxygen.

b) MPO-mediated antimicrobial systems

The combination of myeloperoxidase, $H_2O_2$and halide produces a very effective microbicidal system acting on a wide range of cells and microorganisms including bacteria, fungi and viruses, (Klebanoff, 1970a; Lehrer, 1969; Lehrer et al., 1970; Belding et al., 1970).

Role in the intact cell:

The implication of the MPO-mediated antimicrobial system as a defence mechanism in the neutrophil has stemmed from the following observations:

(i) Myeloperoxidase, $H_2O_2$and the oxidizable cofactor necessary for the antimicrobial system are all present in the neutrophil.

(ii) H_2O_2 production and myeloperoxidase degranulation take place during phagocytosis.

(iii) The pH of the phagosome (4.5-5.0) is optimum for the MPO-mediated antimicrobial system.

(iv) Iodination of microorganisms during phagocytosis has been detected in the intact phagosome using radioautographic techniques (Klebanoff, 1970a).

(v) The impaired microbicidal activity of CGD neutrophils due to deficient H_2O_2 production (Holmes et al., 1967; Quie et al., 1967), and the ability to correct the defect by introducing H_2O_2 into the phagosome. H_2O_2-generating bacteria are killed normally by CGD neutrophils (Holmes et al., 1972; Klebanoff et al., 1969; Mandell et al., 1969;

Pitt et al., 1974). Latex particles coated with glucose oxidase or addition of extracellular H_2O_2 are both capable of reversing the defective killing (Baehner et al., 1970a; Johnston et al., 1970; Root, 1974).

(vi) Patients with leukocyte myeloperoxidase deficiency are more susceptible to infections (Davis et al., 1971; Klebanoff, 1970; Lehrer et al., 1969; Lehrer et al., 1972; Lehrer et al., 1969). Defective microbicidal activity can be achieved by treating normal leukocytes with myeloperoxidase inhibitors such as azide, cyanide, (Klebanoff, 1970) or sulfonamides (Lehrer, 1971).

Components of the Myeloperoxidase-mediated antimicrobial system:

1. Myeloperoxidase

This is a basic protein with a molecular weight of about 150,000 and contains two haemprosthetic groups per molecule (Ehrenberg et al., 1958). Myeloperoxidase is found in the azurophilic granules of the resting neutrophils of a wide variety of species (Baggiolini et al., 1969; Bainton et al., 1968; Dunn et al., 1968; Jain, 1967; Schultz et al., 1965). Myeloperoxidase concentrations as high as 5% of the dry weight of the cell have been reported (Schultz et al., 1962). Electron microscope studies demonstrate the release of the enzyme into the phagosome during the process of degranulation (Baehner et al., 1969; Klebanoff, 1970a; Stossel et al., 1971).

2. H_2O_2

The H_2O_2 for the MPO-mediated antimicrobial system can be supplied by the leukocyte metabolism and the microbial metabolism.

(i) Leukocytic metabolism: H_2O_2 synthesis in phagocytosing PMNs has been detected in a variety of techniques. These included direct measurement (see page 99) and oxidation of formate by catalase (Iver et al., 1961). Extensive investigations have been carried out to identify the enzyme system responsible for H_2O_2 production (Karnovsky, 1968; Karnovsky, 1973; Klebanoff, 1972; Rossi et al., 1972). The cyanide insensitivity of H_2O_2 production indicates that an oxidase enzyme is involved that is not a haem protein and reduces oxygen to H_2O_2. It has also to be activated by phagocytosis and located at a site which allows the accumulation of H_2O_2 in the phagocytic vacuole. Cytochrome oxidase is not involved as it reduces O_2 to H_2O, is inhibited by cyanide and is located in the mitochondria which are not abundant in the mature PMN. NADPH or NADH oxidases reduce oxygen to H_2O_2, are insensitive to cyanide and are more likely to be responsible for the respiratory burst.

(ii) Microbial metabolism: In certain microorganisms, the final steps in the oxidation pathway are catalyzed by flavo-proteins which reduce O_2 to H_2O_2. These organisms do not have enzymes for detoxifying H_2O_2 and as a result H_2O_2 can be found in the extracellular medium (Avery et al., 1924; McLeod et al., 1922; Whittenbury, 1964). Certain catalase negative bacteria such as the pneumococci, streptococci and lactobacilli can produce varying amounts of H_2O_2 depending on the incubation conditions and the energy source (Avery et al., 1924; Paul et al., 1968; Whittenbury, 1964). In

neutrophils with impaired H_2O_2 production as in the case of CGD, the microbial production of H_2O_2 corrects the cells ability to kill the organism.

3. Cofactor

(i) Chloride: This ion is present in all cells including leukocytes as well as in the surrounding plasma at concentrations which exceed the minimum requirements of the system (Klebanoff, 1968).

(ii) Iodide: This ion is present in the human serum at very low concentrations less than 1 mM/1 compared with 95-105 mM/1 for chloride, but it is the most effective cofactor for the MPO-mediated antimicrobial system. Intracellular concentration of iodide can be actively raised by the leukocytes (Siegel et al., 1964).

(iii) Thyroid hormones: An additional supply of iodide for the MPO-mediated antimicrobial system can result from the degradation of thyroxine (T4) and triiodithryonine (T3) by the phagocytosing leukocytes (Klebanoff et al., 1973; Klebanoff et al., 1972; Woeber et al., 1972; Woeber et al., 1973). The ability of the thyroid hormones to bind to the leukocyte plasma membrane (Klebanoff et al., 1972; Roche et al., 1962) as well as to microorganisms (Klebanoff et al., 1972) facilitate their entry to the phagosome.

Chloride, iodide and thyroid hormones as well as any other unidentified cofactors add up to a significant pool which is unlikely to be limiting in the MPO-H_2O_2 cofactor antimicrobial system (Klebanoff, 1975).

4. Acid

It is well established that the acidity inside the phagocytic vacuole drops from neutrality to an acidic pH within the first ten minutes of phagocytosis (see page 28). It is also well known that myeloperoxidase has a pH optimum of about 4.5 (Klebanoff, 1967; Klebanoff, 1968; McRipley et al., 1967).

Inhibitors:

1. Catalase

Catalase competes with peroxidase for H_2O_2 and renders the MPO-mediated antimicrobial system ineffective by depriving it of H_2O_2. Catalase positive organisms such as Staphylococcus aureus are likely to be more virulent and less susceptible to attack by normal neutrophils than catalase negative ones (Mandell, 1974a).

2. H_2O_2

The mechanism by which H_2O_2 reacts with Myeloperoxidase was proposed by Agner in 1963. This involves the formation of a complex between the haem iron groups of the MPO and the H_2O_2, the degradation of H_2O_2 to O_2 and H_2O and the inactivation of the enzyme. This mechanism could explain the observed inhibition of MPO by excess H_2O_2 (Agner, 1941; Agner, 1963: De Chatelet et al., 1971) and provide possible control of the antimicrobial system.

Mechanism:

The current theory regarding the MPO-mediated antimicrobial system involves the formation of a complex or complexes between the H_2O_2 and the haem iron groups of the

MPO. These complexes oxidize the halides which in turn exert a strong antimicrobial action.

1. Iodide

(i) Iodination: A large iodine atom replacing hydrogen in vital locations can be very damaging to the metabolism of microorganisms. Microbial proteins, in particular tyrosine, can be easily iodinated leading to the death of the organism (Klebanoff, 1967).

(ii) Oxidation: The oxidation of iodide produces strong oxidizing agents which are capable of oxidizing vital components of the microorganisms. Lipid peroxidation has been described (Welton et al., 1972). Inactivation of enzymes by oxidation of essential sulfhydryl groups has also been proposed as the mechanism for the microbicidal action of iodine (Green et al., 1946).

2. Bromide

The possible contribution of bromide to the antimicrobial system is very similar to that of iodide.

3. Chloride

The action of chloride in the MPO-mediated antimicrobial system has been attributed to the formation of "activated chloride" as follows:-

$$\text{MPO}\begin{cases}\text{Fe}\\ \text{Fe}\end{cases} + Cl^- + H_2O_2 \rightarrow \text{MPO}\begin{cases}\text{Fe}\ {}^{\cdot}\text{HOOH}\\ \text{Fe}\ {}^{\cdot}\text{Cl}\end{cases} \rightarrow \text{MPO}\begin{cases}\text{Fe}\\ \text{Fe}\end{cases} + \text{"activated chloride"}$$

The "activated chloride" within the phagosome could act in a similar manner to that of hypochlorous acid (HClO) which is believed to be the active microbicidal agent produced when

chlorine disinfectants hydrolyze in swimming pools (Sykes, 1965). "Activated chloride" can exert its germicidal action in a number of ways:-

(i) Oxidation of sulfhydryl groups: Optimum activity of enzymes containing sulfhydryl groups have been found to be adversely affected by the presence of bactericidal concentrations of chlorine derivatives (Green et al., 1946; Knox et al., 1948). The inhibition of such enzymes will interfere with the metabolic pathways of the organism and lead to cell death (Babior et al., 1973; Knox et al., 1948).

(ii) Iodination: Iodide can be oxidized to iodine by the "activated chloride" (Sykes, 1965). Chloride, therefore, can stimulate microbial iodination (see page 46) under conditions of low iodide concentrations (Klebanoff, 1970a).

(iii) Degradation of the parent compound: Deamination and decarboxylation of amino acids at acid pH by the MPO, H_2O_2 and chloride with the formation of aldehydes has been demonstrated (Zgliczynski et al., 1971). Microbicidal aldehydes have been implicated in the mechanism of action of the MPO-H_2O_2-chloride antimicrobial system (Sbarra et al., 1972; Strauss et al., 1971).

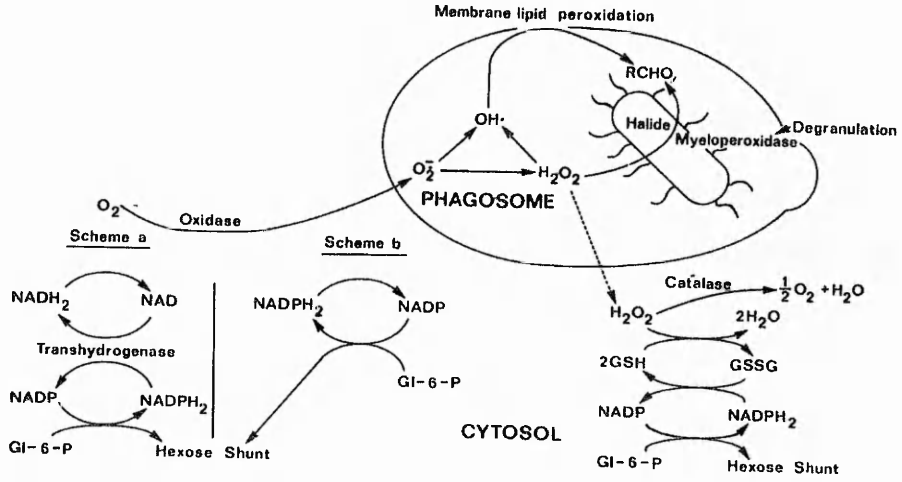


Fig 1 Possible mechanisms for the production, action and detoxification of peroxides in polymorphonuclear neutrophils.

Our knowledge of the mechanism of action of the MPO-mediated antimicrobial system is by no means complete and no doubt other mechanisms will come to light.

Pages 49 to 53 inclusive have been deleted.

C H A P T E R T W O

THE MYELOPROLIFERATIVE DISORDERS

The hypothesis of the myeloproliferative disorders is based on the functional unity of the marrow, not only as it functions from birth onwards, but also including the periods which precede the participation of marrow in blood production in foetal life. The tissues involved in foetal blood production may regain their capacity to produce blood cells in response to certain stimuli.

In 1951, Dameshek proposed that the term Myeloproliferative disorder should be used, pointing out that the bone marrow cells, erythroblasts, granulocytes, megakaryocytes and fibroblasts often proliferate as a unit rather than as a single element. Different combinations of disordered cell growth can occur in individual patients during the course of the disease. Spontaneous remissions are unlikely while progressive deterioration is more common.

1. Chronic Granulocytic Leukaemia (CGL)

Chronic Granulocytic Leukaemia is perhaps the most uniform, well-defined and "typical" entity in the Myeloproliferative group of diseases. It was the first type of leukaemia to be discovered and for many decades it was known as "splenic" leukaemia (Craige, 1845).

a) Definition:

CGL can be defined as a neoplastic disease associated with a unique chromosome abnormality in which the major clinical manifestations relate to abnormal, excessive, and apparently unrestrained overgrowth of granulocytes in the bone marrow, large numbers of immature myeloid cells characteristically circulate in the blood, and extramedullary myelopoiesis produces gross enlargement in the spleen and liver. During the terminal phase, all therapeutic agents are comparatively ineffective and death finally results.

b) Incidence:

It accounts for about 20% of leukaemia cases in Western countries and the death rate from it is about 1 per 100,000 population per year (Dameshek et al., 1964). The incidence of CGL in recent years does not seem to have increased like that of acute leukaemia and chronic lymphocytic leukaemia (Cutler et al., 1967). CGL occurs rarely during the first few months of life but, when it does, its course tends to be relatively acute. There is no well-defined hereditary liability and children born of mothers who have the disease are not affected (Ask-Upmark, 1964). The typical disease is occasionally seen in adolescents but the

great majority of patients are in their middle years (25-60). The peak incidence of CGL is in the fifth decade of life (Dameshek, 1964; Cutler et al., 1967; Shimkin et al., 1951). In recent decades, it seems that the appearance and course of the disease are identical in both sexes (Shimkin et al., 1951) and not influenced by racial and geographical factors.

c) Clinical findings:

The disease may be present for a long time, even with increased white blood count and enlarged spleen, before there are clinical manifestations. The earliest signs of disease come from one or more of the following changes: pressure of an enlarged spleen or liver, symptoms from increased metabolism, loss of weight, nervousness, perspiration and deterioration of the nutritional state. Fever and bleeding may occur later. In a patient found to have a neutrophil leukocytosis without obvious cause the possibility of early granulocytic leukaemia should always be considered. Enlargement of the spleen is most pronounced in Chronic Granulocytic Leukaemia.

d) Blood:

(i) Microscopic appearance: In advanced cases the blood is thick and sticky and even smears are difficult to prepare. In the haematocrit tube there is a thick buffy coat and a thick layer of platelets.

(ii) Red cells: In early stages there may be no anaemia but it appears sooner or later. Its intensity varies and, as a rule, it is normocytic with anisocytosis. Poikilocytosis is rare. Normoblasts, polychromatophilia,

basophilic stippling and reticulocytosis may be present.

(iii) Platelets: Early in the disease the platelet count is normal or slightly increased, later it may be markedly increased. An increase from subnormal to normal, or a fall from excessively high to normal, is indicative of clinical improvement. A persistently low count is a poor prognostic sign.

(iv) White cells: The white cell count is greatly increased, ranging from 50,000 to 300,000 mm^{-3} and even higher. Myeloblasts are usually few; myelocytes and metamyelocytes are numerous with myelocytes may be increasing to 50%. The blood smear may be as cellular as a smear of a normal sternal marrow. There is an increase of eosinophils and basophils, the latter 3 to 20%. The number of basophils is usually higher than in any other disease. A leukocytosis with fewer immature white cells offers a better prognosis than when the outpouring of undifferentiated cells is more pronounced. The presence of large numbers of undifferentiated white cells early in the disease is a particularly ominous prognostic indicator. In the terminal phase, large numbers of myeloblasts appear in the blood and marrow - an acute "blastic crisis".

(v) Leukocyte alkaline phosphatase: (LAP) is greatly reduced or absent in most patients with Chronic Granulocytic Leukaemia (Dameshek et al., 1964). Although a low LAP is characteristic of CGL, it is not specific. In some cases, during remissions of CGL with a normal blood picture, the LAP continues to be low. In others it returns to normal and

may increase in response to infection as it does in normal individuals.

e) Chromosomal abnormalities:

In direct bone marrow preparations and in metaphases of cultured peripheral blood, most patients with CGL have an abnormally small acrocentric chromosome in the 21 group. This was first described in 1960 by Nowel & Hungerford and is called the Philadelphia (Ph^1) chromosome. The Ph^1 chromosome is present in blood and marrow cells during relapse and can be found in the marrow but not the blood during remission. It appears that the Ph^1 chromosome is present in precursors of granulocytes, in erythroblasts and in megakaryocytes, but not in lymphocytes or skin cells.

The small proportion of patients with CGL who lack the Ph^1 chromosome are characteristic in most other respects: average age, spleen size, marrow and blood picture and LAP values. On average, however, the patients in this Ph^1-negative group have less elevated white blood cell counts, have lower platelet counts, include a larger proportion of children, respond less well to therapy and have shorter survival (Tijo et al., 1966).

f) Marrow findings:

(i) Gross: Appearance is grey or greyish red and abundant even in locations normally devoid of cellular marrow, for example in long bones.

(ii) Microscopic: Examination shows granulocytic hyperplasia. The E:G (erythrogranulocytic) ratio changes from the highest normal of 1:6 to about 1:10 and higher.

(iii) Cytology: Granulocytic cells of all types are increased with an overabundance of cells at each end of the granulocytic spectrum - myeloblasts and promyelocytes at one end and band and segmented forms at the other - and depression of nucleated red cells late in the disease. Frequently there is an increase of megakaryocytes, usually with an accompanying thrombocythemia. Eosinophilic and basophilic granulocytes are frequently more numerous.

g) Therapy:

Irradiation was first used by Senn in 1903. The life-expectation averaged about 3-5 years. This was local X-ray or radium therapy given over the spleen, long bones or areas of leukaemic infiltration. Radioactive phosphorus (^{32}P) was first used in 1936 and provided internal radiation (Lawrence et al., 1948). Other agents have been used such as Busulphan and Nitrogen mustards (Haddow et al., 1953; Galton, 1953). These agents suppress the growth of some rapidly dividing cells and are the most generally effective in the treatment of CGL. Busulfan is unique in that it exerts virtually no pharmacological action other than myelosuppression. At low doses, selective depression of granulocytopoiesis is evident. Erythroid elements may be suppressed as the dosage is raised and eventually a pancytopenia results. Cytotoxic action does not appear to extend to either the lymphoid tissues or the gastrointestinal epithelium.

2. Myeloid Metaplasia (MM)

There are about 25 synonyms for Myeloid Metaplasia

(MM), reflecting the aspect which appeared most striking to the diagnostician, e.g. Myelofibrosis, Myelosclerosis and Megakaryocytic Splenomegaly. Today, the disease described under these various names is looked upon as a single entity. As early as 1879 Heuck reported bone marrow sclerosis in a patient who appeared to have had "splenic leukaemia".

a) Definition:

This is a chronic, progressive panmyelosis characterized by varying degrees of fibrosis of the marrow, massive splenomegaly due to extramedullary hematopoiesis, and a leukoerythroblastic anaemia with marked red cell abnormalities, circulating normoblasts, immature granulocytes and atypical platelets (Dameshek et al., 1964).

b) Incidence:

MM occurs most commonly in middle-aged and elderly individuals, with a mean age of onset of 58.4 years (Silverstein et al., 1967), with a number of cases in the third and fourth decades and a few in childhood (Rosenberg et al., 1958). Both sexes are equally affected. There are no adequate statistics on its incidence, but experience suggests that it is of the same order as that of CGL. An increased number of cases of MM occurred in Hiroshima in the years after the atomic bombing (Anderson et al., 1964) and benzol poisoning has been stated to cause the condition (Rawson et al., 1941). MM may arise late in the course of Polycythaemia Vera, and a number of MM patients terminate with acute Granulocytic Leukaemia. Both of these facts indicate a close connection between MM and other

Myeloproliferative disorders.

c) Clinical findings:

The disorder has an insidious onset with weight loss, signs and symptoms of anaemia and abdominal discomfort due to the large spleen. Often the liver is also enlarged. On X-ray, diffuse or patchy osteosclerosis may appear in ⅓ to ½ of patients; osteoporosis may also be seen.

d) Blood:

A moderate normochromic, normocytic aneamia (frequently with some hypochromic cells and basophilic stippling), moderate anisocytosis and marked poikilocytosis, including prominent teardrop forms and elongated red cells, are characteristic. Normoblasts are often present in numbers out of proportion to the degree of anaemia and a slight reticulocytosis is frequently found. The anaemia may have a complicated origin, with components of marrow failure, ineffective erythropoiesis and hemolysis. The leukocyte count is normal or, more commonly, moderately increased, immature neutrophils and occasionally even myeloblasts are present. The leukocyte alkaline phosphatase is often elevated, may be normal but rarely is decreased. Chromosomal studies haye not shown the presence of the Philadelphia (Ph^1) chromosome which is so characteristic of Chronic Granulocytic Leukaemia. Basophils are often increased in number. Platelets are normal or decreased in number (rarely increased) and often are atypical with distinct "zones" (a clear hyaloplasm and a central pale chromomere which lacks the usual concentration of azurophilic granules). Small

megakaryocytic fragments, the size of lymphocytes with both nucleus and cytoplasm (dwarf megakaryocytes) or small megakaryoblasts, may usually be found if searched for, and on rare occasions, they are present in considerable numbers.

e) Marrow:

It is usually impossible to aspirate marrow from MM cases and a needle biopsy or a surgical biopsy is necessary for adequate study of the marrow, especially later in the course of the disease. If examined early, the marrow may be hypercellular with panmyelosis and prominently increased megakaryocytes which are frequently abnormal. In histologic sections, there is a diffuse increase in reticulin fibres, demonstrable with silver stains and patchy fibrosis may be present. Later, the marrow becomes more fibrotic, with residual islands of atypical megakaryocytes, erythroid, and granulocytic precursors. The fibrosis is of loose connective tissue with scanty collagen, but reticulin fibers are abundant. Foci of osteoid may be found and the bony trabeculae are sometimes irregularly thickened (Myelosclerosis). The marrow may show a mixture of hyperplasia and fibrosis in one sample, or may differ in different sites of the body (Davidson et al., 1969).

f) Course:

A small but significant proportion of cases of myelofibrosis with myeloid metaplasis are a late stage, (after many years progression) of typical Polycythaemia Vera. The usual course is one of progressive anaemia and enlargement of the spleen. Hemolysis frequently becomes

an increasing element in the anaemia and infections may be a serious problem. The average survival is slightly longer than that of Chronic Granulocytic Leukaemia, but considerably less than that of Polycythaemia Vera. However, patients may occasionally live as long as 10 to 15 years. Frequently, in patients with longer survival, the terminal event is an acute blastic leukaemia.

g) Therapy:

Therapy in general unsatisfactory. The commonest symptom needing relief is anaemia. Radiotherapy and chemotherapy have been used but with little effect in prolonging survival (Silverstein et al., 1967), which averages 2 to 3 years, with 8% of patients living 6 to 10 years.

3. Polycythaemia Rubra Vera (PRV)

Polycythaemia is a condition in which the erythrocyte count is above the normal for the patient's age and sex. Usually, the hematocrit and hemoglobin are also elevated.

a) Definition:

PRV was described in 1892 by Vaquez, early in his career, as a "special form of cyanosis" with persistent and excessive hyperglobulia and splenomegaly. It is one of the myeloproliferative disorders characterized by excessive proliferation of erythroid, granulocytic and megakaryocytic elements in the marrow and also in extramedullary sites, and reflected in the blood predominantly as an absolute increase in the red cell mass and also by leukocytosis and thrombocytosis. The production of red cells appears to be autonomous, but it does respond to erythropoietin when

the patient has become anaemic through blood loss. The cause of this panmyelosis and pancytosis is unknown.

b) Incidence:

Accurate information is not available. If strict diagnostic criteria are used, it would appear to be a rare disorder. The minimal (annual) incidence rate was found to be 4 to 5 new cases per million population per year in the Baltimore, Md area (Modan, 1965), or from 0.6 to 1.6 per 100,000 population (Silverstein et al., 1971). The disease is more frequent in men than women (1.5:1) (Modan, 1965a). It begins in middle age and its prevalence is highest after 50. The influence of racial and geographical factors is not clear. Jews have greater-than-expected incidence, while, in contrast, it rarely occurs in Negroes (Wasserman et al., 1959; Modan, 1965a; Damon et al., 1958). A family history of PRV is rarely obtained.

c) Clinical findings:

Affected patients exhibit a peculiar and striking ruddy cyanosis. Splenomegaly is present in two-thirds of patients. Thrombotic or hemorrhagic phenomena occur in about one-half of patients. Myocardial infarction, cerebral thrombosis, splenic infarction, pulmonary infarcts and thrombophlebitis account for the most frequent thrombotic episodes. Upper gastrointestinal bleeding, often from peptic ulcer, is the most common bleeding problem (Wasserman & Gilburt, 1966). Pruritus, especially after bathing, is common. Polycythaemia, occuring with hypertension but without splenomegaly, has been referred to as Gaisböck's syndrome. Polycythaemia in

combination with hepatic cirrhosis is known as Mosse's syndrome.

d) Blood:

The erythrocytes number 7-12 x 10^6 mm^{-3}, and the hemoglobin is 18-24 g. $100cm^{-3}$. The MCV, MCH, and MCHC are normal or low. The red cells are hypochromic and microcytic if chronic blood loss has occurred. Macrocytes, microcytes, polychromatic cells and normoblasts may be found but are not a prominent feature of the disease. Red cell production is increased. Red cell destruction is normal during the period of erythrocytosis. Later in the disease, as splenomegaly develops, the red cell survival diminishes. The total blood volume is increased, primarily because of the increased red cell mass, though the plasma volume may also be elevated to a lesser degree. Blood viscosity is high and it may be difficult to prepare good smears. The ESR is reduced. The platelet count is increased in about two-thirds of patients, often to levels exceeding 1 x $10^6 mm^{-3}$. Moderate neutrophilic leukocytosis in the range of 10,000 to $30,000 mm^{-3}$ is the rule. Immature granulocytes are seen in about one-half of cases and basophils are often increased. The leukocyte alkaline phosphatase is almost always markedly elevated (Anstey et al., 1963).

e) Marrow:

The marrow is characteristically hypercellular, with all of the elements (erythroid, granulocytic and megakaryocytic) sharing the hyperplasis. Fat is decreased. Storage iron is decreased or absent.

f) Course and therapy:

Polycythaemia Vera is a chronic disease, patients usually live 10 to 20 years under good control. Phlebotomy, Busulphan and P^{32} have been used to control the manifestations of the disease.

In about 10 to 20 per cent of patients, progressive anaemia, gradual splenic enlargement and further elevation of the leukocyte count, with more immature granulocytes and more circulating nucleated red cells, may occur. Bone marrow aspiration becomes impossible because of myelofibrosis and splenomegaly is due to increasing extromedullary hematopoiesis. The manifestations at this stage of the disease are indistinguishable from myelofibrosis with myeloid metaplasia. The latter, therefore, is not commonly a sequel of Polycythaemia Vera (Dameshek et al., 1964).

Another late complication of Polycythaemia Vera is acute blastic leukaemia. At the present time, it is not clear whether this is aetiologically related to prior therapy with X-ray or P^{32} (Gardner, 1966).

CHAPTER THREE

METHODS

1. Background

The technique used for measuring the rate of phagocytosis and killing of Candida, (that provided the basis for the methods used in this work), was first described by Lehrer & Cline (1969) and later modified by Lehrer, (1970). It involves differential staining of living and dead Candida organisms so that ingestion and killing can be measured by direct microscopy and avoids laborious and inaccurate plating and colony counting. Lehrer & Cline (1969a) showed that for Candida albicans, exclusion of methylene blue, correlates well with the ability of organisms to form colonies in culture. More recently Lehrer (1970) showed that uptake of Romanowsky stains using fixed preparations also correlates with colony formation and with exclusion of methylene blue. Leishman staining of fixed preparations has been used in this study and pink staining cell ghosts are considered to be dead, and dark blue staining organisms to be living. Failure to stain dark blue with Leishman is probably due to degradation of the cell wall and loss of nucleotides (Lehrer, 1970).

When phagocytosis is measured by the technique of Lehrer & Cline (1969a) there should be an excess of organisms so that every neutrophil has the opportunity to ingest.

By contrast, when measuring Candida killing an excess of organisms is usually avoided so that all the Candida are rapidly ingested and none remains outside the phagocyte at the end of the incubation (Goldman & Th'ng, 1973). However, if the ratio of organisms to phagocytes is too low then some neutrophils will ingest several organisms while others fail

to ingest any, and a defect affecting part of the cell population may not be detectable. Thus, for any particular experimental system the optimum ratio of organisms to cells must be determined to give complete phagocytosis within a relatively short incubation and at the same time allow ingestion of organisms by all or almost all the neutrophils.

Lehrer & Cline (1969a) and other investigators (Rosner et al., 1970; Goldman & Th'ng, 1973) have used Candida albicans because it is a pathogenic organism, but if the ratio is greater than two Candida albicans per neutrophil there is an increasing tendency for the Candida to grow into hyphal forms within the neutrophils and then to kill the cells (Rosner et al., 1970). This is one reason why a ratio of 1:1 or 2:1 has been used with the associated problem of not testing the whole of the neutrophil population. The difficulty can be avoided by using a less pathogenic species, Candida guillermondii, which always remains in the yeast form and does not kill the neutrophils (Goldstein et al., 1965). Using this organism we have determined the optimum ratio and timing for measuring the rate of both phagocytosis and killing by normal cells.

Differential characters of Candida species are identified according to Martin et al. (1937). The mycology reference laboratory, Public Health Laboratory Service (London School of Hygiene and Tropical Medicine) carried out the identification of the Candida species used in this study.

2. Reasons for the use or determination of the parameters investigated

a) Candida guillermondii as the test organism:

Candida albicans has been used by many workers to study leukocyte function, that is phagocytosis and killing. Its relatively large size (4μm) facilitates observation. Also it is a potential pathogen in patients with leukaemia or myeloproliferative disorders. Candida guillermondii, however, even though less pathogenic, has other advantages over C. albicans:

1. Filamentous growth does not occur on exposure to human serum.
2. It does not form filaments when within the neutrophil.
3. It does not kill the neutrophils.
4. It does not give false low colony counts caused by aggregation of "germinating" Candida cells into large clusters of pseudomycelia.

b) Specific staining rather than colony counting or dye-exclusion:

If Leishman-stained slides are prepared shortly after viable Candida guillermondii have been ingested by leukocytes, the intracellular yeast appears spherical and their cytoplasm takes a uniform dark blue stain. If such slides are prepared after several hours of incubation have elapsed, two classes of Candida cells can be discerned within the phagocytes. One is composed of organisms that have maintained

their cytoplasmic basophilia and clearly have not been digested despite their intracellular location. The other class consists of oval organisms that have lost cytoplasmic basophilia and appear pink. These "altered" Candida cells, called ghosts, represent nonviable organisms that have been depleted of their cytoplasmic RNA after death within the phagocyte. The percentage of phagocytozed organisms that are converted into ghosts can provide an indicator of the Candidacidal ability of the neutrophils.

In the dye-exclusion method, aqueous methylene blue stains the cytoplasm of nonviable Candida cells blue, whereas viable organisms remain unstained.

The "specific staining" method has a number of advantages. First, it avoids the conventional colony counting method which can be laborious, inaccurate and expensive in terms of time, money and space. Unlike colony-count methods which register loss of an organism's replicative potential, "specific staining" detects major alterations in the permeability or metabolic state of an organism, which under the test conditions, are indicative of its death. In addition, whereas colony count methods register changes in the colony-forming units (each unit being composed of one or more individual cells), the "specific staining" method records changes in the state of individual cells. Despite such differences, the two methods yield concordant results in studies with human neutrophils and Candida guillermondii. (Unpublished observations).

Secondly, unlike the dye-exclusion method which requires observation of a wet preparation under the microscope, within a short period of the end of the incubation, hence limiting the number of investigations per day, the "specific staining" method uses dry preparations which can be examined after any length of time, even days. This provides flexibility, and allows a larger number of patients to be tested each day.

Thirdly, unlike colony counting, "specific staining" does not require incubation of plates, thus eliminating waiting and fear of contamination and mislabeling.

Also, in addition to being the simplest method, the "specific staining" technique is cheap and reliable.

c) Qualitative and quantitative estimation of leukocyte alkaline phosphatase:

Many studies have shown striking and consistent differences in the concentration of leukocyte alkaline phosphatase (LAP) in normal as compared with abnormal leukocytes, both by chemical and cyto-chemical methods. LAP is contained in the secondary or specific granules of the mature neutrophils and although its real function is unknown, it is assumed to be concerned with the integrity of lipid membranes as it binds to them very rapidly. Normally, about 20% of mature granulocytes are alkaline phosphatase positive. Wachstein (1949) showed that in infections both the number of LAP-positive cells and the strength of the reaction is greatly increased, but in Chronic Granulocytic Leukaemia (CGL), in striking contrast, there is

a decrease in the LAP-positivity of the cells. Only two of Wachstein's thirteen cases of Chronic Granulocytic Leukaemia showed an activity comparable to that of normal blood. Mitus et al. (1968) found that LAP is much higher than normal in Polycythaemia Vera and in many cases of myelosclerosis with myeloid metaplasia. Mitus et al (1958) have shown that high, normal and low LAP values can be found in individual cases of myelosclerosis. The low LAP concentrations found in CGL are quite striking and serve as an empirical diagnostic criterion. However, a low LAP content does not by itself mean a diagnosis of CGL. It was tempting to find out whether there was any correlation between the LAP values and neutrophil phagocytosis and killing ability.

d) Qualitative and Quantitative estimation of leukocyte peroxidase:

The peroxidase contents of granulocytes and the absence of the enzyme from cells of the lymphocytic series has been known for many years. Peroxidase is contained in the azurophilic granules which can first be demonstrated in promyelocytes, but there is none in the secondary or specific granules which are developed at a later stage of maturation.

From the diagnostic point of view, the peroxidase reaction has a limited usefulness. If positive, it characterizes a given class of cells as belonging either to the granulocytic or monocytic series, but it can not distinguish definitively between the two. A negative peroxidase reaction is against a diagnosis of acute granulocytic leukaemia.

From the functional point of view, peroxidase is much more important. The peroxidase-peroxide-halide system, described by Klebanoff, (1968) appears to be the most important microbicidal mechanism in neutrophils. Therefore, measurements of neutrophils peroxidase levels as well as phagocytosis and killing of Candida might reveal possible correlation between impaired Candidacidal activity and peroxidase deficiency.

e) Qualitative and quantitative estimation of leukocyte acid phosphatase and qualitative estimation of β-glucuronidase:

These two lysosomal enzymes are contained in the azurophil or primary granule of the granulocytic series, that is the same granule containing the peroxidase. It is of interest to find out whether these two enzymes behave in a manner similar to that of the peroxidase.

f) Quantitative determination of leukocyte H_2O_2 production during phagocytosis:

Phagocytosis of microorganisms by polymorphonuclear neutrophils (PMN) is followed by stimulation of oxygen consumption (Baldridge et al., 1933). ——→ H_2O_2 production (Iver et al., 1961), and oxidation of glucose through the hexose monophosphate pathway (Sbarra et al., 1959). Together with halides and myeloperoxidase, H_2O_2 forms a bactericidal system which eventually results in the killing of the ingested organism (Klebanoff, 1968). It would be very helpful if the resulting H_2O_2 could be measured quantitatively and related to the

peroxidase values and the ability of the PMN to phagocytose and kill efficiently.

3. The estimation of neutrophil function method

a) Candida preparation:

Candida guillermondii was cultured overnight in glucose broth. The suspension was sonicated for 30 s at setting 5 to break up any clumps into individual yeast cells (see fig. 2).

Fig 2. A Leishman stained preparation of Candida guillermondii before sonication. (X1000).

Candida was resuspended in cold ($4^{o}C$) tissue culture medium (TC 199) at a concentration of 4×10^{7} organisms per cm^{3}. This suspension was used immediately to avoid further budding. Trypan blue exclusion confirmed more than 99% viability.

b) Leukocyte preparation:

Venous blood (20cm^3) was taken into 500 I.U. preservative free heparin. This was allowed to mix in a sterile plastic universal bottle on a rotating wheel. A smear was prepared and stained with Leishman's stain prior to a differential white cell count.

Plasmagel (5cm^3) was added to the blood (20cm^3) and mixed for 5 minutes.

The mixture was drawn into a 20 cm^3 plastic syringe. This was inverted at an angle of 45^o and incubated at 37^{o}C for 45', allowing the red cells to sediment. (See fig. 3).

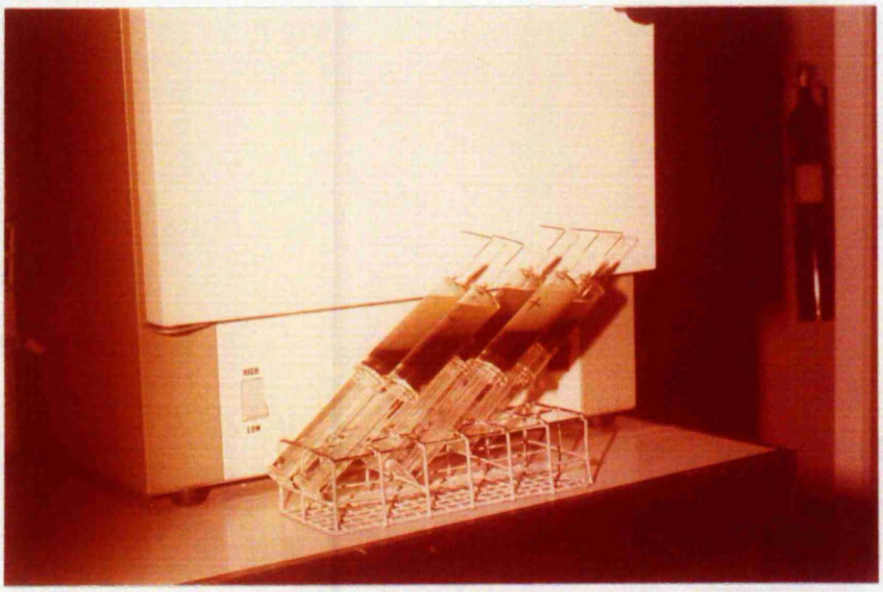


Fig. 3. Blood sedimentation for leukocyte rich plasma preparation.

The leukocyte rich plasma was removed by forcing it out through a bent needle into a flat-bottomed plastic cuvette. The volume of the leukocyte rich plasma was

noted. A total leukocyte count was carried out using a Coulter counter. The leukocyte rich plasma was centrifuged at 150g for 5 min and resuspended in the plasma and plasmagel mixture at a concentration of 9 x 10^6 neutrophils per cm^3. The flat bottom cuvette and low centrifuge speed were necessary to avoid cell clumping. Only mature and band forms were counted. The leukocytes were kept at 37°C in a water bath ready for use. Viability by trypan blue exclusion was greater than 95%.

c) Incubation of Candida and leukocytes:

Leukocytes and Candida suspensions were allowed to equilibrate at 37°C for 5 min. In a round-bottomed plastic tube, 0.5 cm^3 of TC199 was added and also allowed to equilibrate for 5 min at 37°C. Candida suspension, 0.25 cm^3 was added to the tube and mixed gently. At zero time, 0.25cm^3 of leukocyte suspension was added, mixed gently and incubated without agitation. Three replicates were used.

d) Phagocytosis:

After 15 min incubation, each tube was mixed and a 0.1 cm^3 aliquot was transferred into 1 cm^3 of cold saline containing 0.006M EDTA to stop further phagocytosis. 0.1 cm^3 of this diluted suspension was used to prepare slides using the cytocentrifuge. These slides were stained with Leishman's and assessed microscopically under oil. At least 200 neutrophils were observed from each tube, and the results were the average of the three replicates.

Two separate measurements can be made for phagocytosis: first, the proportion of total neutrophils that have engulfed

one or more Candida at 15 or 90 min, which is called the percentage phagocytosis, and secondly, the proportion of total Candida that have been engulfed at 15 or 90 min, which is called the percentage uptake. (See fig. 4).

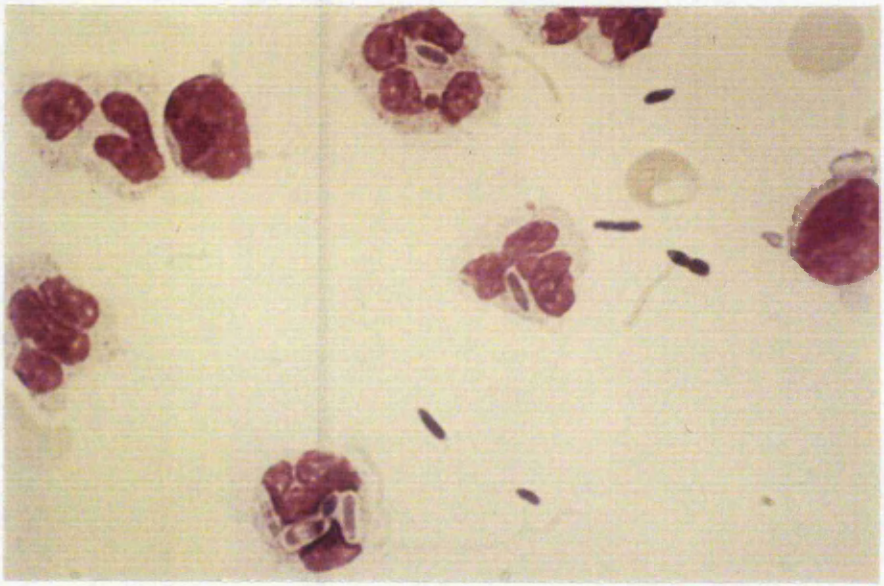


Fig. 4. A Leishman stained preparation of normal leukocytes and Candida after 15 minutes incubation. Some Candida has been phagocytosed. (X1215).

e) Killing:

After the 15 min incubation for the phagocytosis measurements, the tubes were sealed with plastic stoppers, and transferred onto a rotating wheel inside a 37°C incubator, on which they were inverted 24 times per min (see fig. 5).

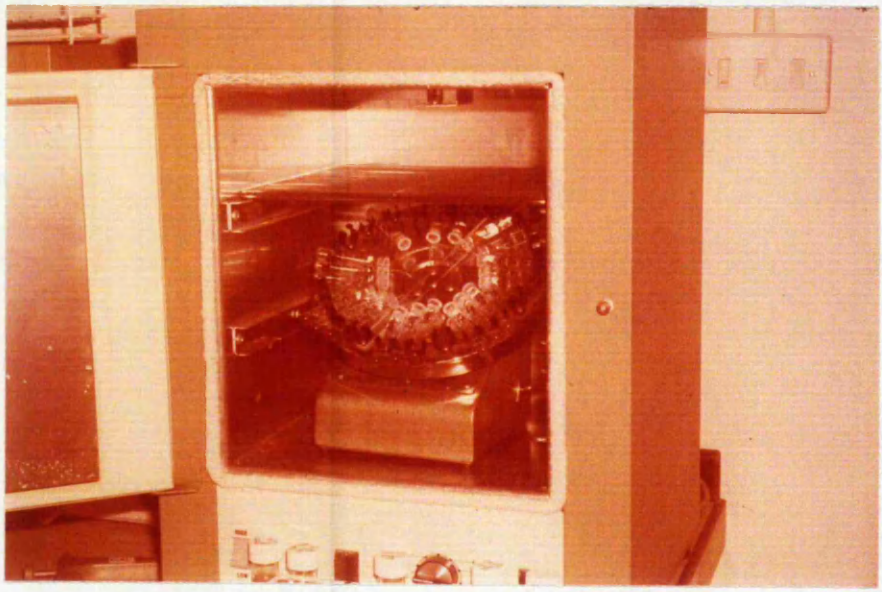


Fig. 5. Sealed incubation tubes rotating at 24 r.p.m. inside a 37°C incubator.

Incubation at 37°C was continued for 75 min making a total of 90 minutes. At 90 minutes tubes were placed in a water bath at 4°C and a 0.1cm³ aliquot was removed and diluted as before in cold saline containing EDTA. A slide was prepared and stained as before. The remaining 0.8cm³ were sonicated for 10 seconds at setting 5 to disrupt the leukocytes and release the Candida. A 0.1cm³ was removed, diluted in cold saline and a further slide was prepared and stained with Leishman.

Microscopic examination of the first slide confirms total uptake and phagocytosis, while the second slide reveals the percentage kill. This is scored by expressing the pale pink degraded organisms or ghosts as a percentage of the total Candida (see Fig. 6).

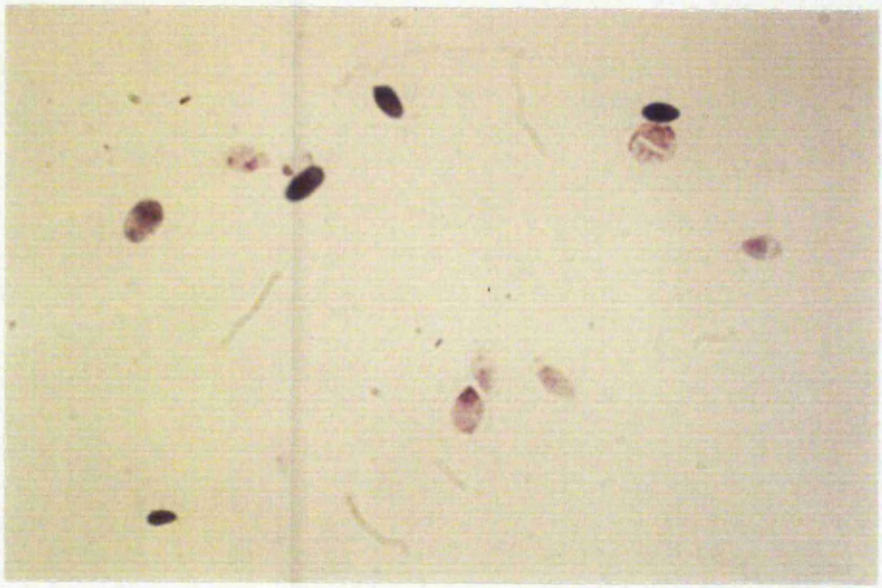


Fig. 6. A Leishman stained preparation of Candida which has been phagocytosed by normal neutrophils for 90 minutes and then released by sonication. The pink ones or "ghosts" are dead. (X1215).

4. Leukocyte peroxidase: Histochemical demonstration

The peroxidase reaction is positive in cells of the neutrophilic and eosinophilic series, and in monocytes, and can be used to differentiate cells of these types from lymphoid or erythroid cells, which are peroxidase negative. Kaplow's method (1965) using benzidine dihydrochloride was used for it's demonstration.

a) Test Principle:

The peroxidase activity of leukocytes transfers hydrogen from benzidine to hydrogen peroxide, yielding a blue derivative of the dye which is localized at the site of the enzyme activity.

b) Preparation of solutions:

(1) Fixative:

formalin (40%)	$10cm^3$
ethanol (absolute)	$90cm^3$

(2) Staining mixture was prepared by sequential addition of the following:

Ethanol, 30% (V/V) in water	$100cm^3$
Benzidine dihydrochloride	0.3g
$ZnSO_4.7H_2O$, (0.132M) (3.8%w/v)	$1.0cm^3$
Sodium acetate trihydrate ($NaC_2H_3O_2.3H_2O$)	1.0g
H_2O_2, 3%	$0.7cm^3$
NaOH (1.0N)	$1.5cm^3$
Safranin-O,	0.2g

The reagents were mixed in the order given. The benzidine dihydrochloride may contain some insoluble material. Upon addition of the $ZnSO_4$ a precipitate formed which dissolved on addition of the remaining ingredients. The pH was 6.00 ± 0.05. The solution was filtered, and was stored in a closed container at room temperature.

It can be used repeatedly for months.

c) Performance of the test:

(1) Freshly prepared blood films were used. Leukocyte peroxidase activity is unstable in light, but films stored in the dark are satisfactory for as long as three weeks. Anticoagulants (heparin, EDTA, oxalate) do not interfere with the test, so venous blood may be used.

(2) The film was fixed by immersion for 60 s in fixative at room temperature.

(3) The film was rinsed for 15-30 s in running tap water, drained and the excess water shaken off. The film was then immersed in the staining solution at room temperature for 30 s.

(4) The film was rinsed again for a few seconds in running tap water, allowed to dry, and examined microscopically under oil.

d) Interpretation:

All nuclei are stained red by the safranin, as is the cytoplasm of the red cells. In the neutrophil series, the peroxidase activity is indicated by blue granules within the cells. Peroxidase activity may be present in cells of the neutrophilic series at all stages of development from promyelocytes.

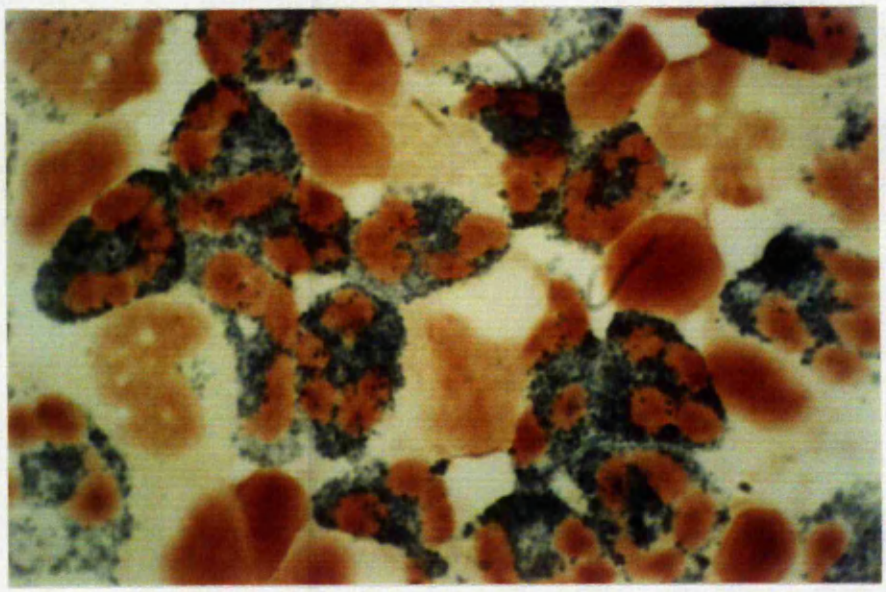


Fig. 7. A peroxidase stained preparation of normal leukocytes. The blue granules indicate sites of enzyme activity. The orange areas indicate the nuclei. (X1215).

e) Results:

Fig. 7 shows a typical peroxidase appearance of normal leukocytes.

5. Leukocyte peroxidase: Quantitative determination

Peroxidase activity was determined by a modification of the o-dianisidine method described in the Worthington Biochemical Corporation manual and by Klebanoff (1965).

a) Test principle:

The rate of decomposition of hydrogen peroxide by peroxidase with o-dianisidine as hydrogen donor is determined by measuring the rate of colour development at 460 nm.

b) Preparation of solutions:

(1) Phosphate buffer 0.01M pH 6.0

KH_2PO_4 0.68g in 500cm^3 H_2O solution (i)

$Na_2HPO_4.2H_2O$ 0.70g in 500cm^3 H_2O solution (ii)

mix 88.0cm^3 of solution (i) plus 12.0cm^3 of solution (ii)

(2) Hydrogen peroxide:

30% H_2O_2 1.0cm^3 (Fresh daily)

H_2O 100cm^3

(3) o-dianisidine 1%

o-dianisidine 0.2g

Methanol 20cm^3

(4) Enzyme (leukocyte sonicate)

1cm^3 cell suspension in 75% medium 199 and 25% plasma, containing about 20 x $10^6 cm^{-3}$ PMNs was sonicated for 30 s, spun at 2000g for 2 min and the supernatant

kept on ice.

c) Incubation solutions:

Into a 1.4 cm^3 cuvette was added:

phosphate buffer	1.0 cm^3
H_2O_2	0.1 cm^3
o-dianisidine	0.025 cm^3
leukocyte sonicate	0.05 cm^3

The contents were mixed by inverting the cuvette with waxpaper over top. The change in optical density at 460 nm was recorded for 16 minutes at room temperature.

The blank contained all components except the enzyme preparation.

d) Results:

Fig. 8 is typical of the change of optical density (OD) against time for peroxidase. The results were expressed as the rate of change of O.D. per minute per 10^6 PMN. This value was later referred to as units per 10^6 PMN.

e) Remarks:

O-dianisidine was freshly prepared and kept in an amber bottle. Fresh H_2O_2 solution was prepared daily,

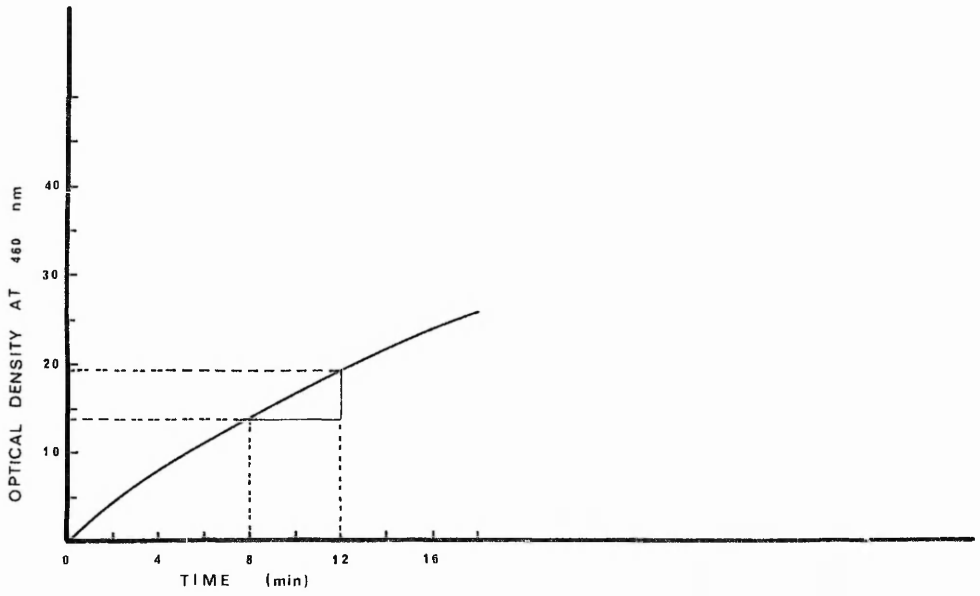


Fig. 8. The kinetics of change of optical density at 460 nm as a function of time for peroxidase of normal neutrophils.

6. Leukocyte Alkaline phosphatase: Histochemical demonstration

(Rutenburg et al., 1965)

a) Test principle:

Naphthol AS-phosphate $\xrightarrow{\text{alkaline phosphatase}}$ Naphthol AS

Naphthol AS+Fast blue BBN salt $\longrightarrow$ insoluble azo dye.

b) Preparation of solutions:

(1) Substrate solution:

Naphthol AS-phosphate	30mg
N:N-Dimethylformamide	0.5cm^3
Tris buffer 0.2M.pH9.1	100cm^3

NaphtholAS-phosphate was dissolved in N:N-Dimethylformamide. Tris buffer was then added and the solution stored at 4^{o}C.

(2) Incubating solution:

Substrate solution (1)	10cm^3
Fast blue BBN salt	10mg

(3) Counterstain:

Safranin O	0.2g
Distilled water	100cm^3

c) Incubating method:

(1) The slides were fixed in 10% formal methanol for 30 sec.

(2) Washed gently in running tap water.

(3) Covered with incubating mixture for 15 mins.

(4) Washed gently in running tap water.

(5) Counterstained for 2 mins.

(6) Dried and examined under oil.

d) Results:

Sites of alkaline phosphatase activity appear blue and nuclei stain red. Fig 9 shows sites of activity of alkaline phosphatase in normal leukocytes.

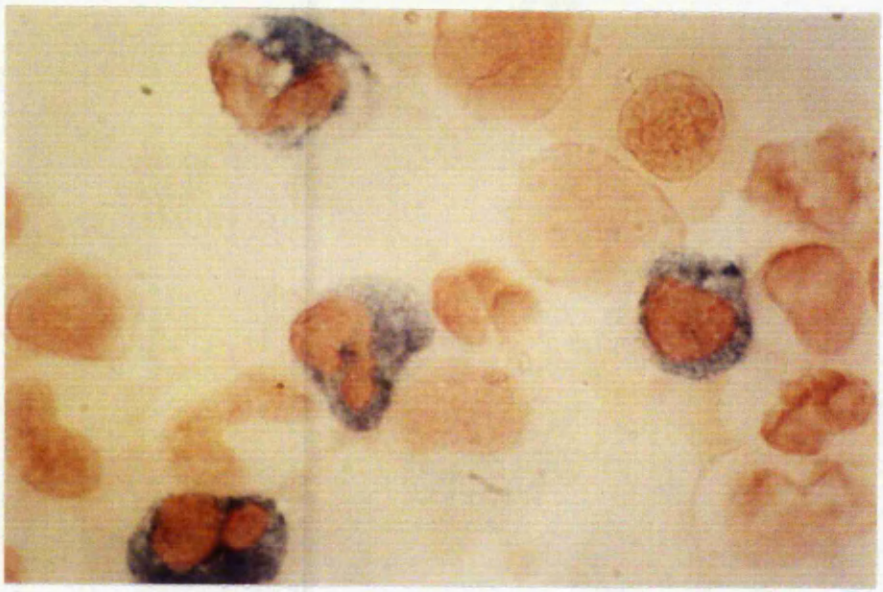


Fig. 9. An alkaline phosphatase stained preparation of normal leukocytes. The blue colouration indicates sites of enzyme activity. The red areas are the nuclei. (X1215).

7. Leukocyte Alkaline phosphatase: Quantitative determination

(Hausamen et al., 1967, modified)

a) Test principle:

Alkaline phosphatase was assayed in an optical test using p-nitrophenyl phosphate as substrate. The pK for p-nitrophenol is 7.16, thus 99% of the product is dissociated in p-nitrophenolate above pH 9.2. The enzyme and substrate were mixed and the change in optical density was recorded at 405 nm over a period of time.

b) Preparation of solutions:

(1)	Diethanolamine	95.8cm^3
	H_2O	904.2cm^3
(2)	HCl (conc.)	100cm^3
	H_2O	920cm^3
(3)	Diethanolanine soln.	1000cm^3
	HCl (dil.)	100cm^3
	adjust pH to 9.8	
	Mg Cl_2 Soln. 1.0M	0.5cm^3
(4)	p-nitrophenyl ortho-phosphate (disodium)	1.0g
	solution (3)	100cm^3

c) Incubating method:

Incubating solution (4)	1.0cm^3
Enzyme (leukocyte sonicate)	0.1cm^3

Placed in 1.4 ml cuvette, mixed, and change in O.D. at 405nm read for 40 minutes at room temperature.

d) Results:

Fig. 10 is typical of the change in optical density against time for alkaline phosphatase. The results are expressed as the rate of change of O.D. per minute per 10^6 PMN. This value was later referred to as units per 10^6 PMN.

e) Remarks:

Solution (3) stored in a dark bottle in the refrigerator. The incubating solution (4) was prepared daily prior to test.

The length of recording period depended on the

activity of the enzyme.

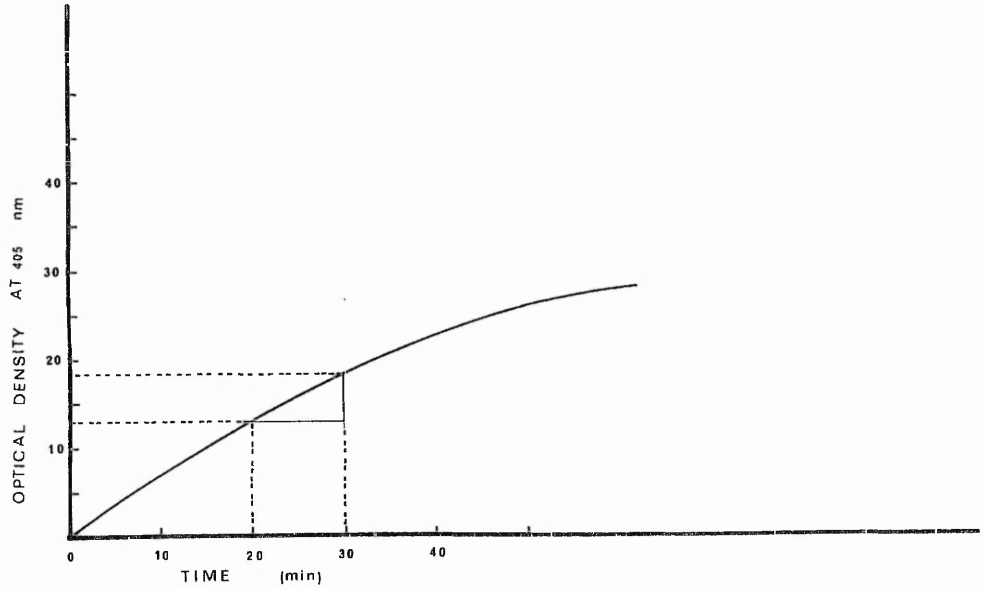


Fig. 10. The kinetics of change of optical density at 405 nm as a function of time for alkaline phosphatase of normal neutrophils.

8. Leukocyte Acid phosphatase: Histochemical demonstration

(Burstone, 1958, modified by Barka, 1960)

a) Test principle:

Naphthol AS-Bl phosphate $\xrightarrow{\text{Acid phosphatase}}$ primary reaction product (naphthol AS-Bl)

Naphthol AS-Bl + hexozonium pararosanilin $\longrightarrow$ Final reaction product (coloured precipitate)

The hexazonium pararosanilin does not inhibit the enzyme at acid pH. The combination of hexazonium pararosanilin and naphthol AS-Bl phosphate allows accurate localization of the reaction product because of its extreme insolubility.

b) Preparation of solutions:

(1) Substrate solution

Naphthol AS-Bl phosphate	50mg
N:N-Dimethyl formamide	5cm^3

(2) Buffer solution

Veronal acetate buffer stock A i.e.:

Sodium acetate crystals CH_3COONa. $3H_2O$	19.4g
Sodium diethyl barbiturate (Veronal)	29.4g
H_2O	1I

(3) Sodium nitrite

$NaNO_2$	400mg
Distilled water	10cm^3

(4) Pararosanilin -- HCl stock

pararosanilin hydrochloride	2g
2N-HCl	50cm^3

Warmed to dissolve, cooled to room temperature and filtered.

c) Preparation of incubation solution:

(1)	Diazo mixture	0.4cm^3 $NaNO_2$ solution (3)
		0.4cm^3 pararosanilin HCl solution (4)
(2)		2.5cm^3 Veronal Buffer (2)
(3)		0.5cm^3 solution (1)
(4)	Distilled water	6.5cm^3

It is necessary for the success of the technique that equal parts of solutions 3 and 4 are mixed together and allowed to

stand for 2 min before proceeding:

0.5cm^3 substrate solution (1) and 2.5cm^3 Veronal buffer (2) were added to the 0.8cm^3 Diazo solution and 6.5cm^3 H_2O. The final pH should be between 4.7-5.0 and was adjusted if necessary with 0.1 N NaOH.

d) Incubating method:

1. Slides were incubated at 37^{o}C for 15-60 min.
2. Washed in distilled water.
3. Counterstained in 2% methyl green (chloroform extracted).
4. Washed in running water.
5. Dried and examined under oil.

e) Results:

Acid phosphatase activity is indicated by red colouration and nuclei stain green. Fig. 11 shows a typical acid phosphatase appearance of normal leukocytes.

f) Remarks:

This was a reliable method giving sharp localization of the enzyme.

Three points to watch in the preparation of the incubating solution:

1. That the $NaNO_2$ solution (3) is fresh,
2. That the pH of the final incubating mixture is correct and
3. That the solution is filtered.

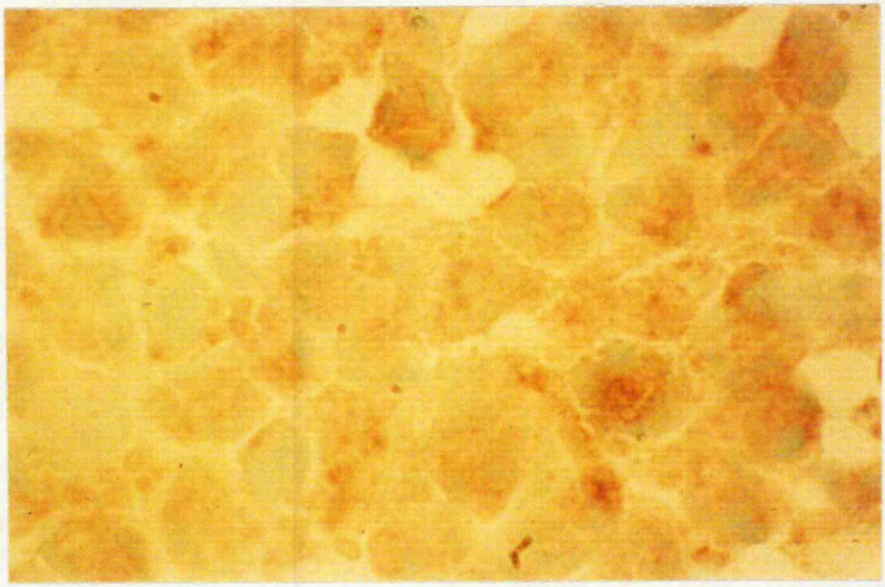


Fig. 11. An acid phosphatase stained preparation of normal leukocytes. The reddish areas indicate sites of enzyme activity. The green areas are the nuclei. (X1215).

9. Leukocyte Acid phosphatase: Quantitative determination

(Eskalab Bulk Reagent acid phosphatase kit product No. 89504, modified)

a) Test principle:

In the presence of the enzyme acid phosphatase, α-naphthyl phosphate is hydrolyzed to α-naphthol and inorganic phosphate at pH 5.0. The rate of hydrolysis is proportional to the enzyme activity present.

$$\alpha\text{-naphthyl phosphate} + H_2O \xrightarrow[\text{phosphatase}]{\text{acid}} \alpha\text{-naphthol} + \text{inorganic phosphate}$$

The α-naphthol produced is coupled with a diazonium compound Fast Red TR (Diazotized 2-amino-5-chlorotoluene)

to produce a coloured complex which absorbs at 405nm.

α-naphthol + Fast Red TR ⟶ chromophore.

Since the coupling reaction is instantaneous, the rate of colour appearance is limited only by the rate of α-naphthol production in the enzyme mediated reaction.

b) Incubation solution:

α-naphthyl phosphate	3mM
Fast Red TR	1mM
Buffer (pH5.0±0.1)	
Citric acid	30mM
Sodium citrate	50mM

Dissolve in 20cm³ distilled water.

c) Incubating method:

1 cm³ of incubating solution in a 1.4cm³ cuvette.

0.1cm³ of PMN sonicate. (see later).

Read at 405 nm for 5 minutes at room temperature.

d) Results:

Fig. 12 is typical of the change in optical density against time for acid phosphatase. The results were expressed as the rate of change of O.D. per minute per 10^6 PMN. This value was later referred to as units per 10^6 PMN.

Acid phosphatase activity was found in lymphocytes and platelets as well as in neutrophils. Therefore, it was necessary to separate the neutrophils from the rest of the leukocytes on a ficoll-triosil gradient.

Heparin and EDTA interfere with acid phosphatase activity, and therefore neutrophils were washed twice in

0.9% NaCl before incubation.

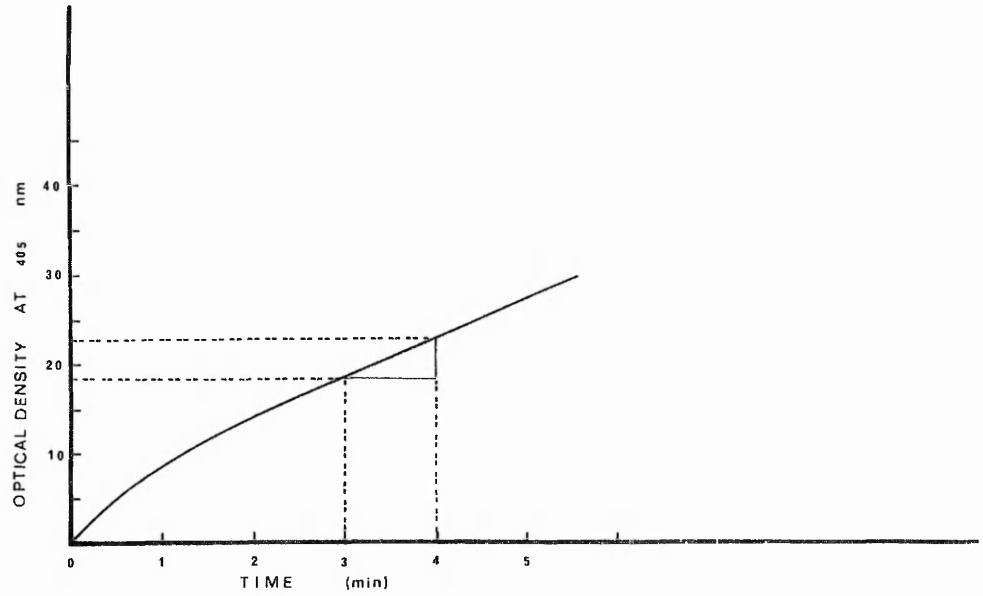


Fig. 12. The kinetics of change of optical density at 405 nm as a function of time for acid phosphatase of normal neutrophils.

10. Leukocyte separation on a ficoll-triosil gradient

a) Preparation of solutions:

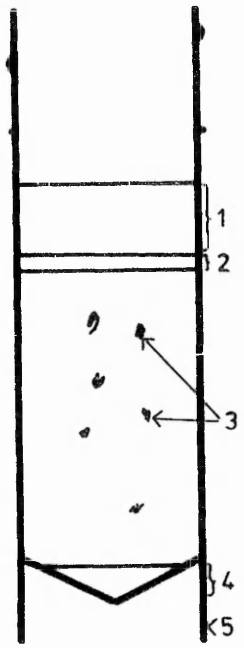
(1)	Ficoll	4.5g
	Distilled water	50cm^3
(2)	Triosil 440	20cm^3
	Distilled water	24.1cm^3

48cm^3 of Ficoll solution (1) and 20cm^3 of Triosil solution (2) were mixed and stored at 4^o.

b) Procedure:

20cm^3 of Ficoll-triosil solution were placed in plastic universal bottle. 5cm^3 of leukocyte rich plasma (see page 77) was layered carefully onto it and the system was centrifuged at 2000 g for 20 min. PMNs and few

erythrocytes were found at the bottom. (See fig. 13).



LAYER	CONTENT
1 =	Few lymphocytes + platelets
2 =	Mainly lymphocytes + few platelets
3 =	Mainly platelets + few PMN's
4 =	Mainly PMN's + few erythrocytes
5 =	Steril plastic universal bottle

Fig. 13. Leukocyte separation on Ficoll/Triosil gradient.

The upper layers (1 and 2) were carefully removed and replaced by 10cm^3 0.9% NaCl, and the system mixed and recentrifuged. The supernatant was removed and PMN's were then washed twice with saline (0.9% w/v) to remove heparin and Ficoll-triosil contamination. The PMN count was adjusted to 10 x 10^6 PMN per cm^3. 1cm^3 of PMN suspension was sonicated for 30 s, spun at 2000g for 2 min and the supernatant kept on ice until use.

c) Remarks:

(1) Ficoll was supplied by Pharmacia Fine Chemicals,

Uppsala, Sweden.

(2) Triosil 440 was supplied by Nyegaard and Co - AS- Oslo. Distributors: Vestric Ltd., Runcorn, Cheshire.

(3) Acid phosphatase kit supplied by Smith Kline Industries, Welwyn Garden City, Herts.

11. Leukocyte β-Glucuronidase: Histochemical demonstration (Hayashi et al., 1964)

a) Test principle:

Naphthol AS-Bl glucuronide $\xrightarrow{\beta\text{- glucuronidase}}$ Naphthol AS-Bl

Naphthol AS-Bl + Hexazonium pararosanilin $\longrightarrow$ insoluble azo dye

The combination of hexazonium pararosanilin and naphthol AS-Bl glucuronide provides accurate localization of the reaction products due to its extreme insolubility.

b) Preparation of solutions:

(1) Sodium bicarbonate:

$NaHCO_3$	210mg
Distilled water	50cm^3

(2) Substrate solution:

Naphthol AS-Bl glucuronide	14mg
sodium bicarbonate solution (1)	0.6cm^3
0.1 M Acetate buffer, pH 5.0	50cm^3

(3) Hexazonium pararosaniline solution:

Pararosanilin HCl	2g
2N-HCl	50cm^3 solution (1)

The mixture was heated gently, cooled to room temperature and filtered.

$NaNO_2$	400mg
Distilled water	10cm^3 solution (2)

0.3cm^3 of pararosanilin solution (1) and 0.3cm^3 of sodium nitrite solution (2) were mixed and allowed to stand for 2 min until the solution became amber.

c) Incubation solution:

Substrate solution, solution (2)	5cm^3
Hexazonium pararosanilin, solution (3)	0.3cm^3
Distilled water	5cm^3

The pararosanilin was added just before use, and the pH of the solution adjusted to 5.2 with 1N NaOH.

d) Incubating method:

1. Slides were placed in the incubating medium at 37^{o}C for 20-40 min.
2. Washed well in distilled water.
3. Counterstained in 2% methyl green (chloroform washed) for 4 min.
4. Washed rapidly in tap water.
5. Dried and examined under oil.

e) Results:

Glucuronidase activity is shown as a red colour and the nuclei stained greeen. Typical β-glucuronidase appearance of normal leukocytes is similar to that of acid phosphatase. See Fig. 11.

f) Remarks:

The pH of the incubating solution should be between 5.0 and 5.3 and the $NaNO_2$ should be freshly prepared. The localization obtained with this technique was good.

12. Leukocyte Hydrogen peroxide estimation during phagocytosis

(Modification of Keston & Brandt, 1965; Homan-Müller, Weening and Roos, 1975).

a) Test principle:

The test is based on the oxidation of a stable non-fluorescent leukodiacetyl-2, 7-dichlorofluorescein to a fluorescent compound by ultramicro amounts of hydrogen peroxide in the presence of peroxidase.

b) Preparation of solutions:

(1) Leukodiacetyl-2, 7-dichlorofluorescein (LDADCF) 0.0244g

pure ethanol 500cm^3

Store in glass in the dark at 4^{o}C. This solution is stable for months.

(2) LDADCF solution (1) 1cm^3

NaOH (0.01N) 4cm^3

Leave for 15 minutes at room temperature for activation.

(3) Activated LDADCF solution (2) 5cm^3

sodium phosphate buffer (pH7.2) (Autoclaved)

(0.025M + 0.04mg/cm^3 $ZnSO_4$) 45cm^3

(4) Enzyme solution:

Horse-radish peroxidase (E.C.1.11.1.7) 0.250mg

phosphate buffer (0.025M pH7.2) 1.0cm^3

Solutions (3) and (4) were freshly prepared each day and kept at 4^{o}C.

(5) Phosphate-buffered salt solution:

NaCl	4.1g
Na_2HPO_4	0.8g
NaH_2PO_4	0.1g
glucose	0.5g
H_2O	500cm^3
Human albumin	2.5g

This solution was autoclaved prior to the addition of human albumin. 0.5g of human albumin was added prior to use to 100cm^3 of the autoclaved solution to prevent wastage.

(6) Leukocytes were washed twice with solution (5) and finally the cells were resuspended in the same medium to a concentration of 5 x 10^6 PMN s/cm^3.

(7) Candida opsinization:

Overnight cultures of Candida were pooled after sonication for 30 s at setting 5 on Ultrasonics Ltd. sonicator. This preparation was boiled for 10 minutes, and resuspended in plasma which had been centrifuged for 5 min at 1500g. The resuspended Candida was left for one hour at 25^{o}C. Candida was then washed twice with 0.9% w/v NaCl and resuspended in the same solution. The final suspension contained 2 x 10^8 Candida/cm^3.

(8)	NaN3	0.650g
	H_2O	100cm^3

(9) Phagocytosis:

The following were mixed in a 5cm^3 plastic tube (twelve tubes) in the following order:-

NaN3 solution (8)	10µl

phosphate-buffered salt solution (5) 0.5cm^3

PMN suspension (6) 0.2cm^3

The mixture was warmed to 37^{o}C for 10 min.

At zero time 0.1cm^3 of Candida (7) also at 37^{o}C was added to the leukocyte suspension (9), mixed gently, and returned to the water bath.

At 15 minutes tubes were placed on a rotating wheel in a 37^{o}C incubator.

(10) H_2O_2 sample preparation:

At intervals tubes were transferred to a 4^{o}C water bath for 2 minutes and then centrifuged at 2000g for one minute. The supernatant was poured into a clean tube and kept at 4^{o}C. 0.2cm^3 was diluted in 3cm^3 cold sterile H_2O for H_2O_2 estimation.

c) Fluorometric analysis of H_2O_2:

(i) Equipment: The excitation and emission spectra of oxidized LDADCF were determined with an Aminco-Bowman spectro photofluorometer. The maximum for excitation was 495nm and for emission 525nm. (see fig. 14).

(ii) Procedure: In the fluorometer cuvette, the following were mixed:

Activated LDADCF, solution (3) 1.0cm^3

Enzyme solution (4) 0.025cm^3

These were mixed and used to zero the fluorometer.

H_2O_2 sample, solution (10) 0.2cm^3

This was added to the cuvette, mixed, and the reading was recorded immediately. This procedure was repeated for each of the 12 tubes.

The excitation spectrum was at an emission wavelength of 200 nM. The emission spectrum was at an excitation wavelength of 495 nM.

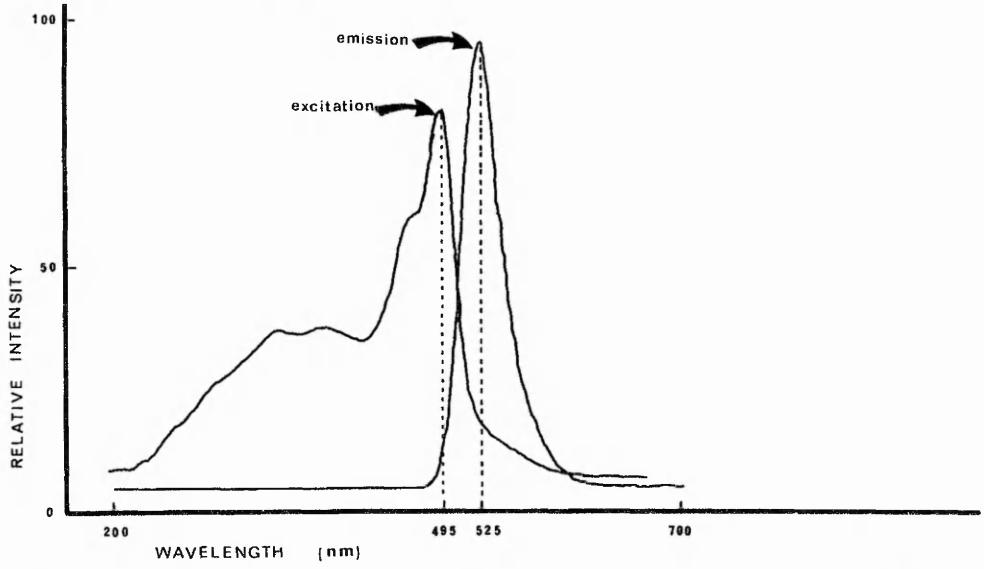


Fig. 14. The excitation and emission spectra of oxidized LDADCF. The maxima were found at 495nm and 525nm, respectively.

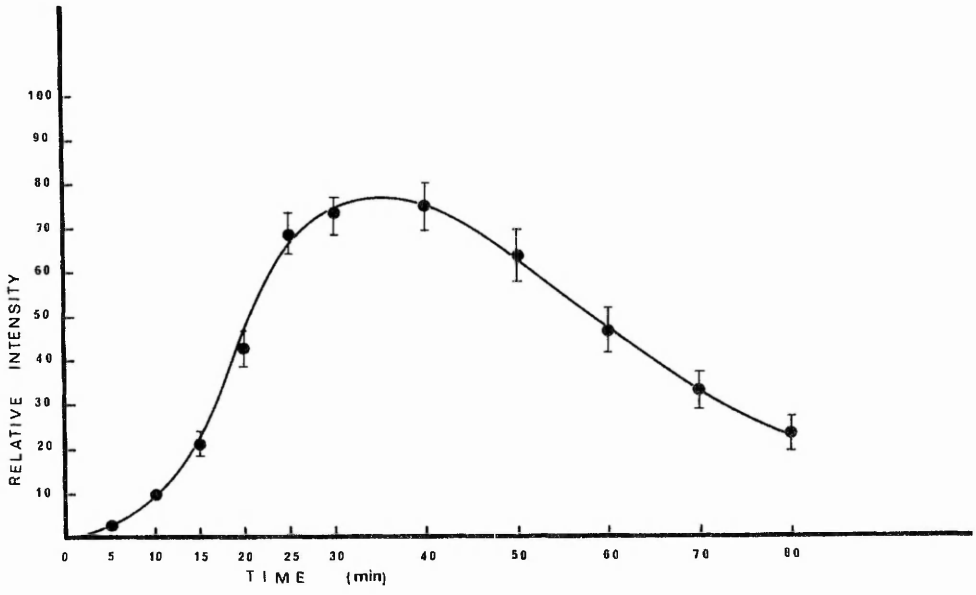


Fig. 15. Average change of relative intensity due to H_2O_2 production by PMN's of 15 normal subjects as a function of incubation time with Candida guillermondii.

d) Results:

H_2O_2 reading from each tube was plotted against length of incubation (see fig. 15).

The shape of the curve represents the average result of two systems, one producing H_2O_2 and another degrading it. The area under that curve is the true representative of the overall amounts of H_2O_2 present over the incubation period. However, it was more accurate, in our case, to cut out the area under the curve and weigh it accurately. This weight was taken to represent the value of H_2O_2 production by 1×10^6 PMN during 80 minutes phagocytosis, and is later referred to as units/10^6 neutrophils/80′.

e) Remarks:

H_2O_2 was very unstable and the following points were observed to enable its accurate measurement:

1. Samples must be kept at $4^{\circ}C$ at all stages between the end of incubation and actual determination.
2. All solutions must be kept at $4^{\circ}C$ until the moment needed.
3. Unnecessary delay in H_2O_2 determination must be avoided.

13. Coulter counter use for leukocyte and Candida counting

The Coulter counter registers the change in current caused by particles moving through an electric field. A known volume of fluid containing particles to be counted is drawn through an aperture of known size across which there is an electric field. When a particle passes through the aperture, the changes in current are visualised as a spike on an oscilloscope, and counted. They thus represent the particle count of the fluid.

The Model D Coulter counter has two controls which affect counting accuracy. One is the "aperture current" switch which controls the current passing between the two electrods. An increase in current produces a larger pulse height for a given size of particle. The correct aperture current setting for white cell counts was found to be position 2, and for Candida position 4.

The threshold discriminator controls the level of response, and eliminates pulses caused by cells below a certain size, debris and base line interference, thus permitting only pulses exceeding the set level to be counted.

A suitable threshold setting was 25 for white cell count on the white threshold control, and 10 for Candida on the red threshold control.

Acceptable threshold settings were found by counting a fluid containing leukocytes or Candida and plotting the resulting counts against the threshold settings.

The white cell counts fell as the setting rose until a plateau was reached and the setting for use was selected along the plateau (see Fig. 16). With Candida a plateau was not observed (see Fig. 17).

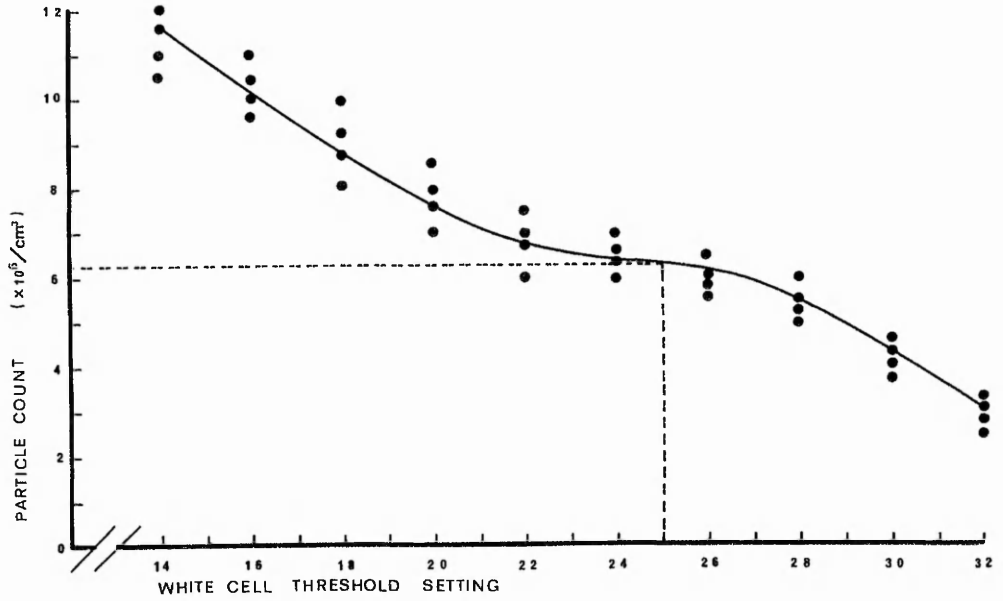


Fig. 16. Particle count at different white cell threshold setting.

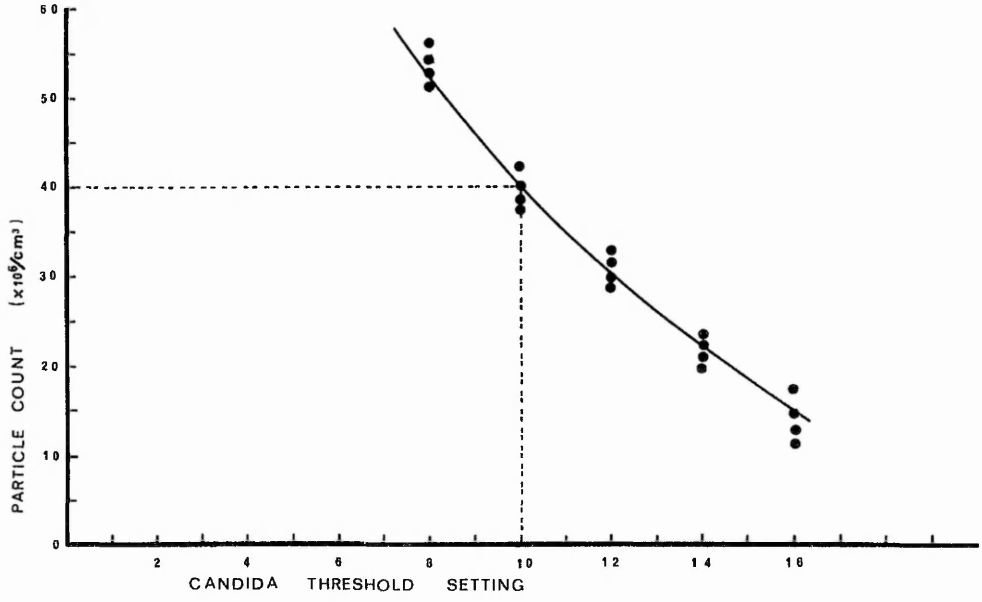


Fig. 17 Particle count at different Candida threshold setting.

To overcome this a Candida suspension was counted using the improved Neubauer counting chamber and the mean of five counts used to adjust the Coulter counter to the correct

setting.

Duplicate counts were performed on the Coulter counter and the mean was taken to represent the actual count.

Difficulties:

When heparinized blood was sedimented with plasmagel and the resulting leukocyte rich plasma was counted after red cell destruction using Zaponin, it was found that the counts dropped very drastically over a short period of time. It is, however, claimed by the manufacturers of the Coulter counter and Zaponin that a sample ready for counting should be stable for at least 30 min. After various investigations into what might be causing this drop it appears that the combination of heparin, plasmagel and Zaponin, inhance white cell shrinkage and lyses. As heparin and plasmagel had to be used to maintain the functional ability of the leukocytes, it was essential to find a way of determining the real count. This was achieved by adding less Zaponin to the diluted sample (one drop instead of six) and by counting immediately twice and then taking the mean of these two readings as the real count.

With Candida there were no such difficulties.

14. Observation recording sheet

A tabulated recording sheet was essential to keep a clear and accurate record of all the observations. These may be as many as thirty for each patient, and it was usual for four subjects to be tested each time. This emphasizes the necessity for a recording sheet. A master copy of the

recording sheet was made and photocopies then prepared and used. A typical record sheet is shown in Fig. 18.

The recording sheet contains the following:

a) Date on which experiment was carried out (top right).

b) Name of the patient or normal control, as well as age, sex, diagnosis and hospital number (bottom right). Each subject will be identified by the figure beside it's name.

c) Space for any relevent comments (right hand column).

d) The ratio of Candida to PMN used (bottom left).

e) Columns for data records:

1. Tube number. The figures .1, .2, .3, or .4 refer to the blood sample of patients 1, 2, 3 or 4. Each test had three replicates and the 2nd and 3rd replicats were indicated by the presence of one dash (') or two ("). e.g. (15.1', 15.1"). The presence of an O before the sample number (e.g. O.1) refers to the fact that this tube is a control having high spun plasma instead of PMN. The presence of 15 before the sample number (e.g. 15.1) refers to the incubation time in minutes.

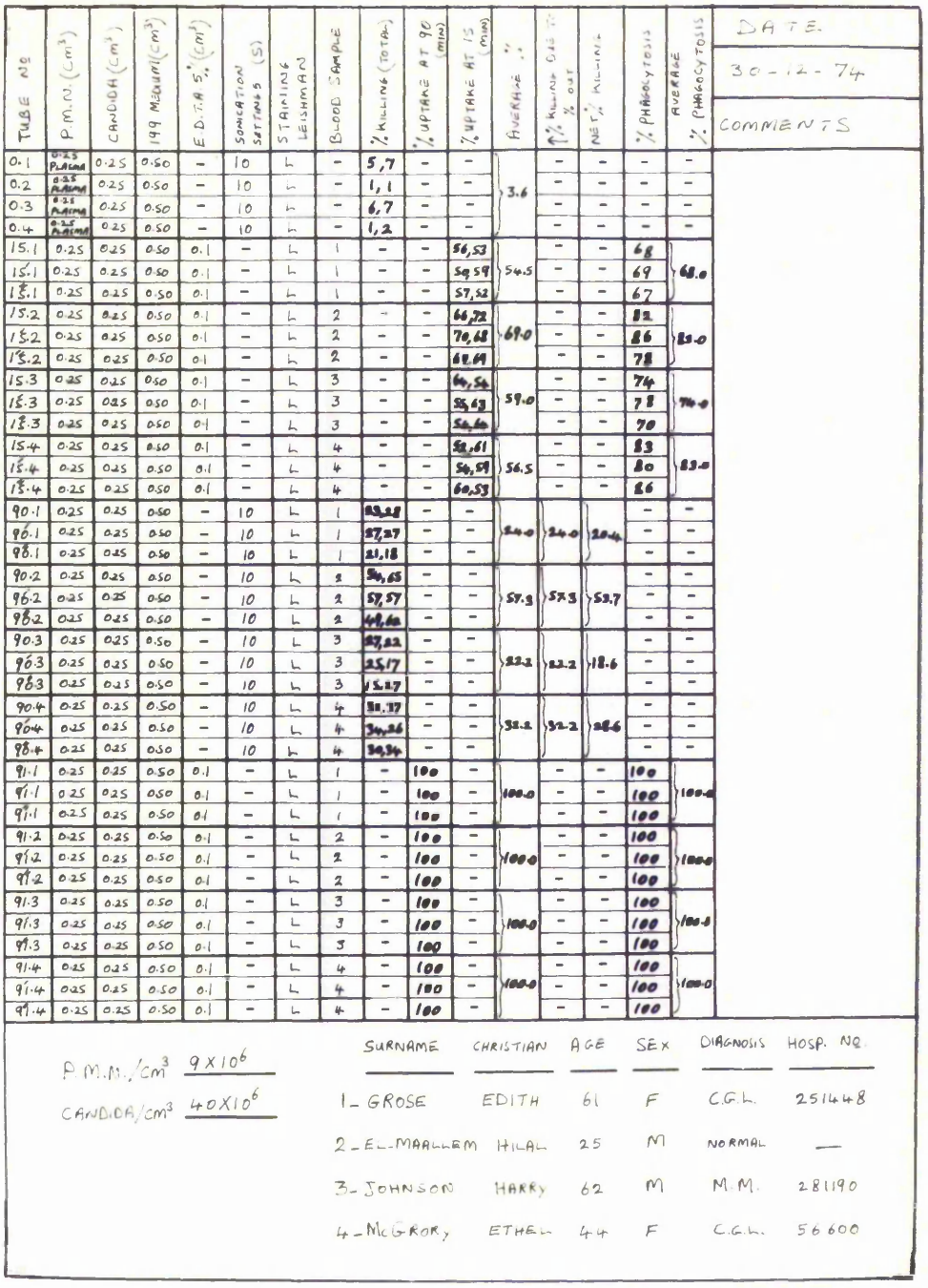
The presence of 90 before the sample number (e.g.90.1) indicates that the tube was incubated for 90 min and then sonicated for 10 seconds.

The presence of 91 before the sample number (e.g. 91.1) indicates that the tube was incubated for 90 min but not sonicated.

2. Volume of PMN suspension added (cm^3)

3. Volume of Candida suspension added (cm^3)

4. Volume of Medium 199 added (cm^3)
5. Volume of EDTA added to $1cm^3$ cold saline to stop phagocytosis at the end of 15 and 90 min incubation
6. Length of sonication(s) at setting 5
7. Staining
8. Blood sample - number 1, 2, 3 or 4 refers to patient or control identity.
9. Percentage killing at 90 min.
10. Percentage uptake at 90 min.
11. Percentage uptake at 15 min.
12. Average percentages from columns 9, 10 and 11
13. Increase in percentage killing due to percentage of Candida which might not have been taken in. This represents an adjusted percentage killing, which may be a figure higher than that recorded if percentage uptake at 90 min was not 100%. As it turned out there was no need for this adjustment.
14. Net percentage killing. This is the figure obtained by subtracting the background percentage killing (top entry in column 12) from the total percentage killing (column 13 - entries 90.1 down to 90.4)
15. Percentage phagocytosis at 15 min and 90 min.
16. Average percentage phagocytosis from column 15.



TUBE NO	P.M.N. (cm³)	CANDIDA (cm³)	199 MEDIUM (cm³)	E.D.T.A. 5% (cm³)	SONICATION SETTINGS (S)	STAINING LEISHMAN	BLOOD SAMPLE	% KILLING (TOTAL)	% UPTAKE AT 90 (min)	% UPTAKE AT 15 (min)	AVERAGE %	% KILLING OUT OF % OUT	% KILLING NEF	% PHAGOCYTOSIS	AVERAGE % PHAGOCYTOSIS	DATE: 30-12-74
0.1	0.25 PLASMA	0.25	0.50	–	10	+	–	5,7	–	–		–	–	–	–	COMMENTS
0.2	0.25 PLASMA	0.25	0.50	–	10	+	–	1,1	–	–	3.6	–	–	–	–	
0.3	0.25 PLASMA	0.25	0.50	–	10	+	–	6,7	–	–		–	–	–	–	
0.4	0.25 PLASMA	0.25	0.50	–	10	+	–	1,2	–	–		–	–	–	–	
15.1	0.25	0.25	0.50	0.1	–	+	1	–	–	56,53		–	–	68		
15.1	0.25	0.25	0.50	0.1	–	+	1	–	–	54,59	54.5	–	–	69	68.0	
15.1	0.25	0.25	0.50	0.1	–	+	1	–	–	57,52		–	–	67		
15.2	0.25	0.25	0.50	0.1	–	+	2	–	–	66,72		–	–	82		
15.2	0.25	0.25	0.50	0.1	–	+	2	–	–	70,68	69.0	–	–	86	83.0	
15.2	0.25	0.25	0.50	0.1	–	+	2	–	–	68,69		–	–	78		
15.3	0.25	0.25	0.50	0.1	–	+	3	–	–	64,54		–	–	74		
15.3	0.25	0.25	0.50	0.1	–	+	3	–	–	55,63	59.0	–	–	78	76.0	
15.3	0.25	0.25	0.50	0.1	–	+	3	–	–	58,60		–	–	76		
15.4	0.25	0.25	0.50	0.1	–	+	4	–	–	52,61		–	–	83		
15.4	0.25	0.25	0.50	0.1	–	+	4	–	–	56,59	56.5	–	–	80	83.0	
15.4	0.25	0.25	0.50	0.1	–	+	4	–	–	60,53		–	–	86		
90.1	0.25	0.25	0.50	–	10	+	1	23,28	–	–				–	–	
90.1	0.25	0.25	0.50	–	10	+	1	27,27	–	–	24.0	24.0	20.4	–	–	
90.1	0.25	0.25	0.50	–	10	+	1	21,18	–	–				–	–	
90.2	0.25	0.25	0.50	–	10	+	2	56,65	–	–				–	–	
90.2	0.25	0.25	0.50	–	10	+	2	57,57	–	–	57.3	57.3	53.7	–	–	
90.2	0.25	0.25	0.50	–	10	+	2	48,66	–	–				–	–	
90.3	0.25	0.25	0.50	–	10	+	3	27,22	–	–				–	–	
90.3	0.25	0.25	0.50	–	10	+	3	25,17	–	–	22.2	22.2	18.6	–	–	
90.3	0.25	0.25	0.50	–	10	+	3	15,27	–	–				–	–	
90.4	0.25	0.25	0.50	–	10	+	4	31,37	–	–				–	–	
90.4	0.25	0.25	0.50	–	10	+	4	34,26	–	–	32.2	32.2	28.6	–	–	
90.4	0.25	0.25	0.50	–	10	+	4	34,34	–	–				–	–	
91.1	0.25	0.25	0.50	0.1	–	+	1	–	100	–		–	–	100		
91.1	0.25	0.25	0.50	0.1	–	+	1	–	100	–	100.0	–	–	100	100.0	
91.1	0.25	0.25	0.50	0.1	–	+	1	–	100	–		–	–	100		
91.2	0.25	0.25	0.50	0.1	–	+	2	–	100	–		–	–	100		
91.2	0.25	0.25	0.50	0.1	–	+	2	–	100	–	100.0	–	–	100	100.0	
91.2	0.25	0.25	0.50	0.1	–	+	2	–	100	–		–	–	100		
91.3	0.25	0.25	0.50	0.1	–	+	3	–	100	–		–	–	100		
91.3	0.25	0.25	0.50	0.1	–	+	3	–	100	–	100.0	–	–	100	100.0	
91.3	0.25	0.25	0.50	0.1	–	+	3	–	100	–		–	–	100		
91.4	0.25	0.25	0.50	0.1	–	+	4	–	100	–		–	–	100		
91.4	0.25	0.25	0.50	0.1	–	+	4	–	100	–	100.0	–	–	100	100.0	
91.4	0.25	0.25	0.50	0.1	–	+	4	–	100	–		–	–	100		

P.M.N./cm³ 9X10⁶

CANDIDA/cm³ 40X10⁶

	SURNAME	CHRISTIAN	AGE	SEX	DIAGNOSIS	HOSP. NO.
1	GROSE	EDITH	61	F	C.G.L.	251448
2	EL-MAALLEM	HILAL	25	M	NORMAL	—
3	JOHNSON	HARRY	62	M	M.M.	281190
4	McGRORY	ETHEL	44	F	C.G.L.	56600

Fig. 18. Observation recording sheet.

15. Statistical analysis

The statistical analysis of the results presented in this thesis were performed with a SONY micro computer (Sobax 1CC-2700).

The programmes used were:-

a) Program identifier SO22O: (Maximum, minimum, standard deviation, coefficient of variation)

This program computes the following values for n observations xi (i=1,2,...n), where xi may take any value, negative, positive or zero.

The following ten parameters are computed and printed:

1) maximum value: max (xi)

2) minimum value: min (xi)

3) range: R=max(xi)-min(xi)

4) mean value: $\bar{x} = \frac{1}{n} \sum_{1}^{n} xi$

5) sum of the square deviation: $S=\sum_{1}^{n} (xi-\bar{x})^2$

6) variance: $S^2 = \frac{1}{n} \sum_{1}^{n} (xi-\bar{x})^2$

7) standard deviation: $SD = \sqrt{\frac{1}{n} \sum_{1}^{n} (xi-\bar{x})^2}$

8) coefficient of variation: $c = \frac{SD}{\bar{x}}$

9) unbiased variance: $V = \frac{S}{n-1} = \frac{1}{n-1} \sum_{1}^{n} (xi-\bar{x})^2$

10) square root of the unbiased variance: $\sqrt{V}$

Standard error was calculated individually S.E. $= \frac{S.D.}{\sqrt{n}}$.

b) Program identifier S3O15: Test of the difference between two sample means.

This program computes t for the purpose of ascertaining whether the means of two samples (xi, yi) taken from the two populations N (μ_x, σ_x^2) and N (μ_y, σ_y^2) differ significantly. Two samples xi (i = 1,2..n_x) and yi (i = 1, 2..n_y) may take

any value. It is not necessary for $n_x = n_y$, $\sigma_x^2 = \sigma_y^2$

This program obtains:

$$t = \frac{|\bar{x} - \bar{y}|}{\sqrt{\frac{Vx}{n_x} + \frac{Vy}{n_y}}}$$

where: $Vx = \sigma_x^2$, $Vy = \sigma_y^2$

$$\phi = \left(\frac{(1-c)^2}{nx-1} + \frac{c^2}{ny-1} \right)^{-1}$$

This is equivalent to degree of freedom

where: $c = \frac{Vy}{ny} \cdot \left(\frac{Vx}{nx} + \frac{Vy}{ny} \right)^{-1}$

P was then identified from the significance limits table of the student distribution.

c) Program identifier SO310: Correlation coefficient and regression line.

This program computes the correlation coefficient r for the ungrouped n paired observations (xi, yi) i = 1,2...n, and parameters a_o, a_1 of the linear regression line, $y = a_1 x + a_o$. (The least squares principle is applied).

$$r = \frac{\Sigma xiyi - \frac{1}{n} \Sigma xi \, \Sigma yi}{\left[\left(\Sigma xi^2 - \frac{1}{n} (\Sigma xi)^2 \right) \left(\Sigma y_i^2 - \frac{1}{n} (\Sigma yi)^2 \right) \right]^{\frac{1}{2}}}$$

$$a_1 = \frac{\Sigma xi \; yi - \frac{1}{n} \Sigma xi \, \Sigma yi}{\Sigma xi^2 - \frac{1}{n} (\Sigma xi)^2}$$

$$a_o = \frac{1}{n} (\Sigma yi - a_1 \Sigma xi)$$

where: $xi \geq 0$

degree of freedom = n - 2

significance limits can be identified from the correlation coefficient table.

16. Equipment and chemicals

a) Equipment:

The fluorometric determination of H_2O_2 was carried out on Aminco-Bowman spectrophotofluorometer.

All spectrophotometric determinations were carried out on Gilford spectrophotometer 240.

Leukocyte and Candida counting was carried out on Coulter Model D, in conjunction with Hook and Tucker Diluter I.

Cytocentrifuge preparations were carried out on Shandon Elliott Cytospin.

Ultrasonic disintegration was carried out using Ultrasonics Ltd. U Probe.

All micro-pipetting was done using Becton-Dickinson/Clay Adam's Automatic pipettes and Jencon's Finnpipettes.

b) Chemicals:

Most of the less specialised reagents.	Analytical Supplies Ltd., Little Eaton, Derby.
Hydrogen peroxide 30% w/v Product number 10128	B.D.H. Chemicals Ltd., Poole, England.
Zaponin	Coulter Electronics Ltd., Coldharbour Lane, Harpenden, Herts.
'Alexa' plastic haemolysis Tubes. CM-3, CM-4.	Henleys Medical Supplies Ltd., London. N8 ODL

2'-7'-dichlorofluorescin diacetate Product number 9846	Kodak Ltd., Acornfield, Kirkby, Liverpool. L33 7UF
Plasmagel	Laboratoir Roger Bellon, 159 Avenue Du Roule, Nevilly (Hauts-de-Seine), France.
Naphthol AS Phosphate N5625 N_1N_1dimethyl formamide D4254 Fast blue BNN salt FO250 Peroxidase P8250 Chloroquine C6628 Sodium acetate S8625 Naphthol-AS-Bl-Glucuronide N1875 Naphthol-AS-Bl-Phosphate N2250 Pararosaniline hydrochloride P3750 Sodium azide S2002 Sodium nitrite S2252 Human albumin A9511 Glucose G5000 Sodium diethyl barbiturate (Veronal) B0500 Naphthol AS-TR phosphoric acid N6125	Sigma, Norbiton Station Yard, Kingston-upon-Thames, Surrey.

Acid phosphatase kit 89504	Smith Kline Industries, Welwyn Garden City, Herts.
Sterile Universal Bottles 128A	Sterlin Ltd., Richmond, Surrey.
Preservative free heparin	Weddel Pharmaceuticals Ltd., London, ECiA 9HY

C H A P T E R F O U R

PRELIMINARY INVESTIGATIONS

As there was no method available for assessment of phagocytosis and killing by PMN, which was entirely suitable for the investigations that were to be performed, it was necessary to develop and validate the procedures used before commencing studies on patients.

a) Effect of sonication on Candida viability:

Unicellular Candida cultures were needed (see page 76) and these were produced by sonication. This procedure could have affected the viability of the organism. Candida guillermondii was cultured overnight in glucose broth. The sonicator was set at '5' and the relationship between time of sonication and viability was investigated. The percentage viability was determined by the specific staining methods. (See page 71).

When 10cm^3 of overnight Candida suspension was sonicated in a glass universal bottle the organisms remained fully viable even after 45 s (Fig. 19). To validate sonication after phagocytosis (see page 80) 1cm^3 of the sonicated unicellular suspension was exposed further, in a 5cm^3 plastic tube. Up to 15 s exposure had no effect on viability, but thereafter viability decreased slightly so that after 45 s only 80% of the Candida were viable. (Fig 20).

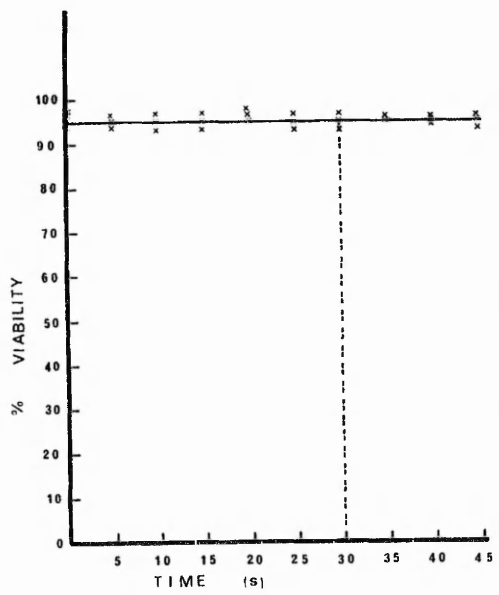


Fig. 19 Percentage viability of Candida as a function of sonication time in universal bottle.

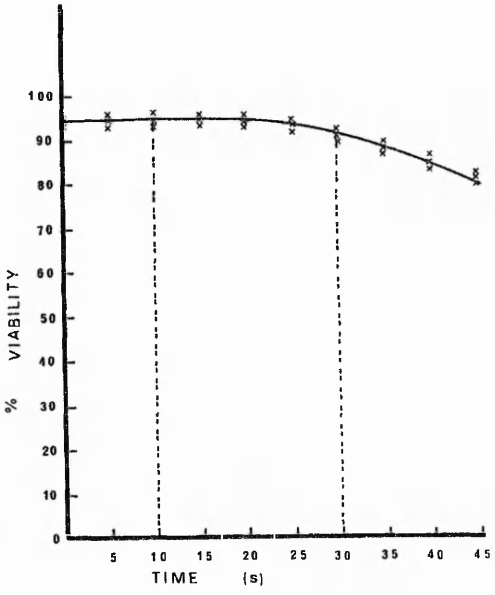


Fig. 20 Percentage viability of Candida as a function of sonication time in a test tube.

b) Release of Candida from PMN after phagocytosis:

Lehrer et al. (1969) used deoxycholate to release ingested C. albicans, but it was found that the detergent killed Candida guillermondii, thus invalidating any assessment of viability. Therefore, sonication was considered as a method for the release of Candida from the PMN.

Before phagocytosis the PMN were disrupted by sonication at a rate which left only 50% cells intact after 6s, and which produced complete disruption after about 25s (see Fig. 21).

After phagocytosis the cells were more fragile, sonication disrupted 50% of the cells after 1s and 100% after about 15s (see Fig. 22).

Leukocyte rich plasma ($1cm^3$) was exposed to sonication at setting '5' in a $5cm^3$ plastic test tube. The number of intact cells was assessed using a Coulter counter and expressed as percentage of total count.

The experiment was repeated with leukocytes that had phagocytosed Candida (ratio of 1:4.4 PMN:Candida) for 90 min.

The effect of sonication after phagocytosis on the viability of Candida has been considered before (see page 116).

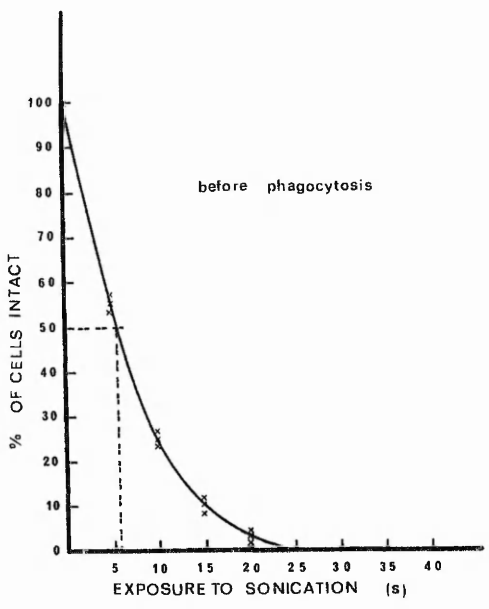


Fig. 21 Percentage of cells intact as a function of sonication time before phagocytosis of Candida

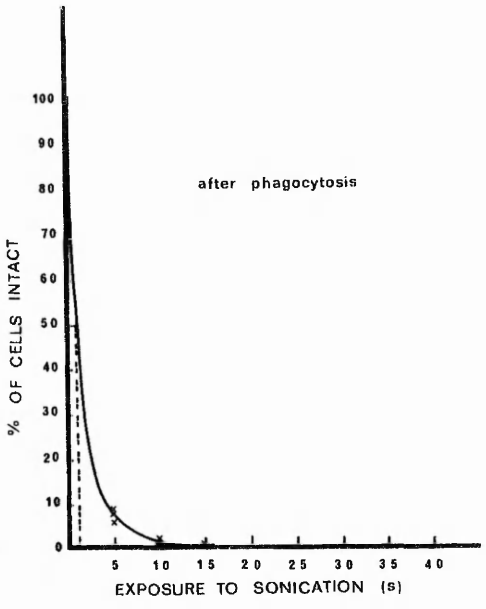


Fig. 22 Percentage of cells intact as a function of sonication time after phagocytosis of Candida

c) Effect of EDTA on PMN and Candida:

Calcium ions are required for phagocytosis by PMN. EDTA, dipotassion salt, was used as a chelating agent to stop phagocytosis by removing free calcium.

When PMN were exposed to 6mM EDTA phagocytosis was abolished and there was no loss of neutrophil viability.

EDTA, (6mM), had no effect on the viability of Candida during a 90 min incubation.

d) Effect of EDTA on Myeloperoxidase, alkaline and acid phosphatase:

EDTA had no effect on the histochemical demonstration of these enzymes or on the biochemical determination of peroxidase.

EDTA can, however, interfere with biochemical determination of phosphatase due to its chelating effects.

e) The kinetics of various Candida to PMN ratios:

It was necessary to establish standard optimum condition for leukocyte-Candida interaction. Two variables were considered. First the ratio of Candida to PMN and secondly the duration of incubation.

Candida and PMN from normal subjects were incubated at 37^{o} in ratios (Candida:PMN) of between 1 and 9.

After 15 min incubation uptake and phagocytosis were assessed (see page 79). Uptake was constant at 60% between C:PMN ratios of 1 and 6 but at higher ratios the uptake decreased (Fig. 23).

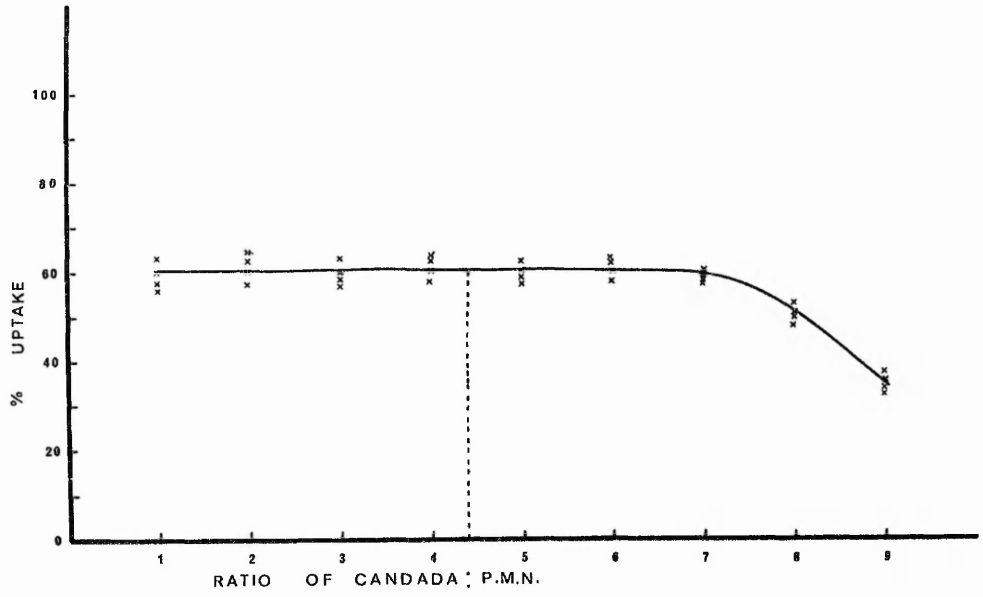


Fig. 23. The kinetics of percentage uptake of normal PMNs at 15 minutes as a function of Candida to PMN ratio..

On the other hand phagocytosis increased from 40% PMNs containing at least 1 Candida at C:PMN ratio of 1 to 100% at a ratio of 9. (Fig. 24).

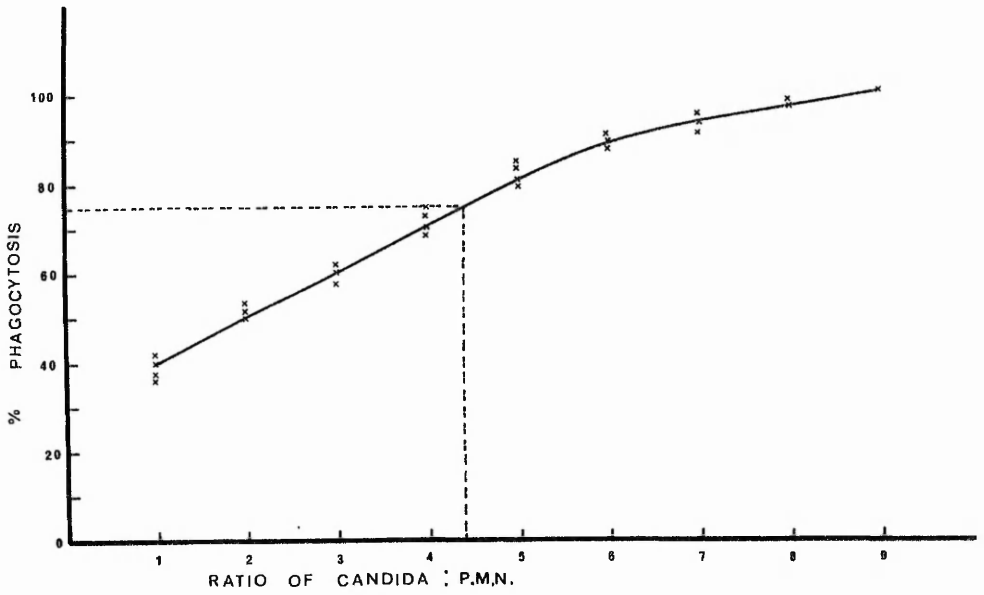


Fig. 24. The kinetics of percentage phagocytosis of normal PMNs at 15 minutes as a function of Candida to PMN ratio.

After 90 min, phagocytosis, uptake and killing were measured. (See page 79). Percentage phagocytosis increased from about 40 at C:PMN ratio of 1 to about 100 at ratio 4. (Fig. 25).

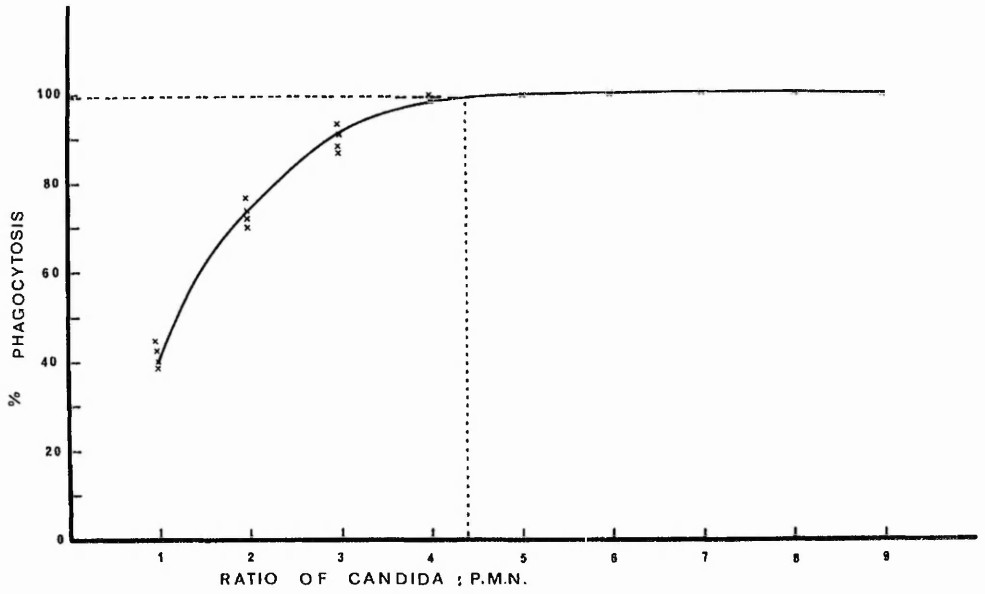


Fig. 25 The kinetics of percentage phagocytosis of normal PMNs at 90 minutes as a function of Candida to PMN ratio.

Percentage uptake was 100 at C:PMN ratios of 1 to 4 and decrease gradually at higher ratios. (Fig. 26).

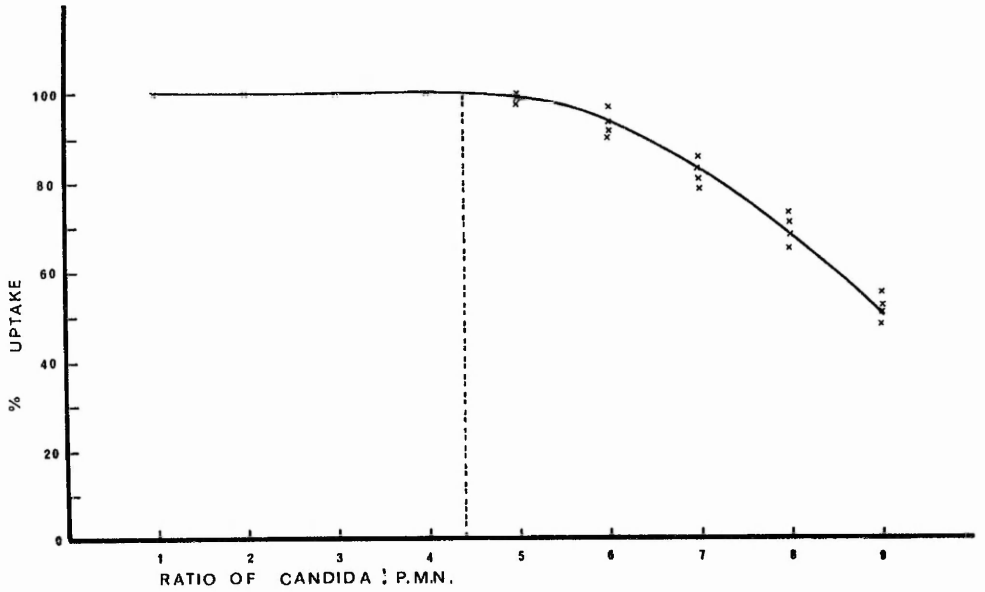


Fig. 26 The kinetics of percentage uptake of normal PMNs at 90 minutes as a function of Candida to PMN ratio.

Percentage kill was nearly 100 at a C:PMN ratio of 1 and decreased gradually at higher ratios. (Fig. 27).

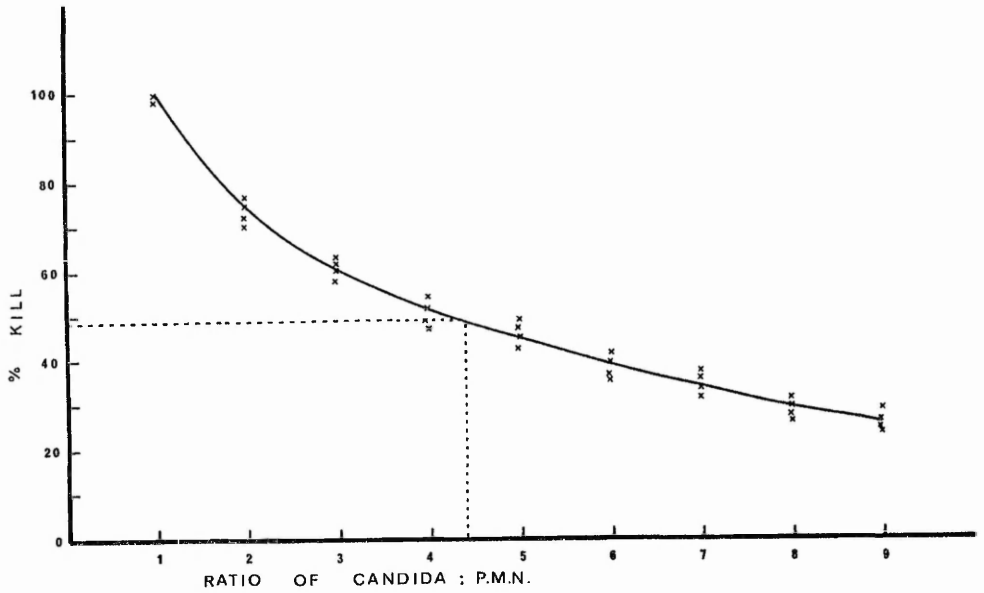


Fig. 27 The kinetics of percentage kill of normal PMNs at 90 minutes as a function of Candida to PMN ratio.

f) Conclusions:

From fig. 23 it seems that PMNs will phagocytose about 60% of Candida after 15 min over a large ratio variations.

From fig. 24 it seems that there is a sort of direct relationship between % phagocytosis after 15 min and ratio of C:PMN. Ratio of 4.5 gives about 80% phagocytosis.

From fig. 25 it seems that some PMNs are more active than others in phagocytosing Candida and a ratio above 4:1 is required to make sure every neutrophil manages to phagocytose at least one Candida.

From fig. 26 it is apparent that the PMNs reached a saturation stage at about the ratio of 5.5:1 C:PMN and any

extra Candida were left outside.

From fig.27thereisa gradual decrease in % kill as the ratio of C:PMN increases. A ratio of 4.5 gives about 50% kill.

From these observations it was concluded that a C:PMN ratio of between 4 and 5 should be used.

g) The kinetics of uptake, phagocytosis and killing against time:

Having chosen the ratio of 4.4:1 Candida:PMN, it was necessary to study the kinetics of % uptake, phagocytosis and killing over time. Leukocytes from6 normal subjects were incubated with Candida at 37^{o} and at a ratio of 4.4:1 C:PMN. Uptake and phagocytosis were measured at intervals up to 45 min, and killing was assessed at intervals up to 150 min.

Fig. 28 shows that about 60% of the total Candida was taken up by normal neutrophils at 15 minutes, and about 100% at 45 minutes.

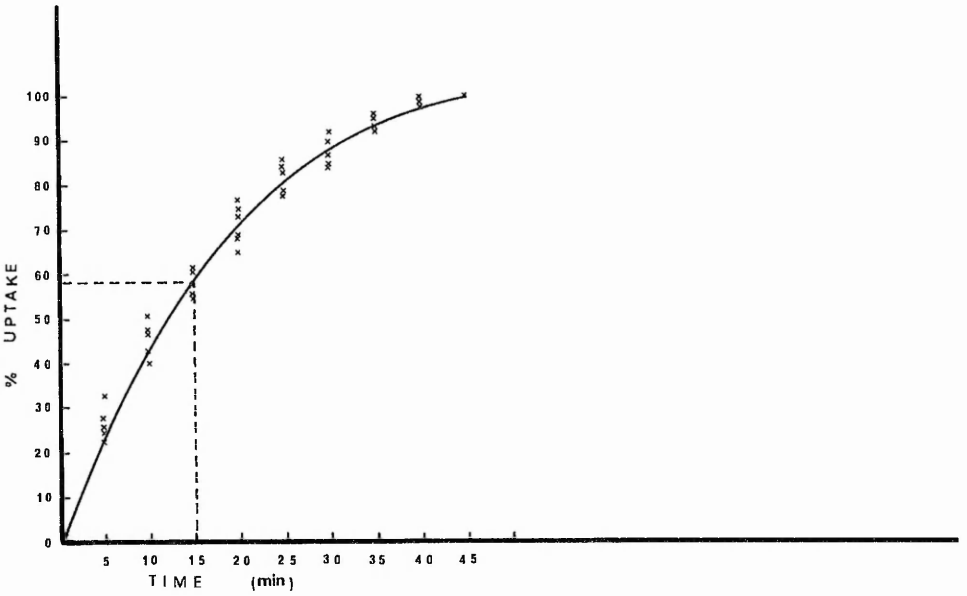


Fig. 28 The kinetics of percentage uptake of Candida by normal neutrophils using a ratio of 4.4 Candida to 1 neutrophil.

Fig. 29 shows that 80% of PMN contain one or more Candida at 15 minutes and nearly every neutrophil contains one or more by 35 minutes.

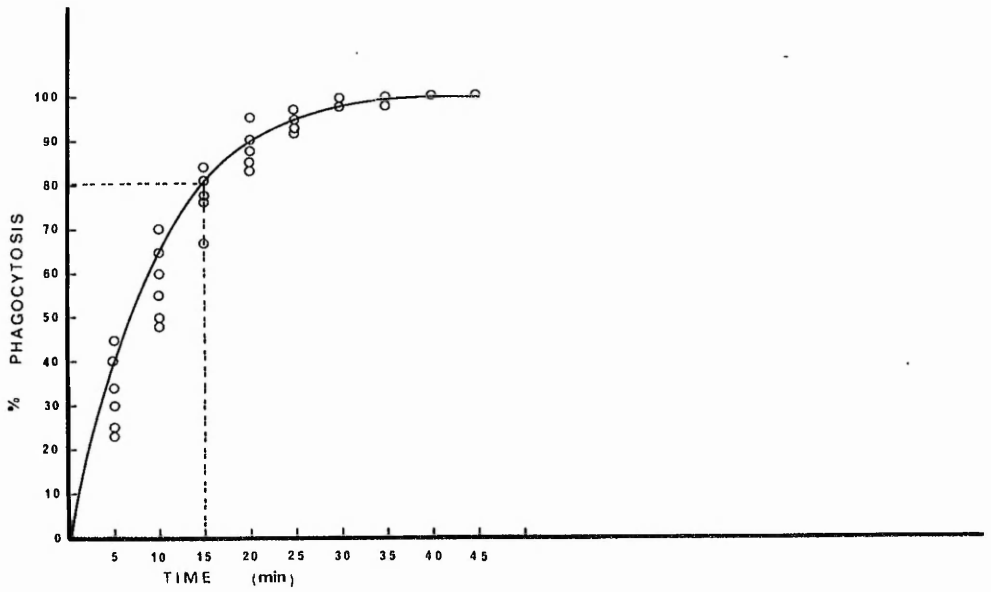


Fig. 29 The kinetics of percentage phagocytosis of Candida by normal neutrophils using a ratio of 4.4 Candida to 1 neutrophil.

Therefore, by choosing the ratio of 4.4:1 and the 15 min timing, it was possible to detect quite small changes in the rate of uptake or phagocytosis.

Fig. 30 shows that about 50% of the phagocytosed Candida was killed by 90 minutes. Only slight increase in percentage kill was achieved by further incubation. (See page 131 for further discussion on the shape of the graph).

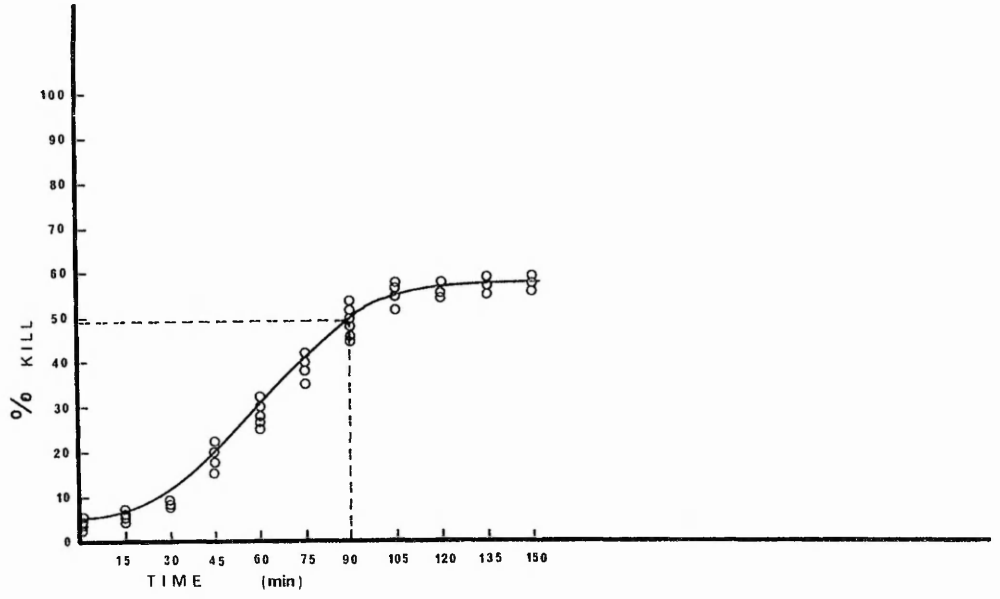


Fig. 30 The kinetics of percentage kill of Candida by normal neutrophils using a ratio of 4.4 Candida to 1 neutrophil.

The choice of ratio and time here allows the detection of impaired or super fungicidal activities.

Thus the following conditions were chosen for use in subsequent investigations:

1. Candida:PMN ratio of 4.4 to 1.
2. Sampling at 15 min for the determination of % uptake and phagocytosis.
3. Sampling at 90 min for the determination of complete uptake and phagocytosis and estimation of % kill of Candida by the neutrophils.

h) Discussion:

Phagocytosis has been extensively studied in vitro in patients with some of the myeloproliferative disorders (mainly CGL) but until recently more attention has been paid to the ingestion potential ("uptake") than to the killing ("cidal") activity of the cell. The latter is as important in defence against infection as the former. The interpretation of experimental results is, however, far from straightforward. A crucial variable is the clinical status of the patient and whether he is being treated or not. Moreover, results may be expected to vary with the nature of the target substance used, such substances have included various living and dead bacteria, fungi, latex particles and indian ink, to mention only a few. It is quite possible that patients with Myeloproliferative disorders could have a phagocytic or cidal defect that is selective for particular species of micro-organisms. It might be identifiable only in tests using one or a few types of microorganism and could reflect a deficiency of a single cytoplasmic enzyme. Thus a normal result in any one laboratory test may be given by neutrophils that would be found to be grossly abnormal in another test. That is why it was decided to monitor as many functions and enzymes as possible on the same patients so that any defect or deficiency which might otherwise be overlooked could be detected.

Assessment of phagocytic capacity:

The conflicting results reported on the phagocytic capacity of neutrophils from normals and patients reflect the different techniques employed as well as the variable clinical

circumstances. An adequate test of phagocytic capacity requires the presence of a clear excess of organisms in the incubation mixture so that no neutrophil is denied the opportunity of demonstrating its activity because of the absence of organisms. An organism-neutrophil ratio of 10 was used by Penny & Galton (1966). However, a ratio of 1:1 would only reveal cases with a very gross impairment of phagocytic capacity.

Assessment of killing capacity:

When killing capacity is being measured, an excess of organisms is usually avoided so that all the organisms are rapidly ingested and none remains outside the neutrophils at the end of the incubation. A ratio of 1:1 has been used by Goldman & Th'ng (1973). However, if the ratio of organisms to neutrophils is too low, then some neutrophils will ingest several organisms while others fail to ingest any, and a defect affecting part of the cell population may not be detectable. Thus, for any particular experimental system the optimum ratio of organism to neutrophil must be determined to give complete phagocytosis within a relatively short incubation and at the same time allow ingestion of organisms by all the neutrophils. Using Candida guillermondii, the optimum ratio and timing for measuring the rate of both phagocytosis and killing by normal cells has been determined. Two separate measurements can be made for phagocytosis; first, the proportion of neutrophils that have engulfed one or more Candida which is called the percentage phagocytosis, and secondly, the proportion of the total Candida that have been

engulfed, which is called the percentage uptake. Measuring these two parameters separately allows us to distinguish the possible presence of two populations of neutrophils. Having chosen the ratio of 4.4:1 Candida to neutrophil and the 15 minute timing it has been possible to detect any slight changes in the rate of uptake or phagocytosis.

In the case of killing, however, the 90 minute incubation will allow the detection of impaired or super fungicidal activities.

It is of interest that active killing takes place between 30 and 90 minutes. The reason for this may be explained by the H_2O_2 finding during phagocytosis where a progressive accumulation of H_2O_2 takes place during the first 30 minutes of incubation followed by a steady decline at about 40. (See Fig. 15). On the whole, the choice of ratio and timing had made sure that every neutrophil contributed to the overall results.

C H A P T E R F I V E

R E S U L T S

Correlation of functional activities and enzymic levels

It is possible to investigate and even to show graphs of possible correlations between any two variables investigated in any of the four conditions in this study. This would result in excess of 180 such graphs and obviously selection was needed.

If statistical correlation between both parameters considered was absent and, in the case of the clinical condition the parameters fell in the normal range, then it was decided that graphical as well as statistical investigations were unnecessary.

To facilitate understanding and comparison, the results are presented under four headings: Normal subjects, Chronic Granulocytic Leukaemia, Myeloid Metaplasia and Polycythaemia Rubra Vera. Studies on patients with severe infection has also been included for reasons discussed on page 204. Presentation of results from these conditions as descrete sections of the thesis is complimented by an integrated discussion of the overall results of the phagocytic mechanism later. (See page 207).

1. Normal subjects

Percentage uptake, phagocytosis and killing were measured on fifty normal subjects, who were mainly hospital staff and patients attending with minor orthopaedic problems. These normal volunteers were free from colds and similar minor conditions. Their age and sex are shown on pages 142 and 140. At least one of these controls was included every time the investigations were carried out on Myeloproliferative patients.

In fig. 31, the percentage uptake was plotted for each of the fifty controls. Uptake ranged between 45 and 70% with a mean of 58.62 and standard error of $\bar{+}$ 0.77.

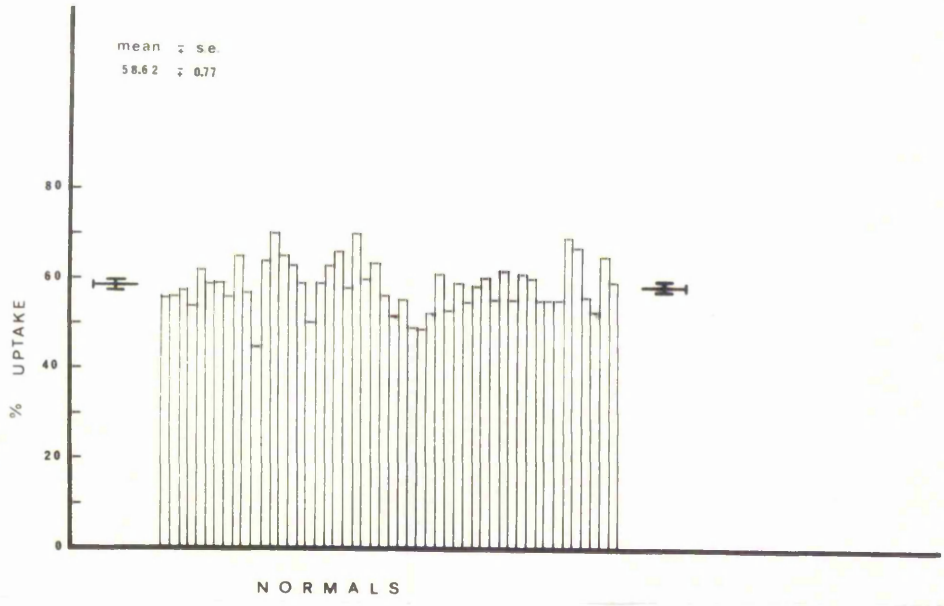


Fig. 31 Percentage uptake after 15 minutes by neutrophils of 50 normal subjects. The results are expressed as mean $\bar{+}$ standard error.

Percentage phagocytosis ranged between 65 and 92% with a mean of 78.90 and a standard error of $\bar{+}$ 0.92 (Fig. 32).

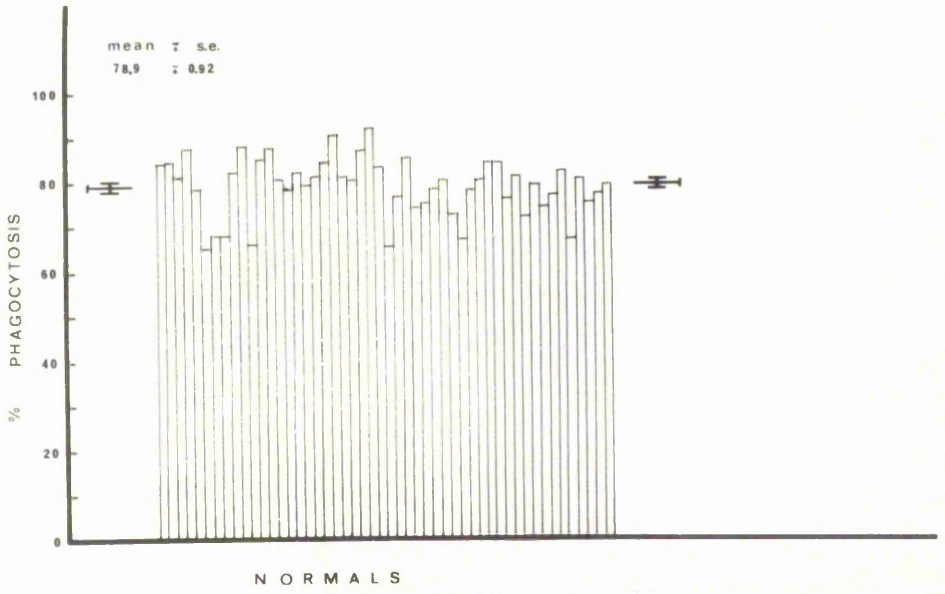


Fig. 32 Percentage phagocytosis after 15 minutes by neutrophils of 50 normal subjects. The results are expressed as mean $\bar{+}$ standard error.

Percentage kill ranged between 41 and 58% with a mean of 49.10 and a standard error of $\bar{+}$ 0.58 (Fig. 33).

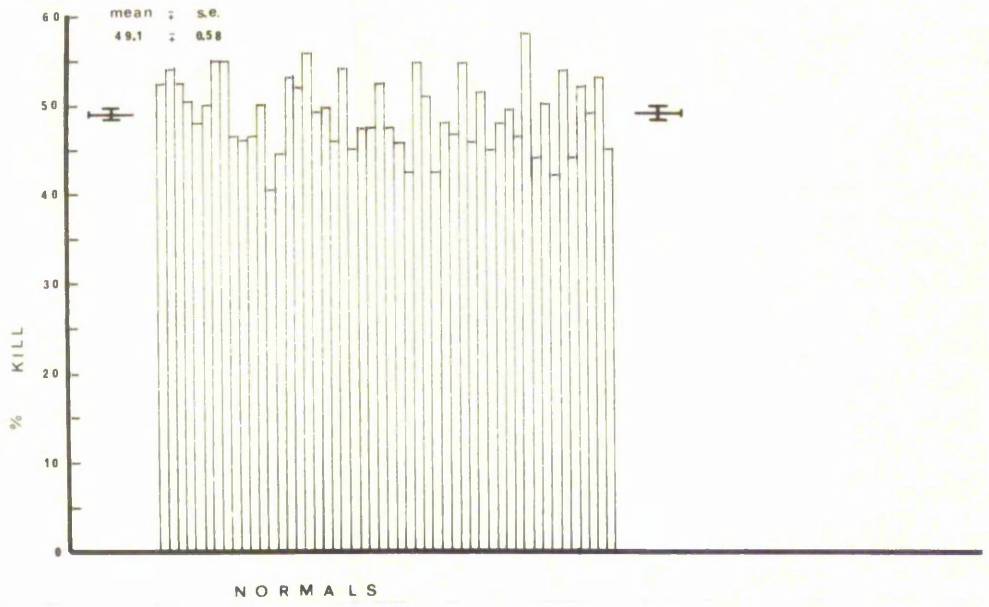


Fig. 33 Percentage kill of Candida after 90 minutes by neutrophils of 50 normal subjects. The results are expressed as mean ∓ standard error.

Quantitative alkaline phosphatase and peroxidase measurements were carried out on 13 of the control subjects.

Normal alkaline phosphatase ranged between 0.11 and 0.39 units/10^6 neutrophils. Mean of 0.22 and standard error of ∓ 0.02. (Fig. 34).

Normal peroxidase values ranged between 0.30 and 0.91 units/10^6 neutrophils. Mean of 0.62 and standard error of ∓ 0.05. (Fig. 35).

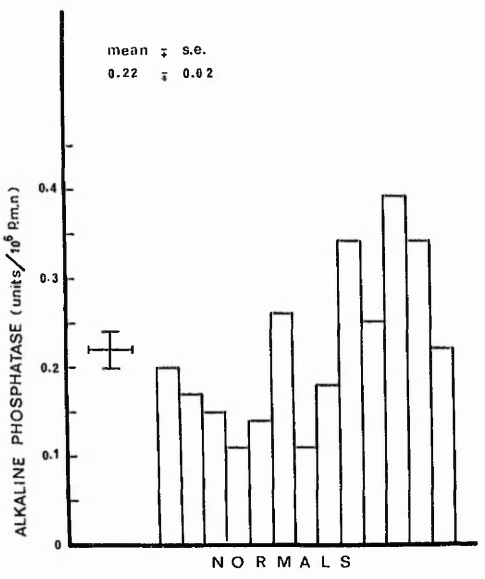


Fig. 34 Neutrophil alkaline phosphatase values of 13 normal subjects. Results expressed as mean ∓ standard error.

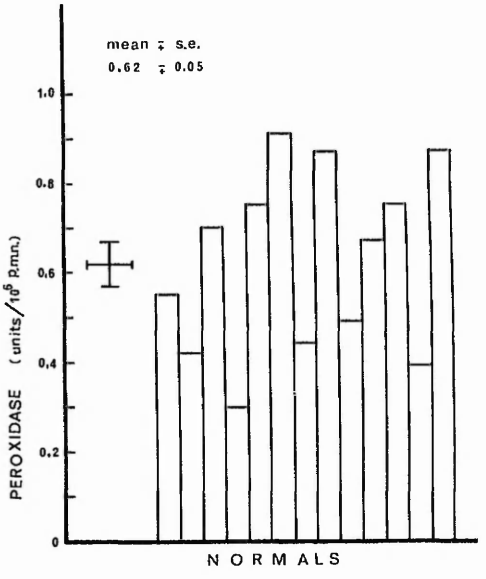


Fig. 35 Neutrophil peroxidase values of 13 normal subjects. Results expressed as mean ∓ standard error.

Quantitative acid phosphatase measurements were carried out on ten normal subjects, giving values ranging from 2.75 to 3.70 units/10^6 neutrophils, with a mean of 3.08 and standard error of ∓ 0.11. (Fig. 36).

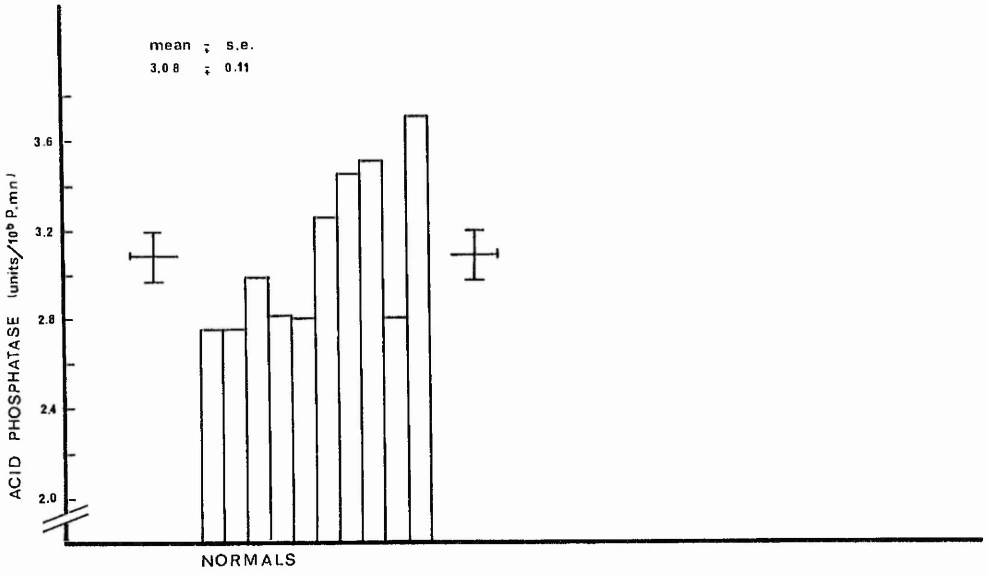


Fig. 36 Neutrophil acid phosphatase values of 10 normal subjects. Results expressed as mean ∓ standard error.

Quantitative H_2O_2 measurements during 80 minutes of phagocytosis were carried out on 15 normal subjects and the normal range of H_2O_2 production varied between 1.10 and 3.20 units/10^6 neutrophils over 80 min, with a mean of 2.34 and standard error of ∓ 0.16. (Fig. 37). (For definition of H_2O_2 unit see page 103).

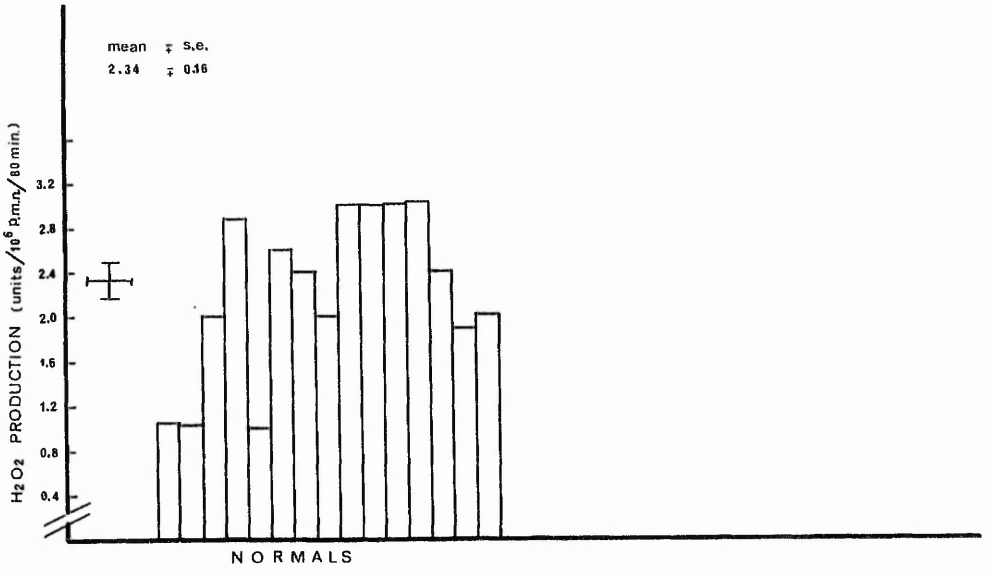


Fig. 37 Hydrogen peroxide production by neutrophils from 15 normal subjects during 80 minutes phagocytosis. Results expressed as mean ∓ standard error.

These normal findings are summarised in Table 1.

	No. of subjects	Range	Mean	S.E.	Fig.No.
Percentage uptake	50	45-70	58.62	∓0.77	31
Percentage phagocytosis	50	65-92	78.90	∓0.92	32
Percentage kill	50	41-58	49.10	∓0.58	33
Peroxidase units/10^6 neutrophils	13	.31-.91	0.62	∓0.05	35
Alkaline phosphatase units/10^6 neutrophils	13	.11-.39	0.22	∓0.02	34
Acid phosphatase units/10^6 neutrophils	10	2.75-3.70	3.08	∓0.11	36
H_2O_2 production units/10^6 neutrophils/80′	15	1.10-3.20	2.34	∓0.16	37

Table 1. A summary of previous normal results.

There was no significant difference between the values of normal males and females for percentage uptake (Fig. 38),

percentage phagocytosis (Fig. 39) and percentage kill (Fig. 40).

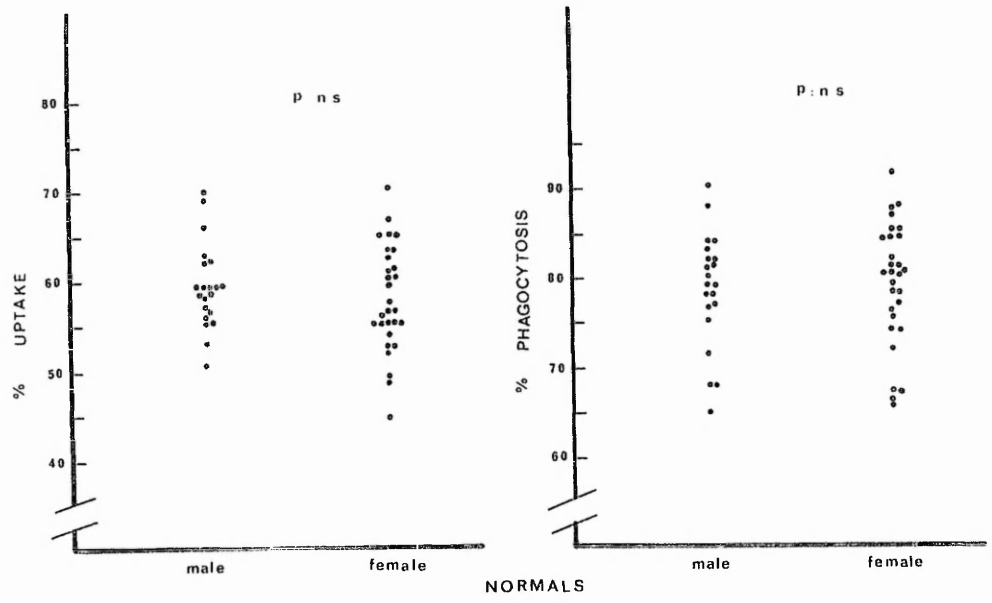


Fig. 38 Percentage uptake by neutrophils from 21 male and 29 female normal subjects. No significant difference.

Fig. 39 Percentage phagocytosis by neutrophils from 21 male and 29 female normal subjects. No significant difference.

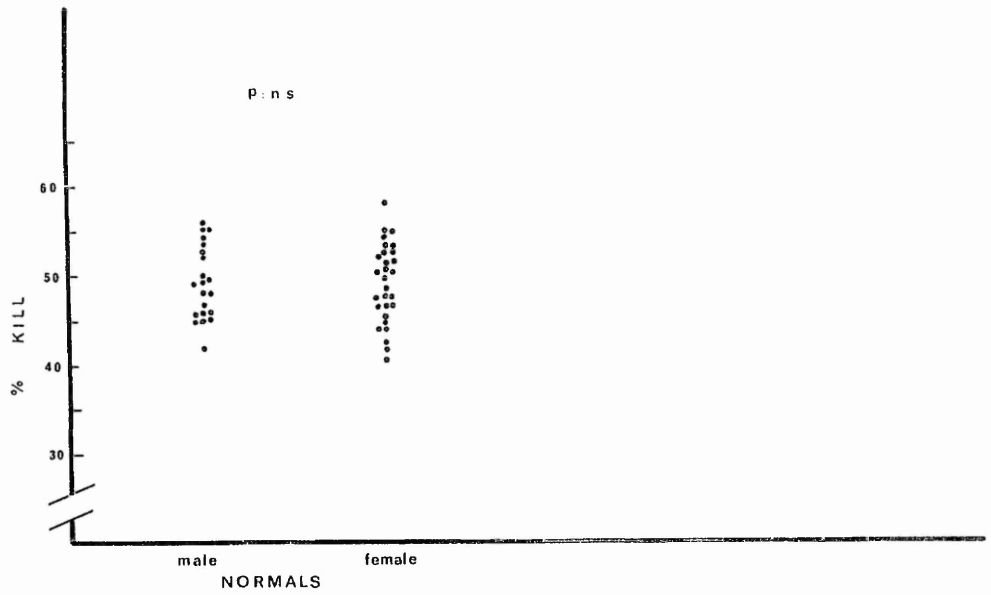


Fig. 40 Percentage kill of Candida by neutrophils from 21 male and 29 female normal subjects. No significant difference.

There was no correlation between the age of the controls and percentage uptake (Fig. 41), percentage phagocytosis (Fig. 42) and percentage kill (Fig. 43).

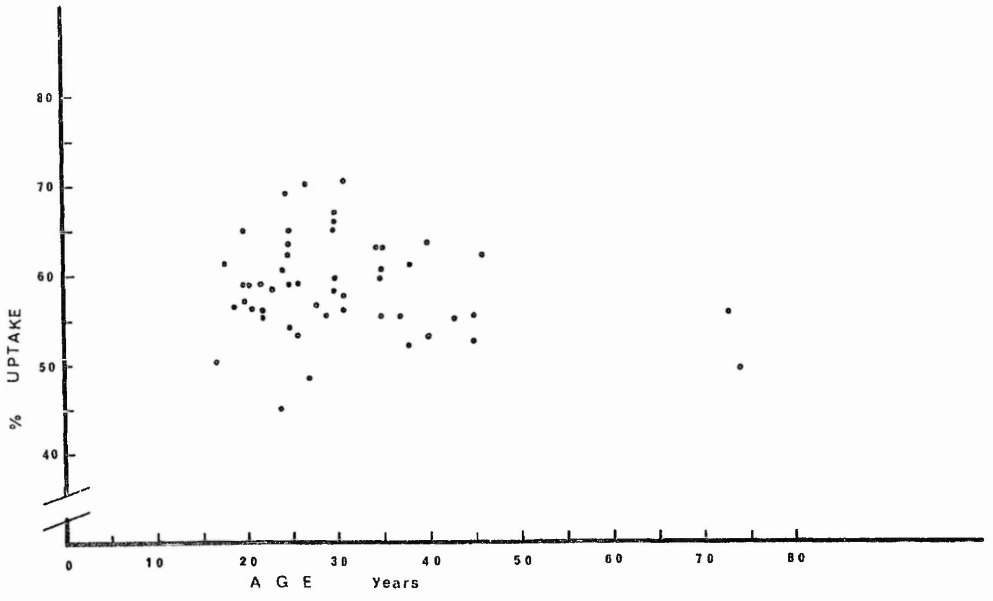


Fig. 41 Percentage uptake by normal neutrophils of 50 donors as a function of the age of donors. No significant correlation.

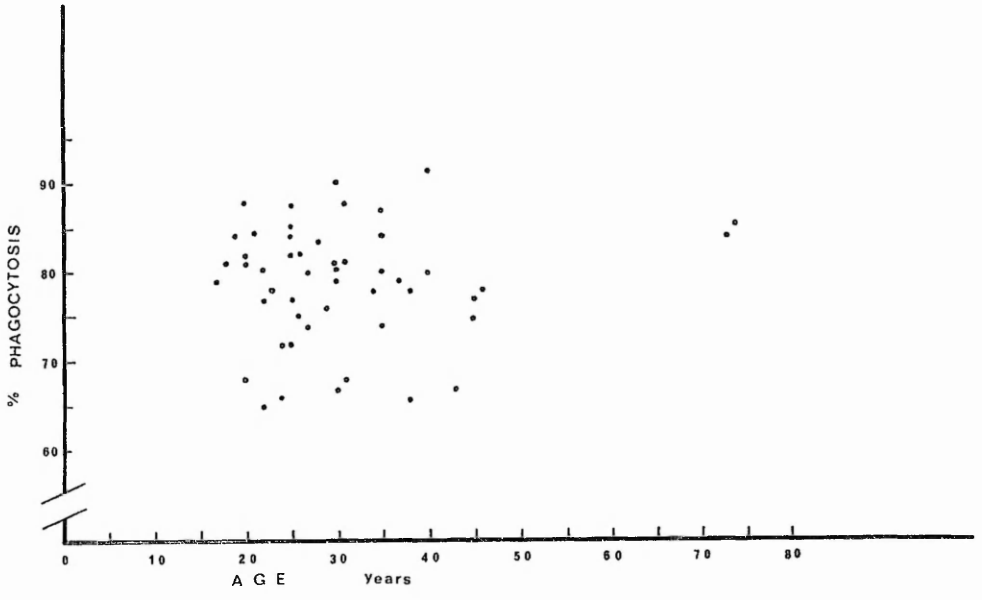


Fig. 42 Percentage phagocytosis by normal neutrophils of 50 donors as a function of the age of donors, No significant correlation.

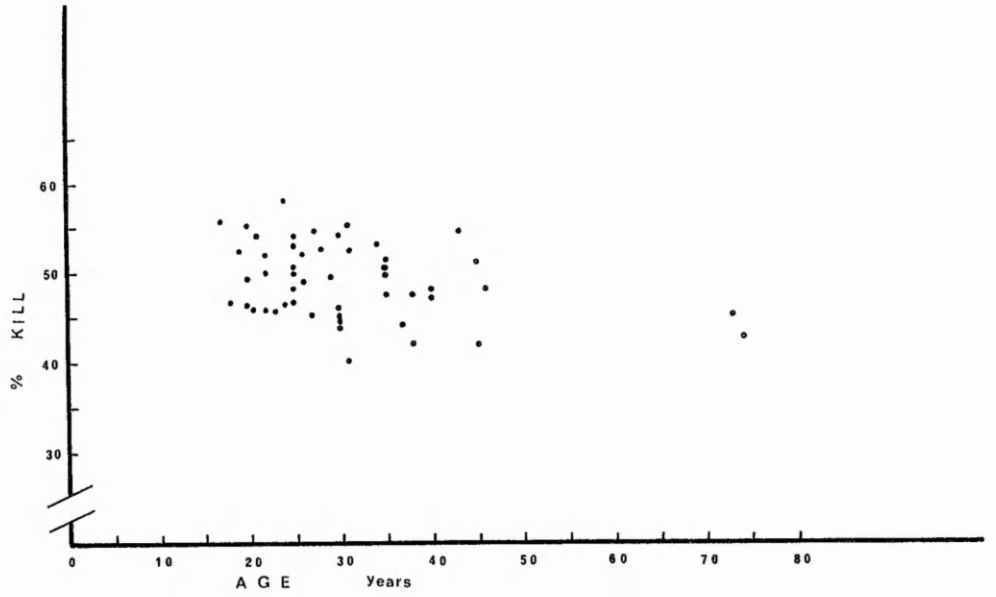


Fig. 43 Percentage kill by normal neutrophils of 50 donors as a function of the age of donors. No significant correlation.

In a few cases, the same subject was tested over a period of time to establish any drift in their neutrophil function. There was no correlation between percentage uptake, phagocytosis, killing and time (Figs. 44, 45 and 46). (See appendix 2, page 216 for decimal date system).

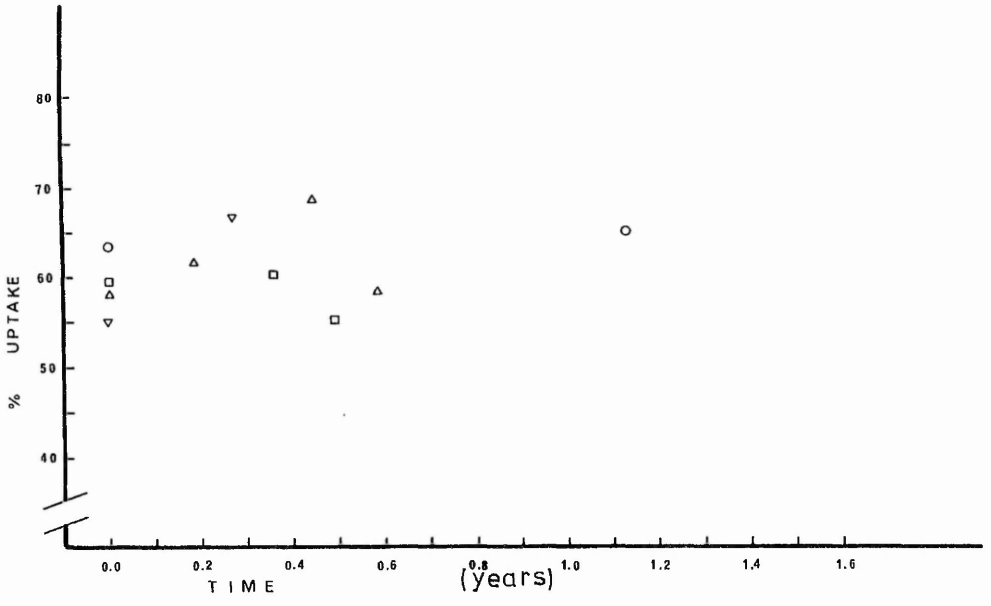


Fig. 44 The reproducability of percentage uptake by normal neutrophils of 4 donors when repeated over a period of time. No significant change.

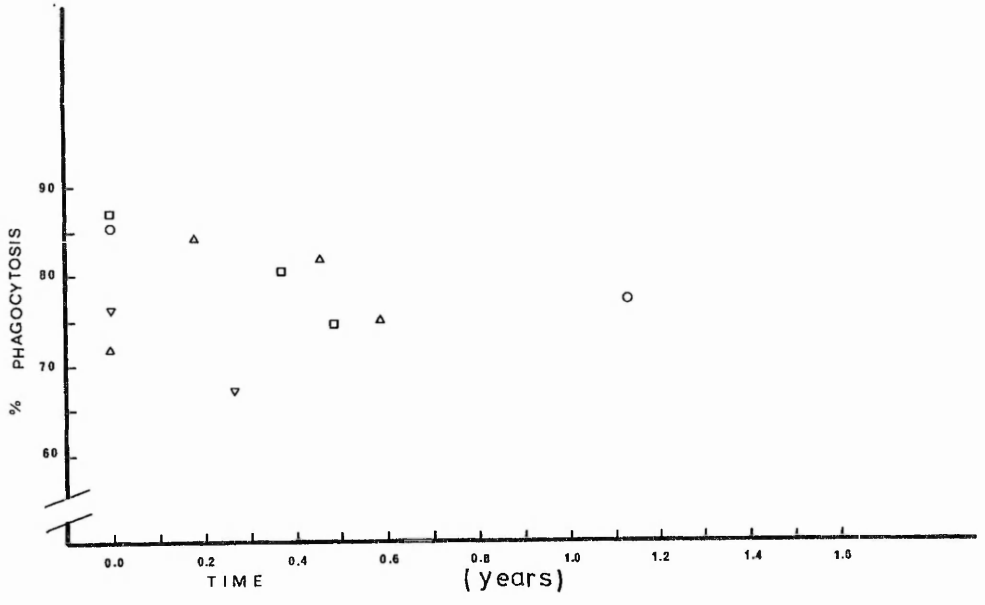


Fig. 45 The reproducability of percentage phagocytosis by normal neutrophils of 4 donors when repeated over a period of time. No significant change.

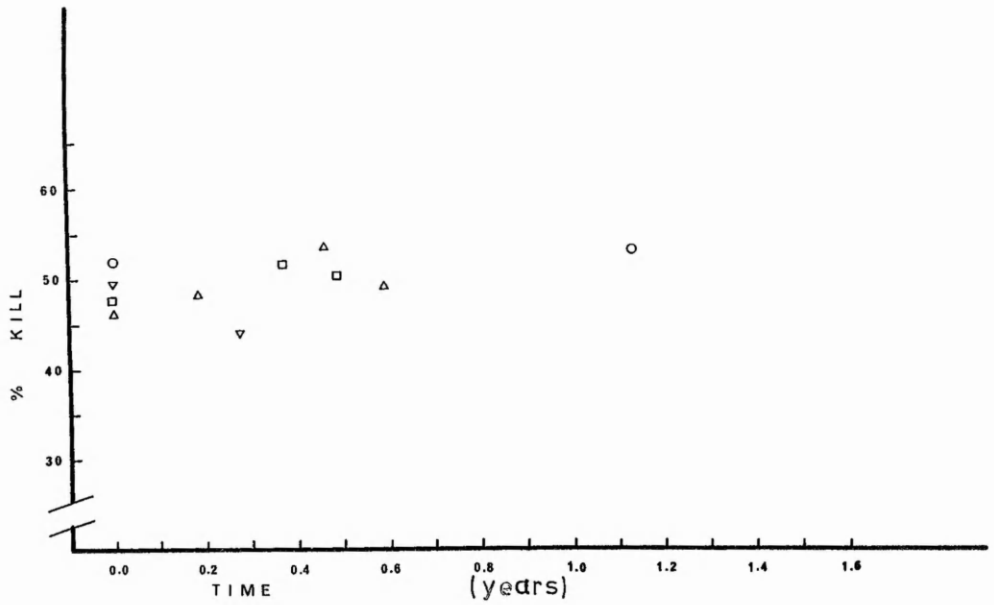


Fig. 46 The reproducability of percentage kill by normal neutrophils of 4 donors when repeated over a period of time. No significant change.

The figures show the decimal date order with 1974 on the left and 1976 on the right.

Observation of normal neutrophils stained for peroxidase shows red stained nuclei and uniformally distributed blue granules throughout the cytoplasm indicating the sites of peroxidase activity. (See Fig. 47).

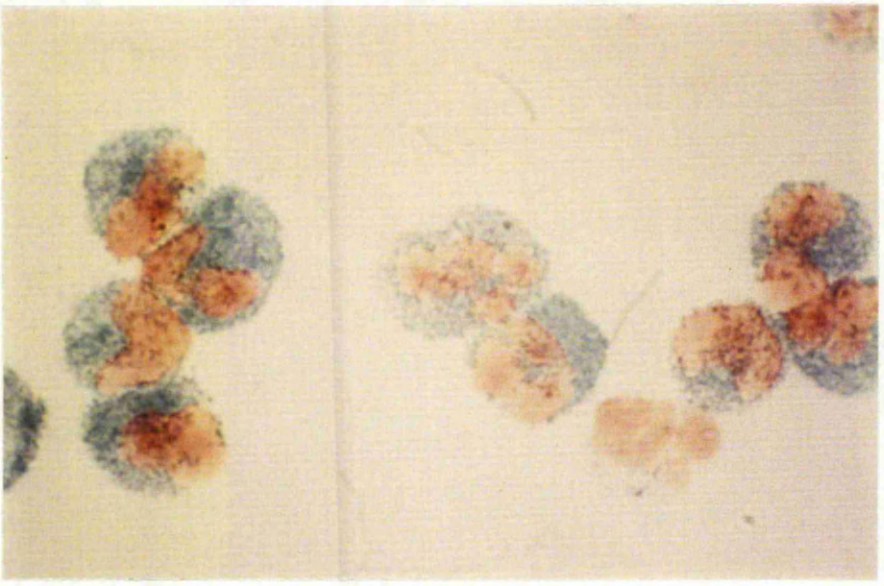


Fig. 47 A peroxidase stained preparation of normal leukocytes before phagocytosis. Note granular reaction products throughout the cytoplasm. (X1215).

When normal neutrophils were allowed to phagocytose for 90 min and were then stained for peroxidase, a significant reduction in the amount and distribution of these blue granules was seen. (See Fig. 48).

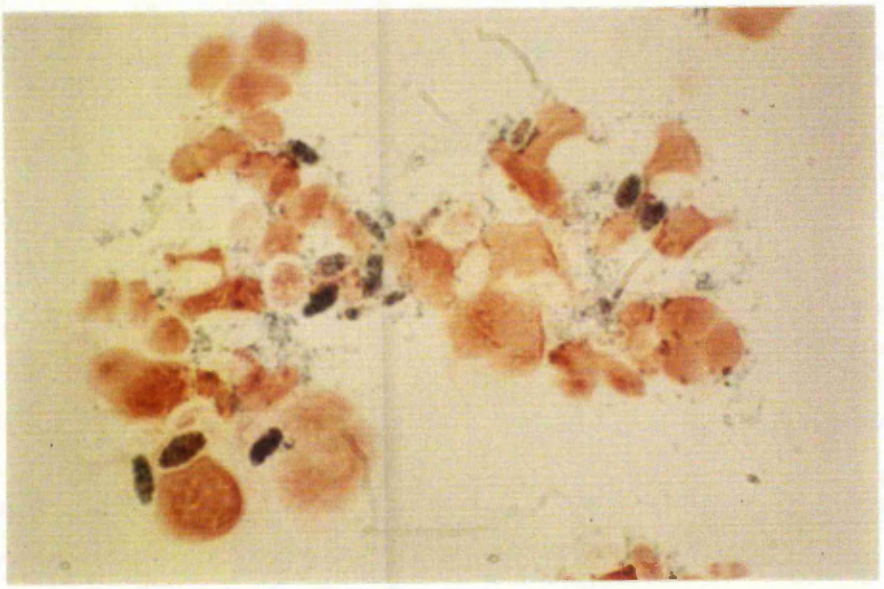


Fig. 48. A peroxidase stained preparation of normal leukocytes after phagocytosis of Candida for 90 minutes. Note loss of reaction products from the cytoplasm. (X1215).

This observation led to quantitative assessment of peroxidase before and after phagocytosis. In eight normal subjects peroxidase levels fell from 0.79 (± 0.12) before phagocytosis to 0.35 (± 0.07) after 90 min incubation with Candida. (See Fig. 49). (See later for CGL part.)

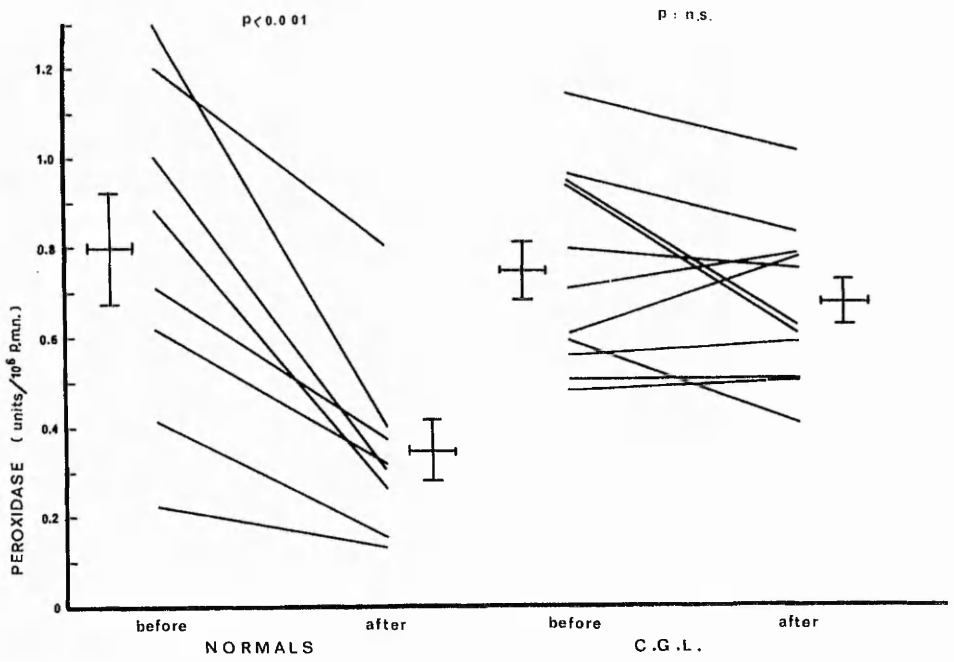


Fig. 49 Peroxidase levels before and after phagocytosis of Candida by neutrophils from normals and from patients with Chronic Granulocytic Leukaemia. Mean ∓ standard error is shown.

Since acid phosphatase is a primary enzyme as well as peroxidase, it was of interest to find out whether it behaved in a similar manner to peroxidase after phagocytosis.

Qualitative observation on normal neutrophils before phagocytosis showed acid phosphatase activity as a uniformally distributed red colouration throughout the cytoplasm while nuclei stained green. (See Fig. 50).

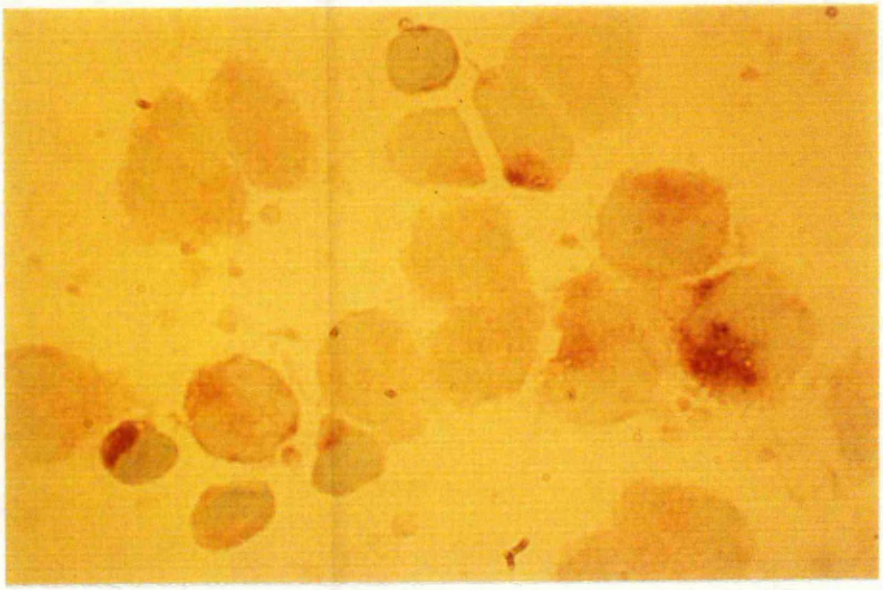


Fig. 50 An acid phosphatase stained preparation of normal leukocytes before phagocytosis. Note reaction products throughout the cytoplasm. (X1215).

Observations after 90 min phagocytosis indicated a considerable loss of the red colouration. This observation reinforces the previous peroxidase observations. (See Fig. 51).

Fig. 51 An acid phosphatase stained preparation of normal leukocytes after phagocytosis of Candida for 90 minutes. Note loss of reaction products from the cytoplasm. (X1215).

Qualitative observations of β-glucuronidase, another primary enzyme, reflected the same picture.

Qualitative alkaline phosphatase observations on normal neutrophils shows blue colouration indicating sites of activity. The density and distribution of these sites varies between complete lack of activity to very high activity. (See Fig. 9).

When alkaline phosphatase observations were made after 90 min phagocytosis there was no detectable change from appearance of activity before phagocytosis.

As a matter of interest gram stain was applied to Candida before and after 90 min phagocytosis by normal neutrophils.

Fig. 52 shows that living Candida guillermondii is gram positive.

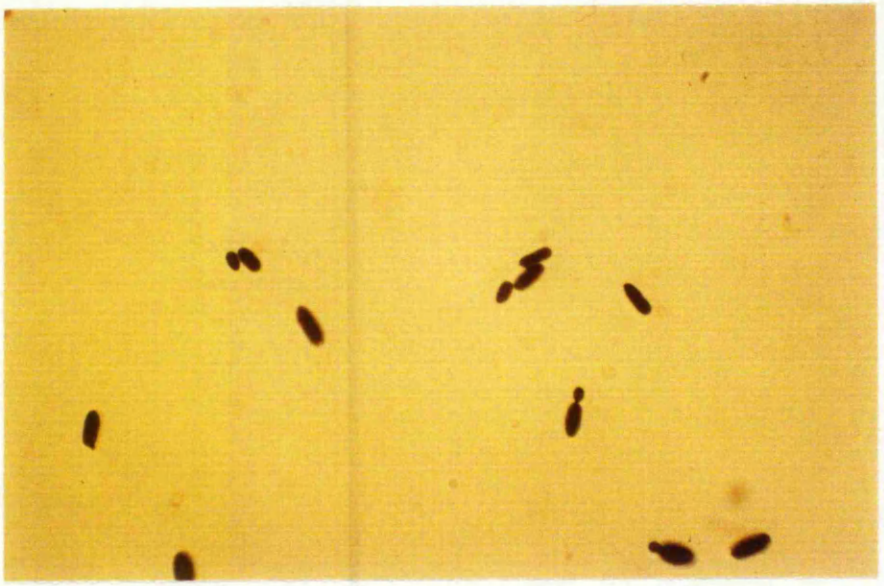


Fig. 52 A gram stained preparation of living Candida guillermondii. Gram positive. (X1000).

The more valuable finding was when gram stain was applied to normal neutrophils which have phagocytosed Candida guillermondii for 90 minutes.

Fig. 53 shows three states of gram stained Candida. Living ones which remained gram positive, gram negative ones and few undergoing localized change from gram positive to gram negative. The possible interpretation of these findings have been discussed on page 209.

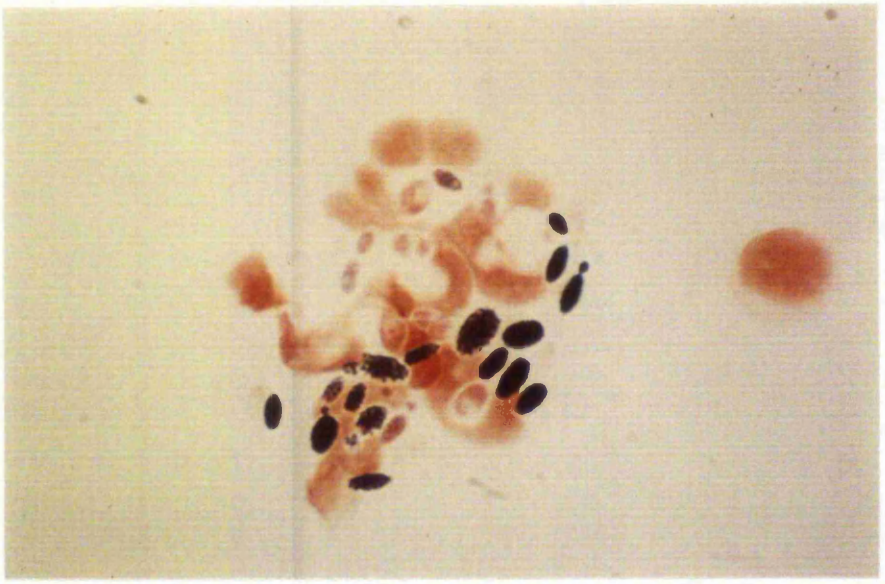


Fig. 53 A gram stained preparation of normal neutrophils and Candida after 90 minutes phagocytosis. Note that some Candida maintained positivity, some have become gram negative and some are undergoing a localized change. (X1215).

a) Discussion:

Results of phagocytosis and killing by normal neutrophils vary from one laboratory to another depending on the nature of the target organism used, the ratio of neutrophil to organism and the length of the incubation time. These differences in the assessment procedure, that is in the case of phagocytosis, whether the percentage of neutrophils containing organisms was quantitated, whether the average number of organisms ingested by each phagocyte was recorded or whether the percentage of organisms taken up by the neutrophils was calculated, all produce variable values.

In the case of killing, normal values vary depending on the method used in lysing the neutrophils to liberate ingested organisms and whether colony counting or specific staining was used in the procedure. All these variables contribute towards a very heterogeneous collection of supposedly normal results. As a result, comparisons of normal values become meaningless.

Similar variations apply to the enzymatic values of normal neutrophils. Factors such as the separation procedure used in obtaining the neutrophils as well as the way of releasing the enzyme could be vital. For example, it was found that peroxidase was stable for at least 12 h in neutrophils which had not ingested particles as long as the cells remained intact, or after gentle sonication, as long as the granules containing the enzyme remained intact. When cells and granules were lysed with zaponin, there was a prompt and steady loss of activity. The units used to express the enzyme level also vary and include units/10^6 neutrophils, units/mg dry weight or units/µg protein, to mention but a few.

On the whole, it is obvious that normal values have to be established specifically in every individual experimental set up. This is a disadvantage when comparing the absolute values of one laboratory with those of another, and therefore a unified approach in all laboratories would be highly desirable. It is less of a problem when interest is concentrated on whether certain populations of neutrophils behave in any way differently to normal ones, which is the

position in the present case. This however, does enable relative comparisons to be made between different laboratories.

There are two important observations regarding the peroxidase level after phagocytosis, and the pattern of H_2O_2 production during phagocytosis. It is well established that degranulation takes place during phagocytosis and enzymic contents of primary and secondary granules enter the phagosome. There was a considerable reduction, qualitatively and quantitatively in the peroxidase after 90′ incubation. This reduction can be due to actual degredation of the enzyme by other hydrolytic enzymes released during the degranulation process or could be due to inhibition by excess H_2O_2. It has been proposed that when H_2O_2 forms a complex with both of the haem iron groups of peroxidase, it is degraded to O_2 and H_2O, and the enzyme is inactivated. We have considered the peroxidase reduction as an indication of normal degranulation.

The other observation is the overall pattern of H_2O_2 production during 80′ phagocytosis (see Fig. 15). As H_2O_2 production reaches a peak round about 35′ and then steadily declines, it is feasible that the observed curve is in fact the net result of H_2O_2 production and H_2O_2 depletion which are taking place at the same time. As H_2O_2 production is stimulated by the actual act of phagocytosis and as we have found that total phagocytosis is achieved by about 30′, it follows that after that time H_2O_2 is being degraded without being generated which explains the decline in the curve. The inactivation of the peroxidase and the degradation of H_2O_2 offers a possible means for control of the peroxidase system

when H_2O_2 is in excess.

The H_2O_2 curve can also explain the killing/time curve. (See Fig. 30).

2. Chronic Granulocytic Leukaemia

Thirteen CGL patients were studied. They were diagnosed by clinical, peripheral blood and bone marrow findings. All were Philadelphia chromosome positive. Most were on Busulphan treatment when first studied. The percentages of uptake, phagocytosis and killing, alkaline phosphatase and peroxidase activities, and the H_2O_2 levels, were all measured.

Percentage uptake ranged between 32.4 and 60.1 with a mean of 49.66 and standard error of $\bar{+}$ 1.89. This is slightly but significantly lower than the normal value. $P < 0.001$. (Fig. 54).

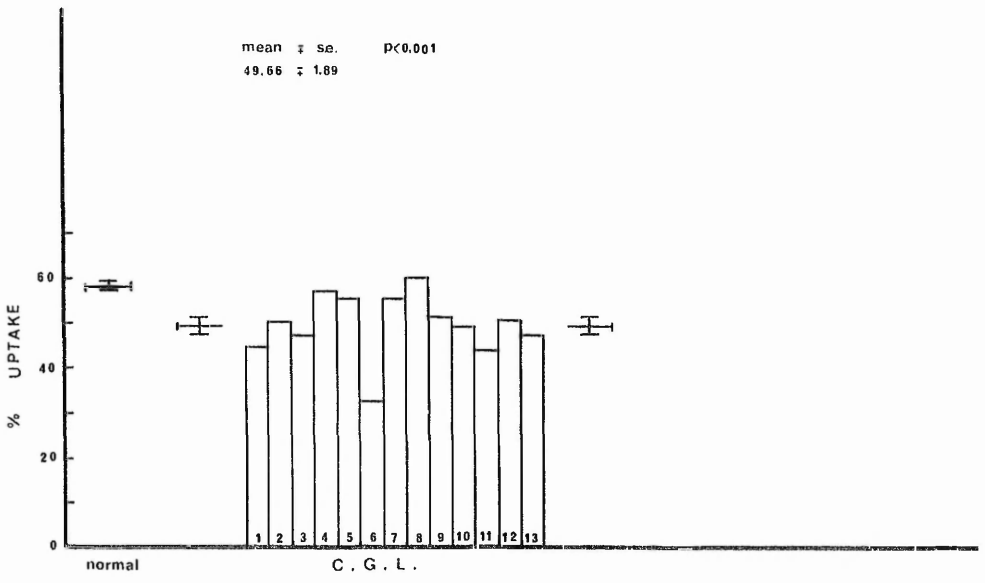


Fig. 54 Percentage uptake after 15 minutes by neutrophils of 13 CGL patients. Results expressed as mean $\bar{+}$ standard error and are significantly different from normal.

Percentage phagocytosis ranged between 34.4 and 81.1 with a mean of 69.70 and standard error of ∓ 3.42. This is also significantly lower than the normal value. $P < 0.025$. (Fig. 55).

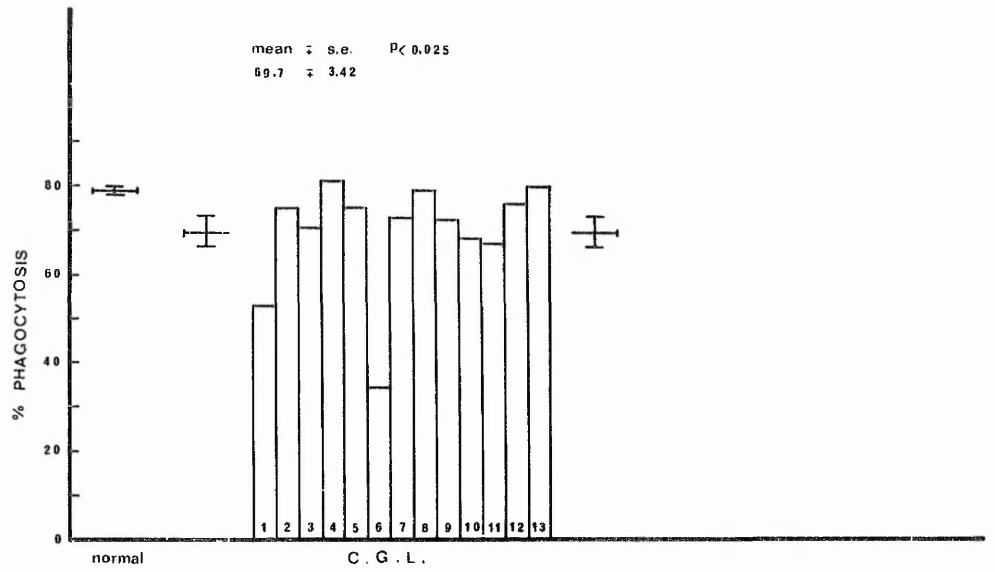


Fig. 55 Percentage phagocytosis after 15 minutes by neutrophils of 13 CGL patients. Results expressed as mean ∓ standard error and are significantly different from normal.

The percentage kill ranged between 13.4 and 40.0 with a mean of 23.67 and standard error of ∓ 2.22. This too is significantly lower than the normal value $P < 0.001$. (Fig. 56).

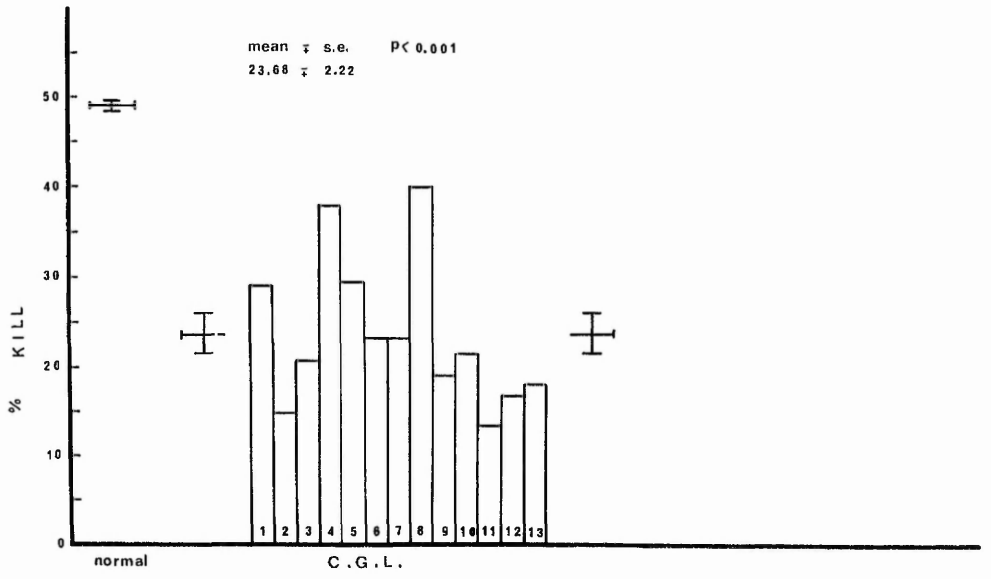


Fig. 56 Percentage kill after 90 minutes by neutrophils of 13 CGL patients. Results expressed as mean ∓ standard error and are significantly different from normal.

Quantitative alkaline phosphatase measurements of seven of the patients ranged between 0.00 and 0.03 units/10^6 neutrophils with an average of 0.01 and standard error of < ∓ 0.01. This was markedly lower than the normal value for alkaline phosphatase $P < 0.001$. (Fig. 57).

Quantitative peroxidase measurements of seven of the patients ranged between 0.37 and 0.61 units/10^6 neutrophils with an average of 0.50 and standard error of ∓ 0.03. This was statistically not significantly different from the normal peroxidase values. (Fig. 58).

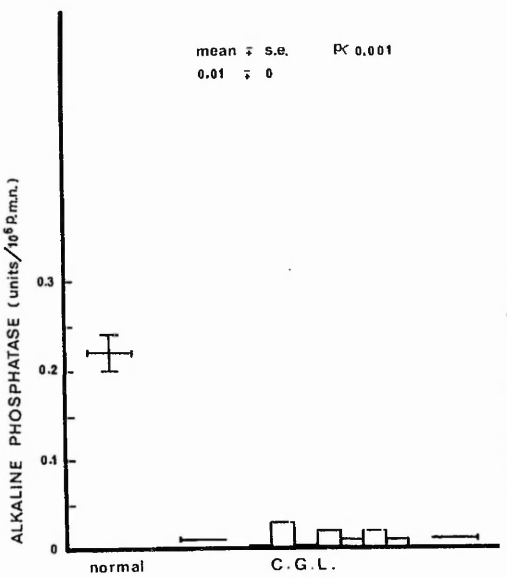


Fig. 57 Neutrophil alkaline phosphatase values of 7 CGL patients. Results as mean ∓ standard error are significantly different from normal.

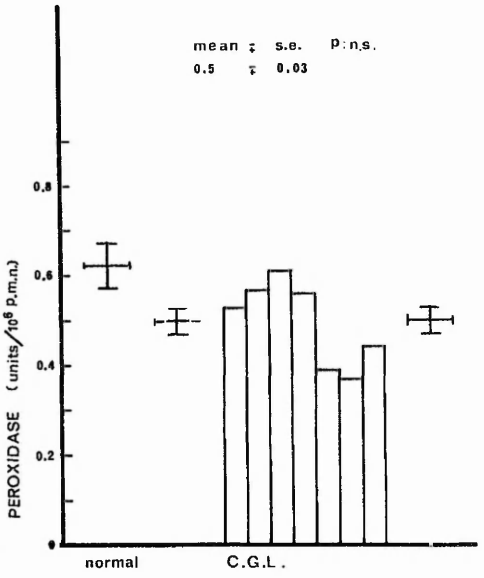


Fig. 58 Neutrophil peroxidase values of 7 CGL patients. Results as mean ∓ standard error are not significantly different from normal.

Quantitative H_2O_2 measurements during 80 minutes phagocytosis ranged between 0.06 and 1.75 units/10^6 neutrophils/80′ with an average of 0.67 and standard error of ∓ 0.16. This is significantly lower than the normal value. $P < 0.001$. (Fig. 59).

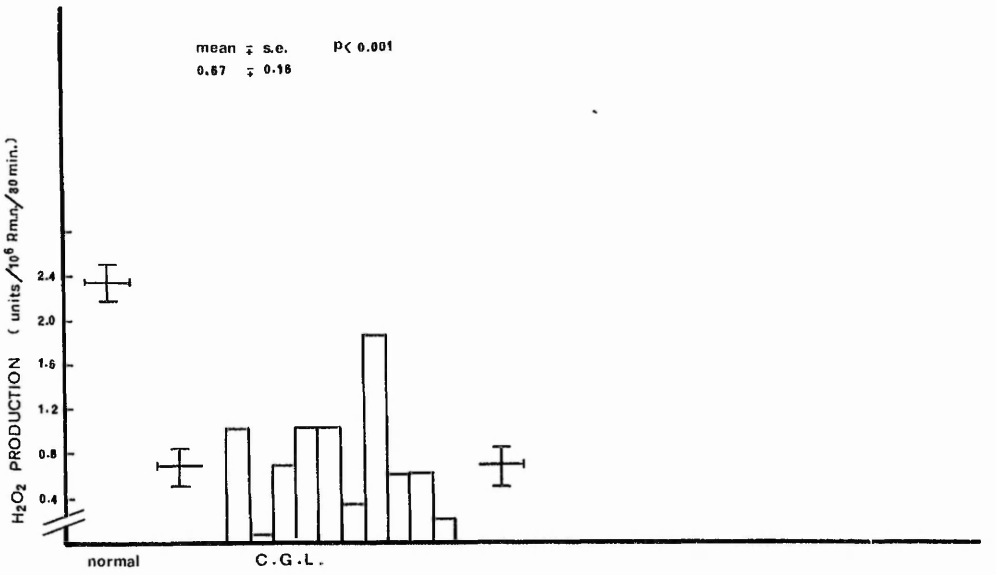


Fig. 59 Hydrogen peroxide production by neutrophils from 10 CGL patients during 80 minutes phagocytosis. Results as mean ∓ standard error are significantly different from normal.

All the previous CGL results are summarised in Table 2.

	No. of subjects	Range	Mean	S.E.	Signif-icance	Fig. No.
Percentage uptake	13	324-601	4966	∓1.89	P<0.001	54
Percentage phagocytosis	13	344-811	6970	∓3.42	P<0.025	55
Percentage kill	13	134-400	2367	∓2.22	P<0.001	56
Peroxidase units/10^6 neutrophils	7	037-061	050	∓0.03	N.S.	58
Alkaline phosphatase units/10^6 neutrophils	7	000-003	001	<∓0.01	P<0.001	57
H_2O_2 production units/10^6 neutrophils 80′	10	096-175	067	∓0.16	P<0.001	59

Table 2. Summary of functional and enzymic values of CGL neutrophils.

Percentage uptake was plotted against percentage kill to find out whether the slight reduction in percentage uptake was in a way responsible for the low percentage kill. However, no correlation was found. (Fig. 60).

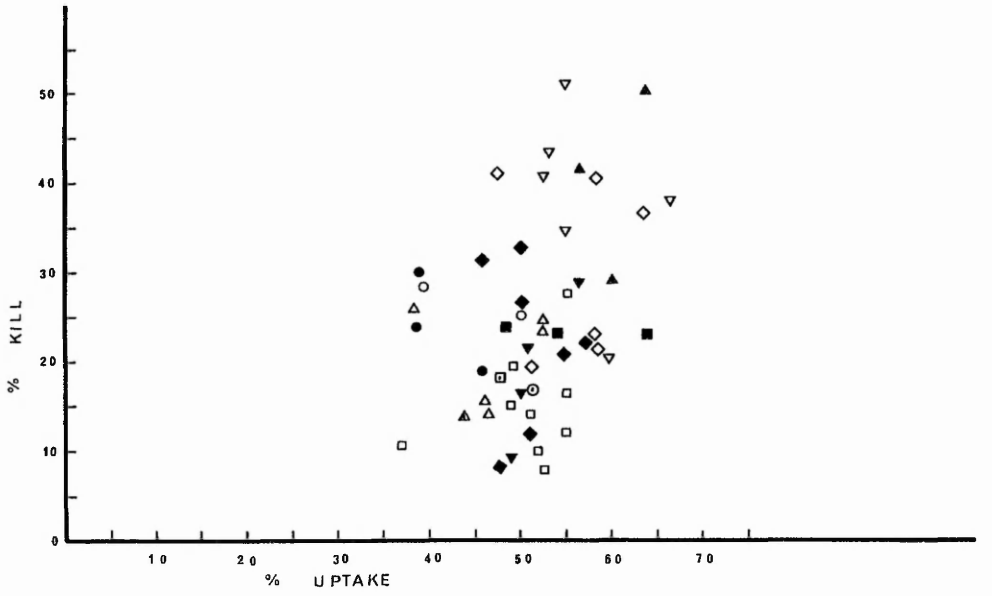


Fig. 60 Percentage kill at 90 minutes against percentage uptake at 15 minutes by neutrophils of 13 CGL patients repeated 52 times. No significant correlation.

Percentage uptake and phagocytosis showed no significant variation with time when the same patients were tested repeatedly over a period of up to 18 months. (See Figs. 61 and 62). This is consistent with normal neutrophils behaviour (see Figs. 44 and 45).

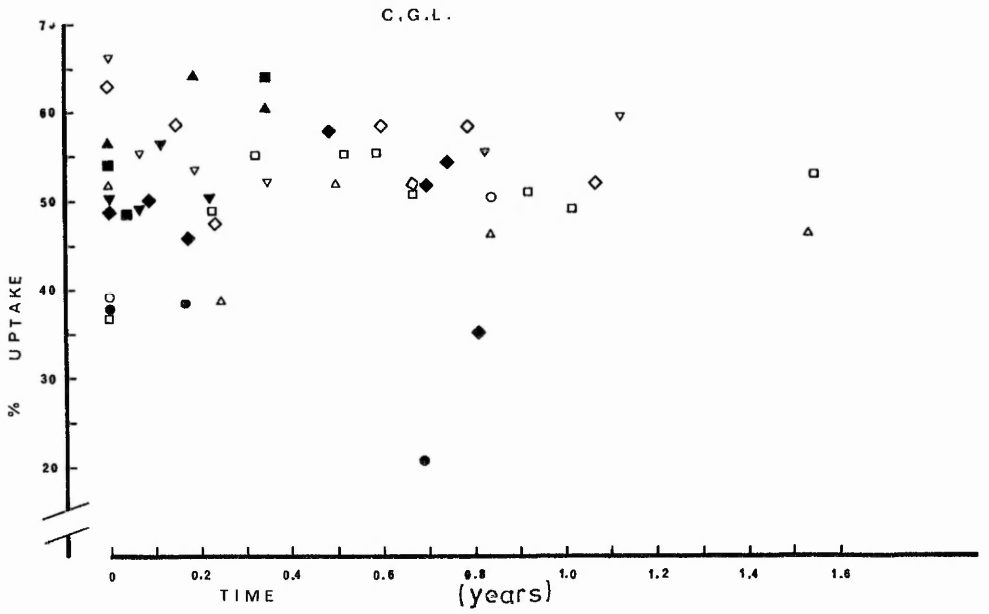


Fig. 61 The reproducability of percentage uptake by neutrophils of 10 CGL patients when repeated over a period of time. No significant change.

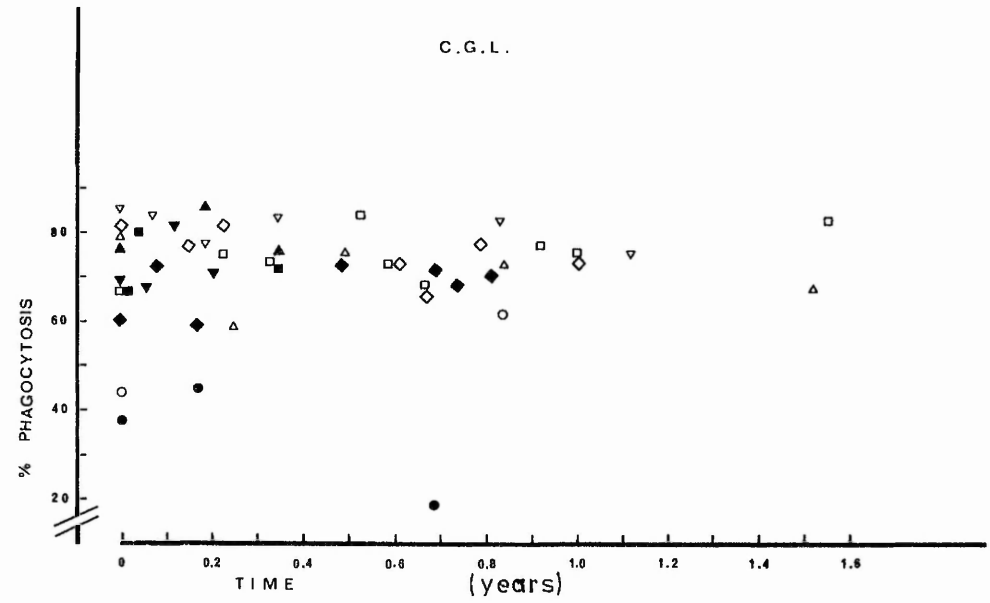


Fig. 62 The reproducability of percentage phagocytosis by neutrophils of 10 CGL patients when repeated over a period of time. No significant change.

There was, however, some correlation between percentage kill and time. (Fig. 63). A reduction of about 10% in percentage kill per year. This reduction was not observed in normal subjects. (See Fig. 46).

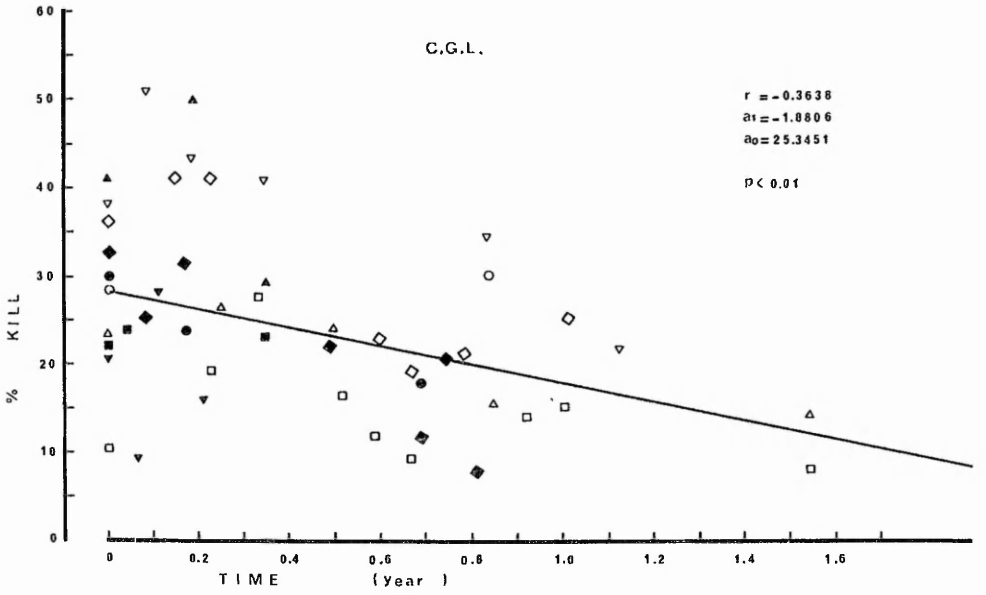


Fig. 63 The reproducability of percentage kill by neutrophils of 10 CGL patients when repeated over a period of time. A significant correlation and a reduction of about 10% in percentage kill per year is observed.

There was no correlation between percentage uptake and alkaline phosphatase values. (Fig. 64).

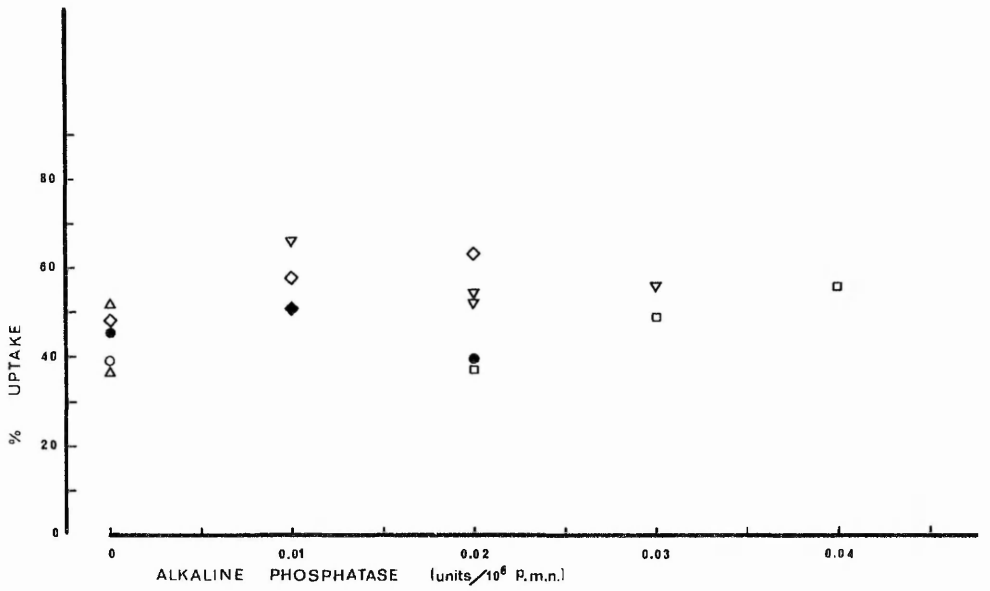


Fig. 64 Percentage uptake of 7 individual CGL patients as a function of their neutrophil alkaline phosphatase level. No significant correlation.

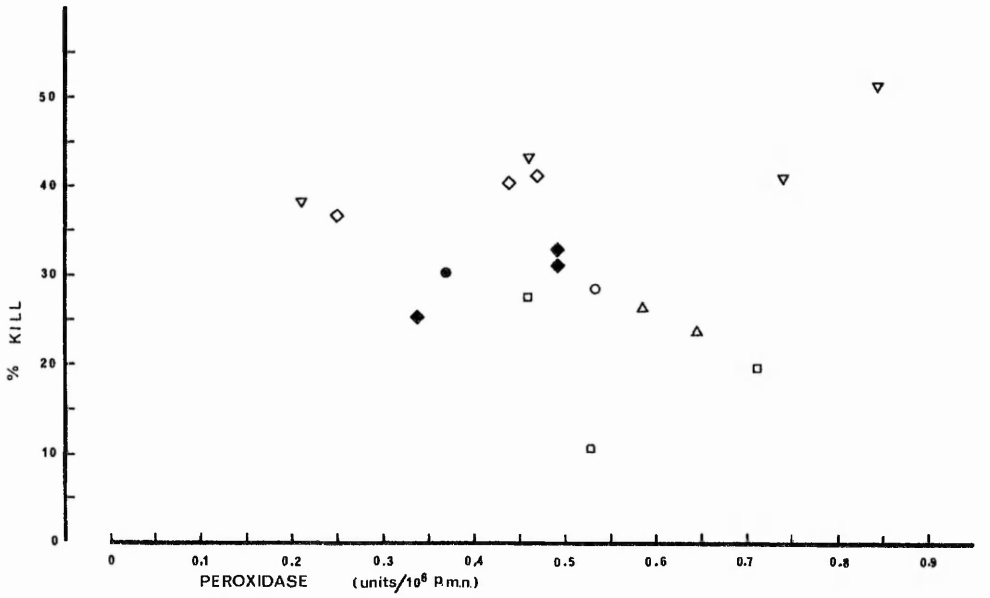


Fig. 65 Percentage kill of 7 individual CGL patients as a function of their neutrophil peroxide level. No significant correlation.

There was no correlation between percentage kill and the peroxidase value (Fig. 65), between percentage kill and alkaline phosphatase value (Fig. 66), or between percentage kill and H_2O_2 level (Fig. 67).

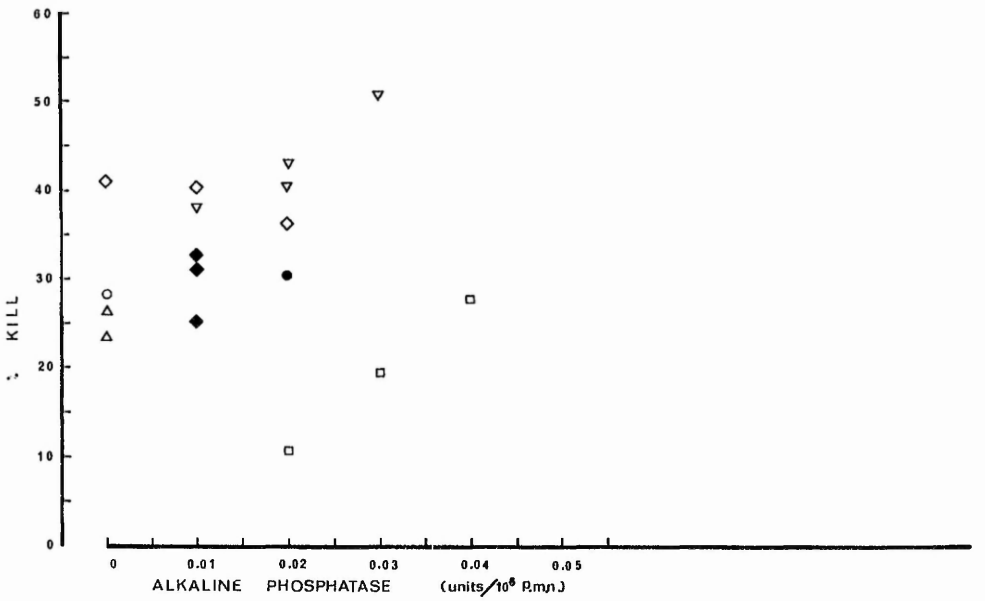


Fig. 66 Percentage kill of 7 individual CGL patients as a function of their neutrophil alkaline phosphatase level. No significant correlation.

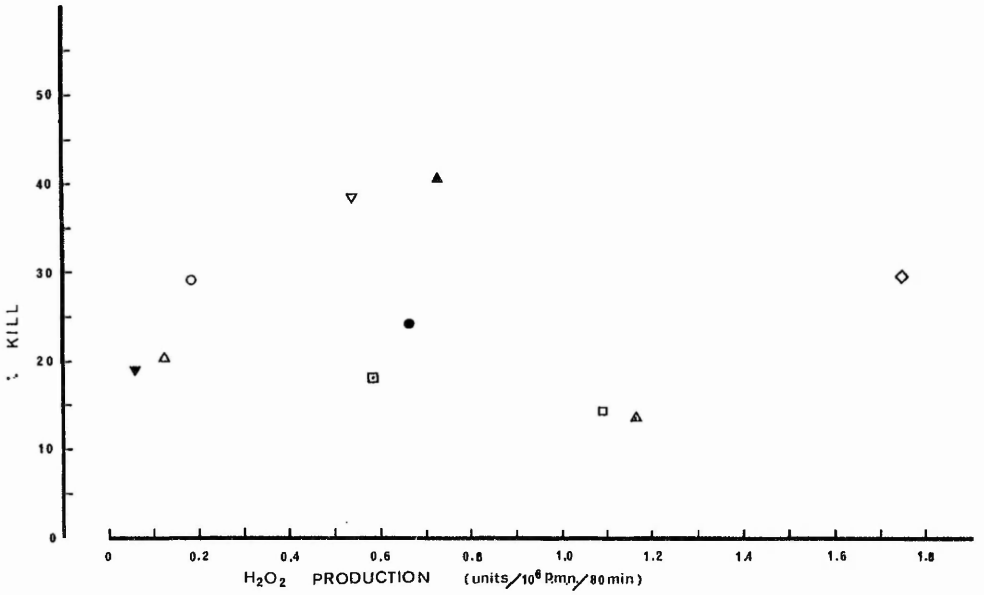


Fig. 67 Percentage kill of 10 individual CGL patients as a function of their neutrophil production of hydrogen peroxide over 80 minutes. No significant correlation.

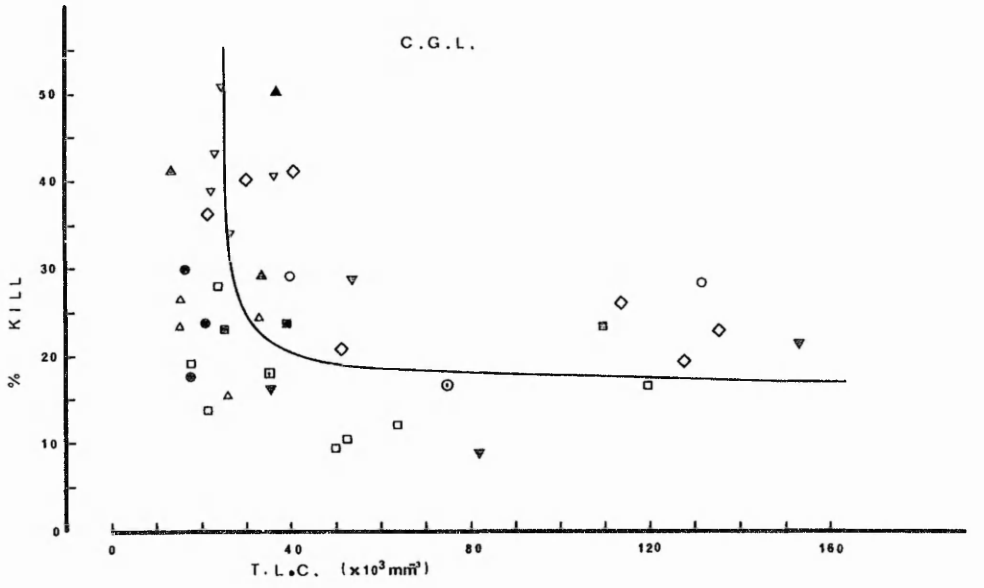


Fig. 68 Percentage kill of CGL neutrophils as a function of the total leukocyte count of the individual patients. A significant correlation is apparent.

Percentage uptake and percentage phagocytosis did not correlate with total leukocyte count. But, when percentage kill was plotted against total leukocyte count, a correlation was found and percentage kill fell as leukocyte count rose. (Fig. 68).

As most of these patients were on Busulphan treatment at one time or another it was necessary to find out any effect of the drug on the neutrophil function.

In Fig. 69 the daily Busulphan dose was plotted against the total leukocyte count. The apparent correlation was due to the fact that higher Busulphan dose was administered when high leukocyte count was detected.

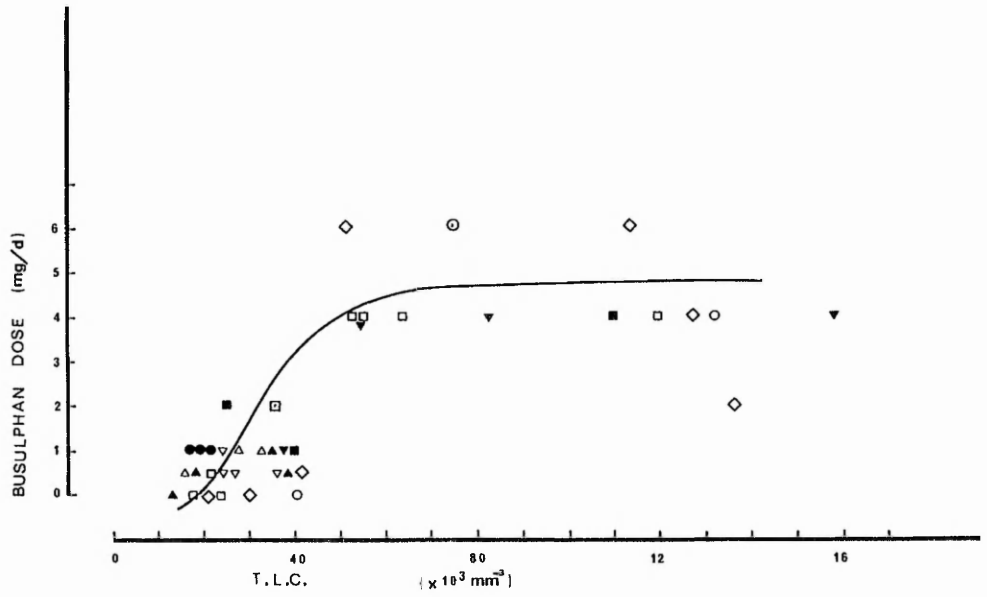


Fig. 69 Daily Busulphan dose as a function of total leukocyte count of the individual CGL patients. A significant correlation is apparent.

In Fig. 70 and Fig. 71, percentage uptake and percentage phagocytosis were plotted against Busulphan dose. No correlation was observed in either case. But when percentage kill was plotted against Busulphan dose, a correlation seemed to be present and percentage kill dropped as the daily dose increased (Fig. 72).

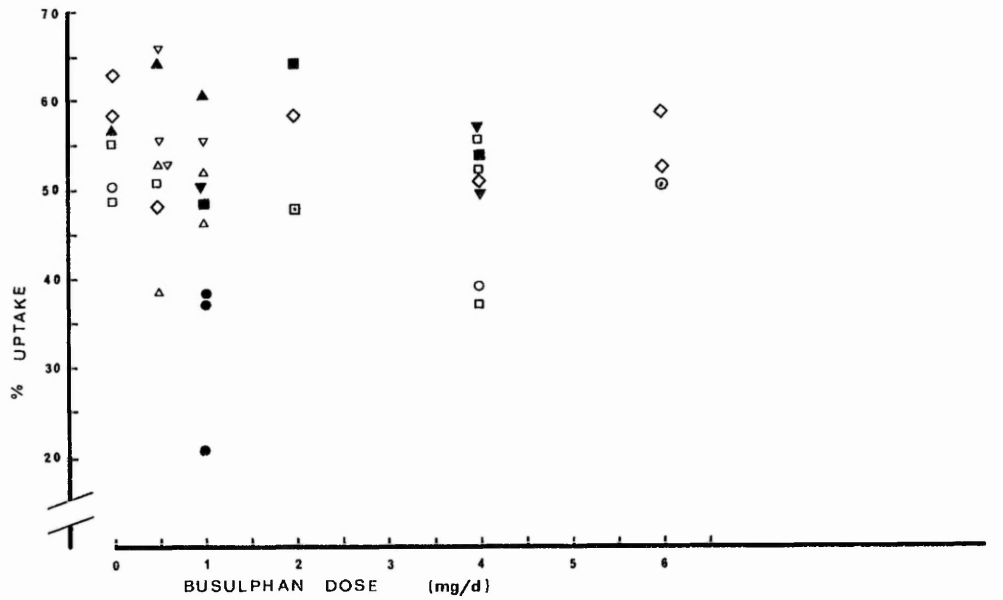


Fig. 70 Percentage uptake by CGL neutrophils as a function of individual daily Busulphan dose. No significant correlation.

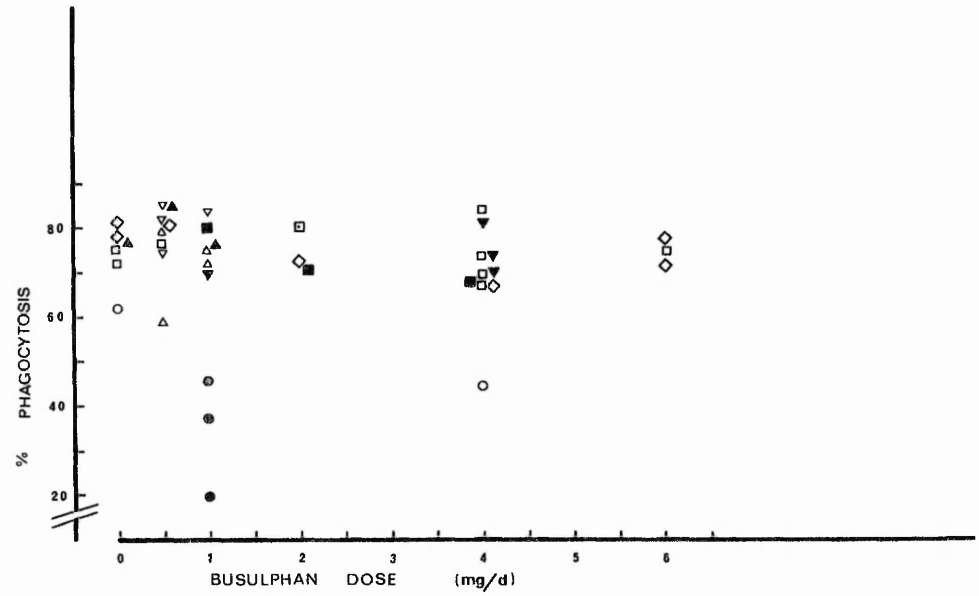


Fig. 71 Percentage phagocytosis by CGL neutrophils as a function of individual daily Busulphan dose. No significant correlation.

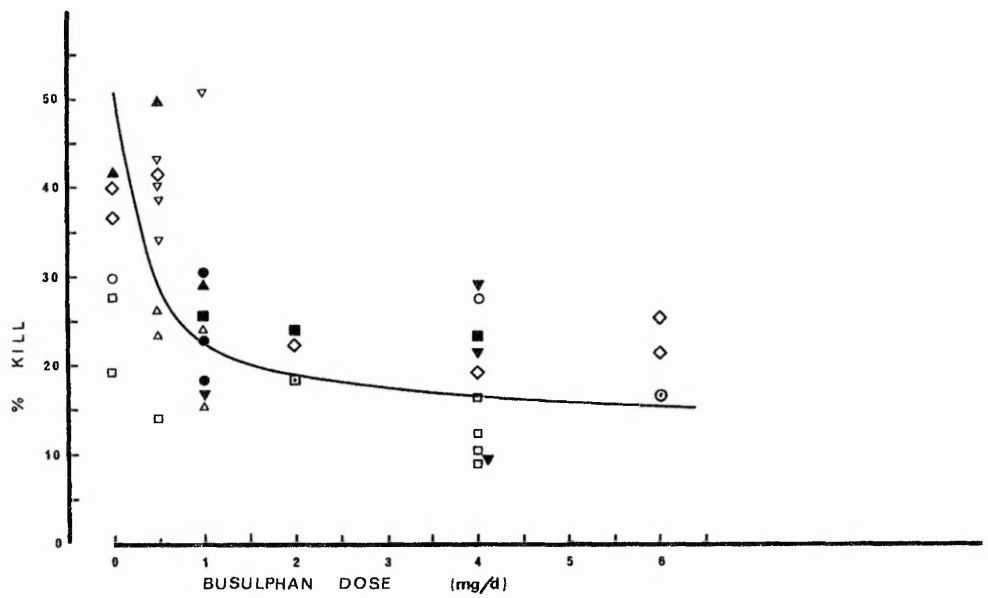


Fig. 72 Percentage kill by CGL neutrophils as a function of individual daily Busulphan dose. A significant correlation is apparent.

Qualitative observations of CGL neutrophils stained for alkaline phosphatase reveals and confirms the striking absence of the demonstratable activity of this enzyme. (See Fig. 73 and compare to normal Fig. 9).

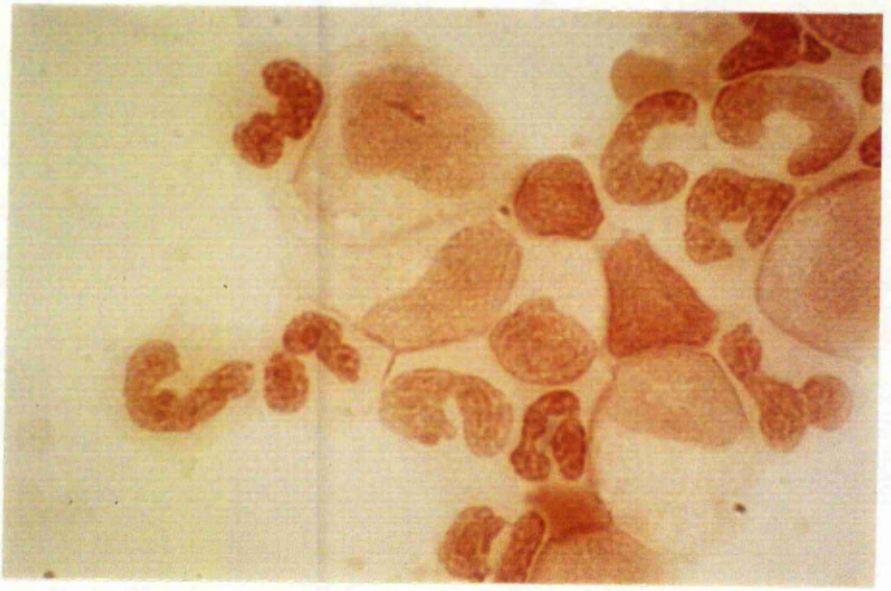


Fig. 73 An alkaline phosphatase stained preparation of CGL leukocytes. Note complete absence of reaction products. Compare with Fig. 9. (X1215).

Observation of peroxidase stained CGL neutrophils shows a similar picture to that of normal neutrophils (see Fig. 74). But when CGL neutrophils were allowed to phagocytose for 90 minutes and then stained for peroxidase, there was no significant change in the amount and distribution of the blue granules (see Fig. 75). This was contrary to what was observed in normal neutrophils (Fig 48).

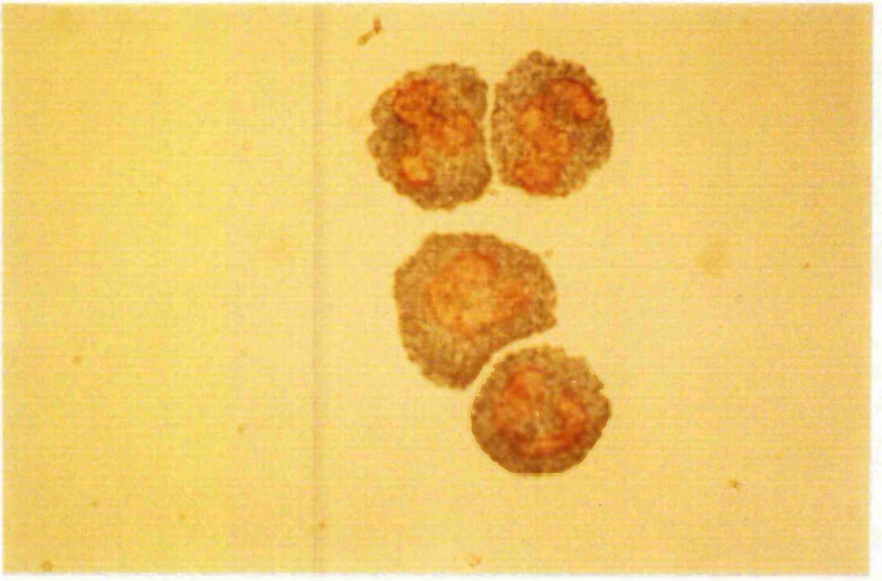


Fig. 74 A peroxidase stained preparation of CGL leukocytes before phagocytosis. Note granular reaction products throughout the cytoplasm. (X1215).

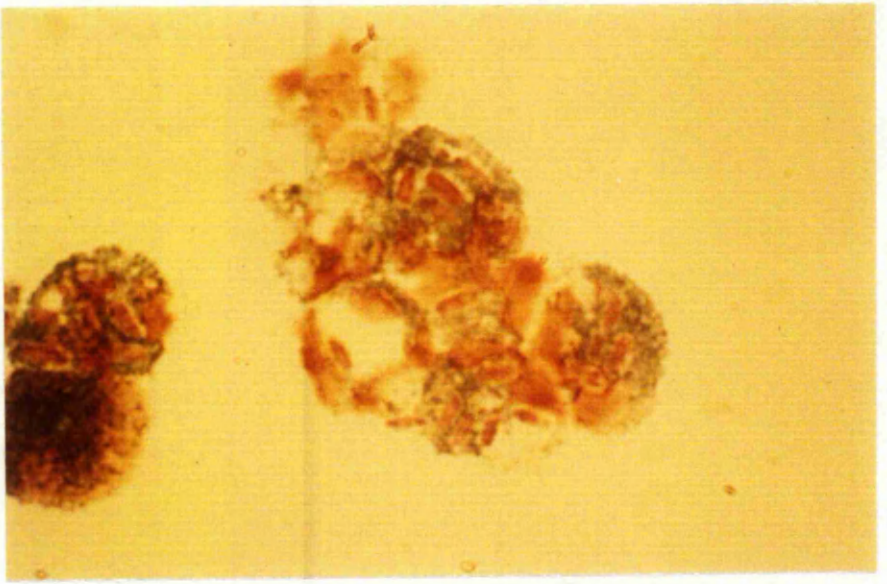


Fig. 75 A peroxidase stained preparation of CGL leukocytes after phagocytosis of Candida for 90 minutes. Note persistence of reaction products in the cytoplasm. (X1215).

Quantitative measurements of peroxidase before and after phagocytosis, (see Fig. 49), in 11 patients gave an average of 0.74 units/10^6 neutrophils and a standard error of $\mp$ 0.06 before phagocytosis, and an average of 0.67 units/10^6 neutrophils and a standard error of $\mp$ 0.05 after phagocytosis. The difference between the peroxidase values before and after phagocytosis was statistically not significant. This reinforces the visual observation that peroxidase is not reduced after 90 min phagocytosis in CGL neutrophils.

Qualitative observation on acid phosphatase and β-glucuronidase before and after 90′ phagocytosis reflected the same picture as that of peroxidase (see Figs. 76 and 77).

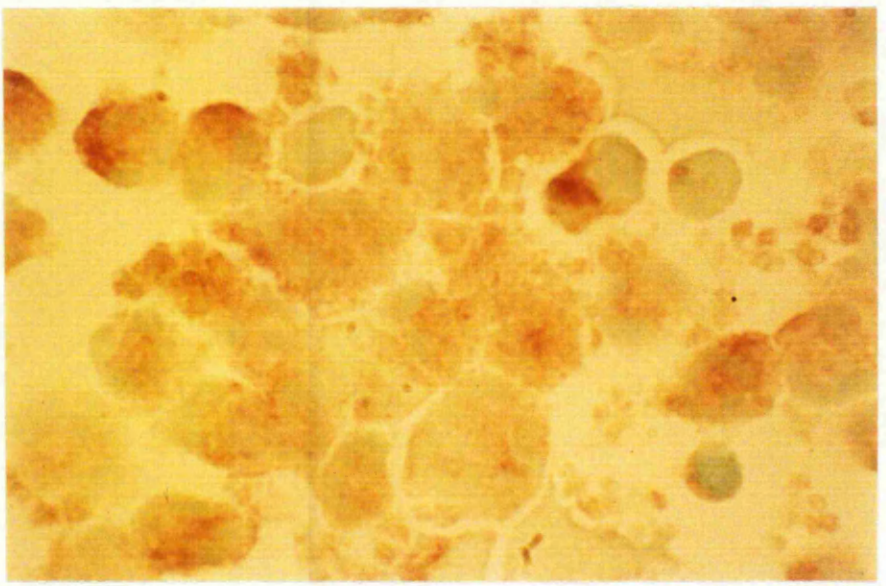


Fig. 76 An acid phosphatase stained preparation of CGL leukocytes before phagocytosis. Note reaction products throughout the cytoplasm. (X1215).

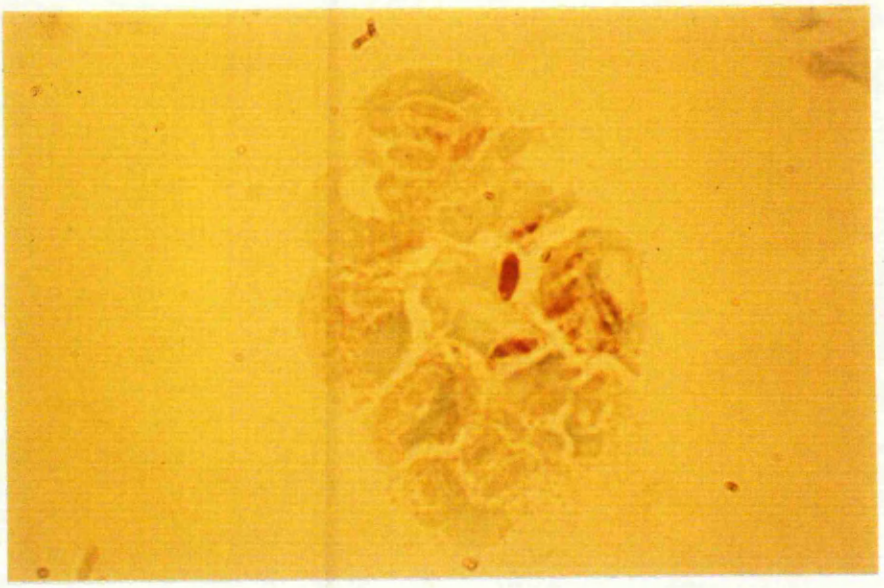


Fig. 77 An acid phosphatase stained preparation of CGL leukocytes after phagocytosis of Candida for 90 minutes. Note persistence of reaction products in the cytoplasm. (X1215).

When gram stain was applied to CGL neutrophils after 90 minutes incubation with Candida guillermondii, only very few of the Candida changed from gram positive (alive) to gram negative (Fig. 78). Compare to normal neutrophils behaviour (Fig. 53). The possible interpreation of these findings is discussed on page 209.

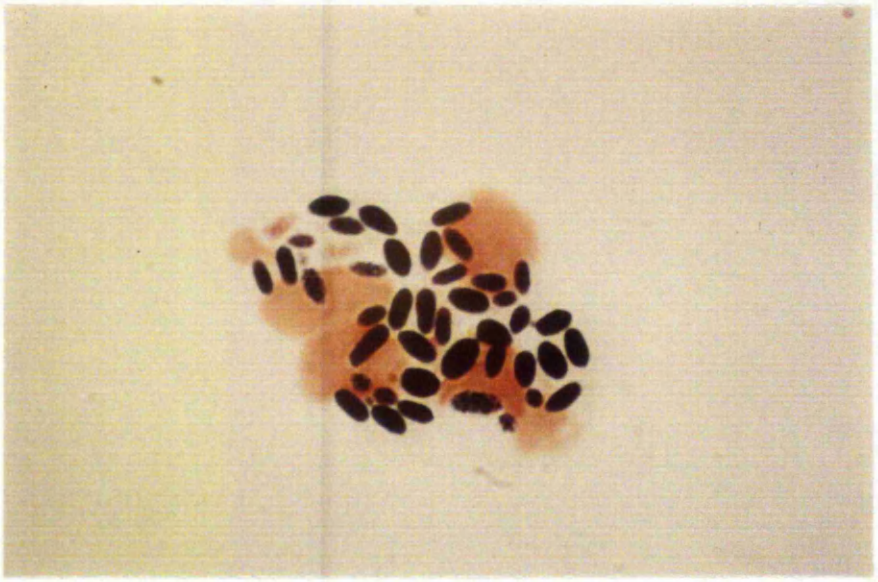


Fig. 78 A gram stained preparation of CGL neutrophils after 90 minute incubation with Candida. Note that the majority of Candida remained gram positive. (X1215).

a) Discussion:

In Chronic Granulocytic Leukaemia, polymorphonuclear neutrophils have an abnormal ultrastructure (Bessis, 1968) and are deficient in glycogen (Gahrton, 1966) and alkaline phosphatase (Wachstein, 1964). The cell line also contains an abnormal chromosome (Ph') (Nowell & Hungerfound, 1960). Studies of functions show defective cell migration (Banerjee et al., 1972) and a decrease of the adhesiveness of the granulocytes to a glass surface (Brandt, 1965). Studies of phagocytic activity have produced conflicting results, some indicating a diminished function (Brandt, 1965; Penny & Galton, 1966; Rosner et al., 1970; Goldman & Th'ng,1973;

Whittaker et al., 1974) others indicating a normal engulfment (Kalinske & Hoeprich, 1969; Odeberg et al., 1975). The bactericidal capability of CGL neutrophils has been little investigated and it is surprising that in view of these abnormalities, it was reported to be almost normal (Sbarra et al., 1965; Kalinske & Hoeprich, 1969; Goldman & Th'ng, 1973) or diminished in some (Odeberg et al., 1975). In view of the diversity of the test systems and the corresponding results it was necessary to have a fresh look at the neutrophil function using the techniques we have established.

The principle abnormalities that have been demonstrated in the CGL neutrophils were the significant reduction in the fungicidal capacity (48% of normals), the impairment of degranulation during phagocytosis, and the significant reduction in H_2O_2 production during phagocytosis (29% of normal). Percentage uptake and phagocytosis were slightly but significantly reduced. This slight reduction could not have been the reason for the highly significant reduction in percentage killing for two reasons. First, there was no correlation between uptake nor phagocytosis and killing, and secondly, further incubation beyond 90′ did not lead to normal Candida killing. The method was chosen so that it was possible to detect even those minor abnormalities in uptake and phagocytosis at 15 min. However, continuous observation beyond that period revealed sluggishness in the rate rather than complete impairment. This sluggishness but otherwise functional phagocytosis in CGL neutrophils

does not support the implication of the importance of the presence of alkaline phosphatase to membrane activity, nor that alkaline-phosphatase-positive polymorphs are more actively phagocytic than negative ones (Pederson & Hayhoe, 1971). The increased number of leukocytes in CGL patients has been related to the abnormally long life-span of neutrophils (Brandt, 1965) and a highly significant correlation was demonstrated in rats between LAP levels and neutrophil age being highest in the youngest cells (Williams, 1975). We have not been able to find any correlation between LAP and total leukocyte count.

The coexistence in the same patient of abnormally low LAP values and the Ph' chromosome gave rise to the speculation that genes controlling LAP metabolism might be situated on chromosome 21. This was strengthened when abnormally high LAP values were discovered in patients with Down's syndrome who were trisomic for the 21 chromosome (Alter et al., 1962). But the presence on the 21 chromosome of structural genes concerned with LAP synthesis was difficult to reconcile with the fluctuations of LAP in certain CGL patients. Also the Ph' abnormality does not arise in the same pair of small acrocentric chromosomes as that which becomes trisomic in Down's syndrome. The possibility that there is a double population of cells, one leukaemic, the other normal, is also untenable as the Ph' chromosome is persistent in all metaphases.

The fact that we have not found any correlation between total leukocyte count and uptake or phagocytosis means that the age of the leukocyte has no effect on its phagocytic

function. This is consistent with the alkaline phosphatase findings.

Total leukocyte count did however correlate with Busulphan dose due to the nature of the treatment, but at the same time, no correlation was observed between the Busulphan dose and uptake or phagocytosis. This is in contrast to Whittaker et al (1974) findings where they reported improved phagocytic index with treatment. Killing which was most significantly reduced, did show slight reduction as the Busulphan dose increased. This does not necessarily mean that Busulphan interferes with the killing mechanism in CGL neutrophils, keeping in mind that high doses of the drug coincided with high leukocyte count, it is more likely that the increased impairment in killing was due to the grossly increased leukocytes which might be older or deficient in some aspects of the killing mechanism. Such a correlation between leukocyte count and percentage kill has been observed. It is also of interest to mention that while there was no change in percentage uptake and phagocytosis with time, there was a slight reduction in killing. This must be due to the overall deterioration of the patients health since such a reduction was not observed in normal people.

As the main defect in CGL neutrophils appears to be in the killing region, investigations were concentrated on the peroxidase-H_2O_2 cidal system. Peroxidase measurements before phagocytosis did not differ from those of normals. This is supported by Odeberg et al. (1975), but the mere presence of the enzyme in the primary granules does not contribute towards

effective killing. It has to be delivered into the phagosome during phagocytosis i.e. degranulation has to take place.

It was observed that normal neutrophils lose most of the peroxidase after 90′ incubation with Candida. This observation was reinforced by quantitative measurements of peroxidase before and after 90′ incubation. The likely reasons for the disappearance of the peroxidase have been discussed before (see page 46). We have used this phenomenon to monitor degranulation in CGL neutrophils, and have found that CGL neutrophils were clearly abnormal as they failed to degranulate and showed no quantitative loss of peroxidase activity. Such behaviour was also observed with acid phosphatase and β-glucuronidase. Impaired degranulation and its relevance to killing in CGL neutrophils had been touched upon by Odeberg et al. (1975) in their discussion, but it was only speculation.

At this stage, lack of alkaline phosphatase appeared as a likely cause for the lack of degranulation. Strauss et al. (1974) reported a boy whose neutrophils lacked specific granules together with alkaline phosphatase, and whose cells were unable to form a normal phagocytic vacuole. These observations suggested that the failure of fusion between azurophil granules and the phagocytic vacuole in CGL cells may be secondary to their lack of alkaline phosphatase. However, a CGL patient with normal alkaline phosphatase showed the same defect of Candida killing and degranulation as other patients with low alkaline phosphatase. The rise in alkaline

phosphatase following splenectomy in another patient did not correct the defect either.

One of the metabolic pathways stimulated by phagocytosis is the production of H_2O_2 and other oxygen metabolites, (singlet oxygen, superoxide, hydroxide radical), (Klebanoff, 1975). The final step in the H_2O_2 production requires an oxidase associated with the membrane of the phagocytic vacuole so that metabolites are concentrated within the vacuole. It may be that the oxygen metabolites change the nature of the vacuole membrane in a way which enables the granules to fuse.

Defective production of active oxygen metabolites would then explain impaired degranulation and killing of organisms.

The most obvious candidate to test the above hyposisis was H_2O_2 production during phagocytosis as it is the final product of the increased oxygen uptake, and one of the vital components of the peroxidase cidal system. It was found that its production in CGL neutrophils over 80 min incubation was about 29% of the normals. This significant impairment could explain the lack of degranulation and hence killing and could also imply decreased oxygen consumption and hexose monophosphate shunt activation, as well as lack of NADH or NADPH oxidase.

Correlation between H_2O_2 levels and killing was not observed in CGL neutrophils. For H_2O_2 to be effective in killing without the inhancing effects of peroxidase, levels which are much higher than normal have to be obtained within the phagosome. On the other hand, there was no correlation between peroxidase levels and killing either which is reasonable since the enzyme was not released to perform its

function.

On the whole, CGL neutrophil did not kill Candida guillermondii as efficiently as normal neutrophils because they did not produce normal amounts of H_2O_2, nor did they degranulate and deliver the peroxidase to its action site. Whether normal H_2O_2 production is responsible for normal degranulation remains to be seen. Alkaline phosphatase did not correlate to any of the neutrophil functions.

3. Myeloid Metaplasia

Twelve MM patients were studied, all have myelosclerosis , enlargement of the spleen and showing metaplastic myeloid tissues in liver biopsy specimens. The majority of these patients have been followed for a number of years and no other cause has been found for their haematological abnormalities.

Percentage uptake was plotted for each of the 12 patients, (Fig 79). Uptake ranged between 26.5 and 58.1% with an average of 49.99 and a standard error of $\bar{+}$ 2.53. This although only slightly lower than the normal value was statistically significant $P<0.01$.

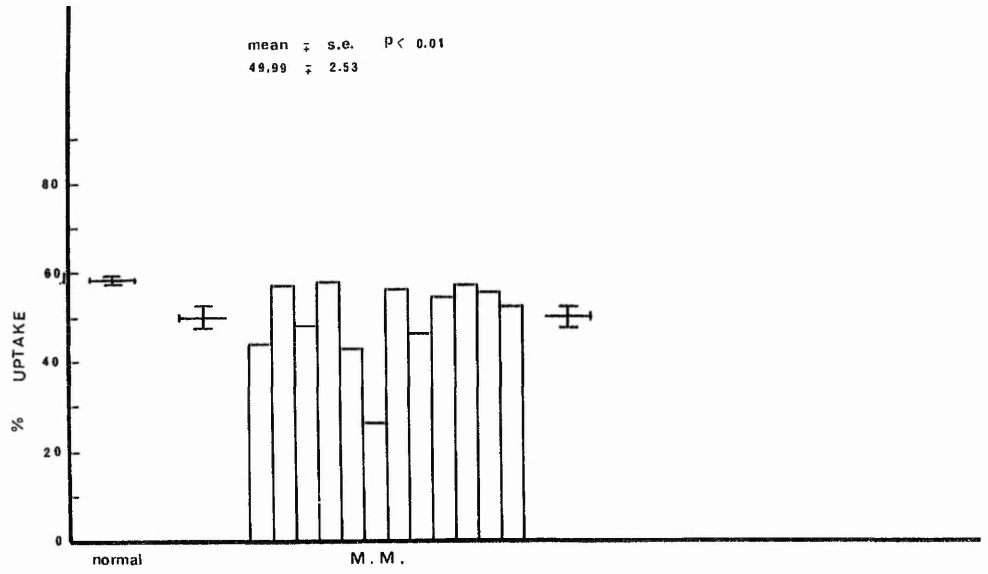


Fig. 79 Percentage uptake after 15 minutes by neutrophils of 12 MM patients. Results expressed as mean $\bar{+}$ standard error and are significantly different from normal.

Percentage phagocytosis was plotted for each of the 12 patients, (Fig. 80). Phagocytosis ranged between 20.2 and 83.2% with an average of 69.21 and a standard error of $\bar{+}$ 4.58. This value was not significantly different from that of normals.

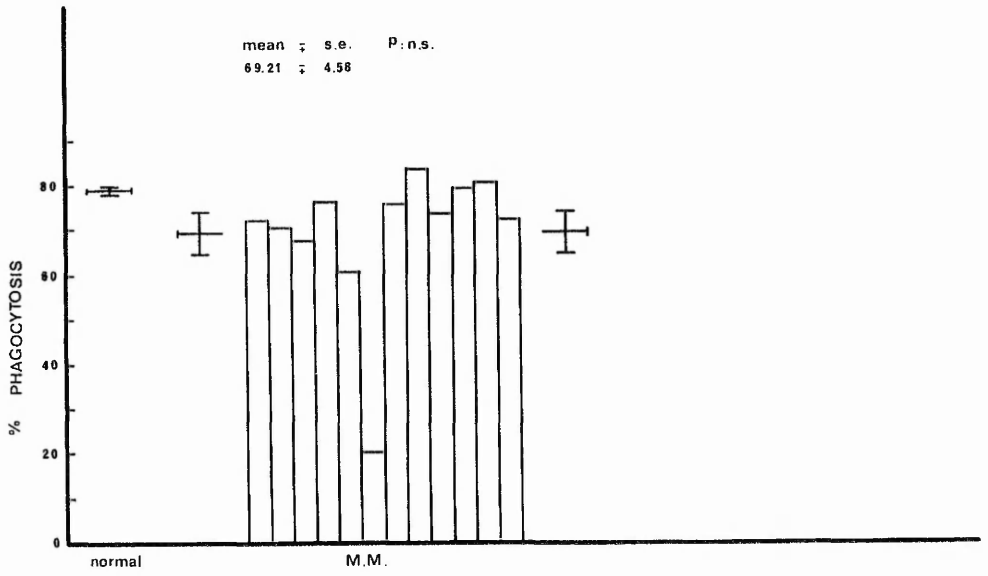


Fig. 80 Percentage phagocytosis after 15 minutes by neutrophils of 12 MM patients. Results expressed as mean $\bar{+}$ standard error and are not significantly different from normal.

Percentage kill was plotted for each of the patients (Fig. 81). Values ranged between 12.3 and 42.0% with an average of 28.29 and a standard error of $\bar{+}$ 2.86. This was significantly lower than the normal value $P<0.001$.

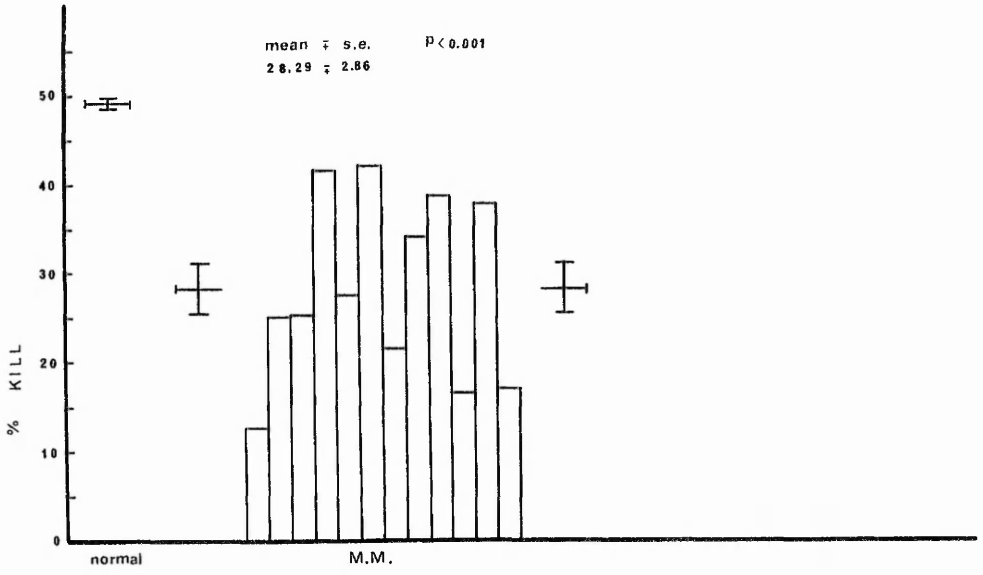


Fig. 81 Percentage kill after 90 minutes by neutrophils of 12 MM patients. Results expressed as mean $\mp$ standard error and are significantly different from normal.

Quantitative peroxidase measurements of 11 of these patients ranged between 0.05 and 0.98 units/10^6 neutrophils with an average of 0.39 and a standard error of $\mp$ 0.08. This was statistically significantly lower than normal. $P<0.05$. (Fig. 82).

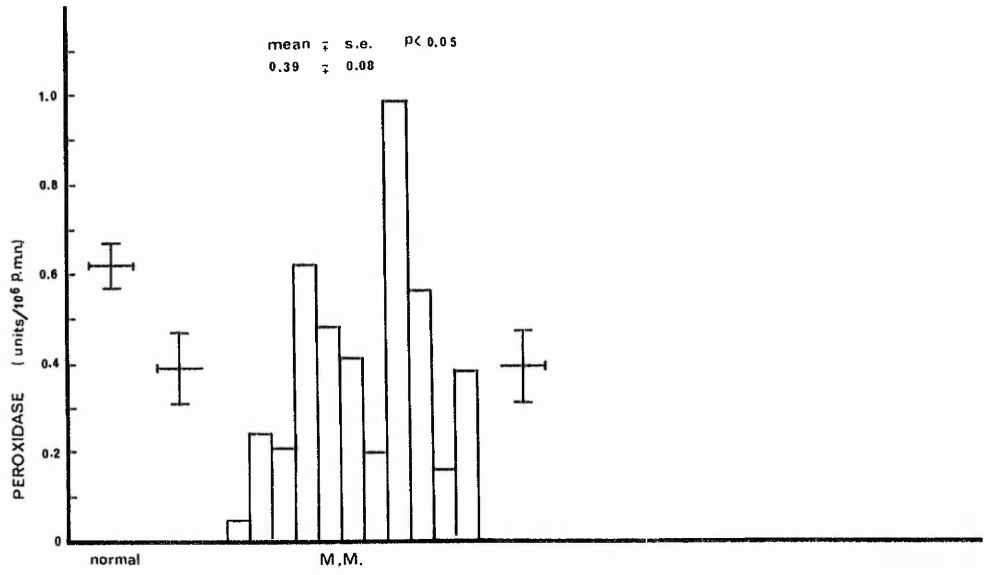


Fig. 82 Neutrophil peroxidase values of 11 MM patients. Results as mean ∓ standard error are significantly different from normal.

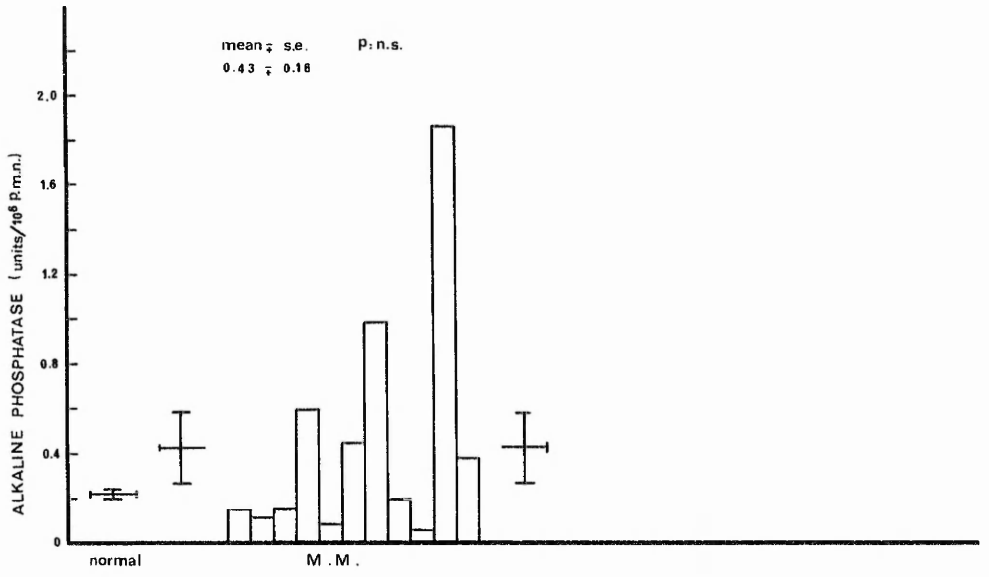


Fig. 83 Neutrophil alkaline phosphatase values of 11 MM patients. Results as mean ∓ standard error are not significantly different from normal.

Quantitative alkaline phosphatase measurements of 11 of these patients ranged between 0.04 and 1.86 units/10^6 neutrophils with an average of 0.43 and a standard error of $\mp$ 0.16. This even though higher than the normal value, was not statistically significant. (Fig. 83).

Quantitative acid phosphatase measurements were carried out on 11 of these patients. (Fig. 84). Values ranged between 2.44 and 4.01 units/10^6 neutrophils with an average of 3.10 and a standard error of $\mp$ 0.13. This value was statistically not significantly different from that of normals.

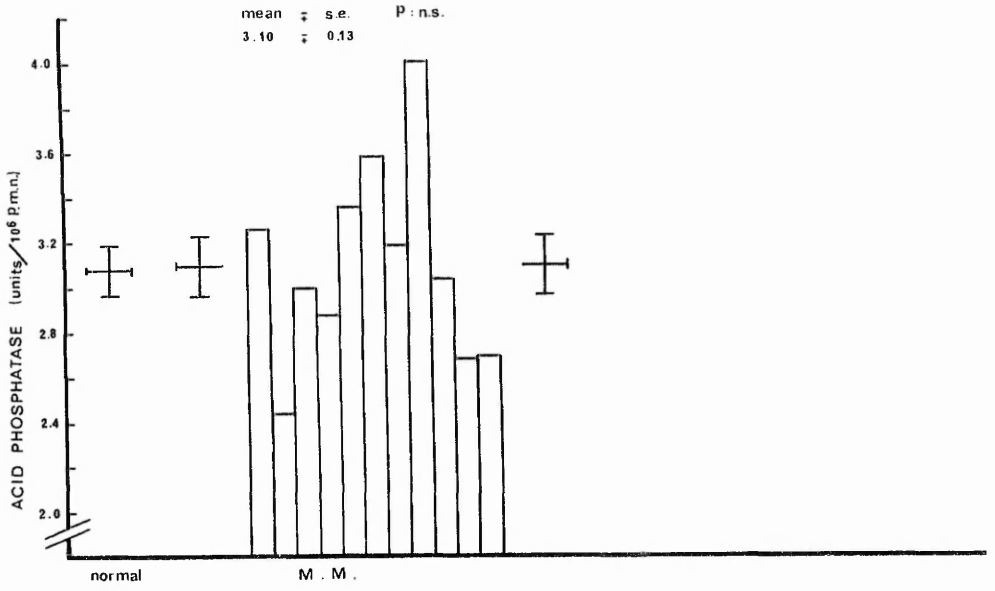


Fig. 84 Neutrophil acid phosphatase values of 11 MM patients. Results as mean $\mp$ standard error are not significantly different from normal.

The results of MM neutrophil function and enzyme levels are summarized in table 3.

	No. of subjects	Range	Mean	S.E.	Signif-icance	Fig. No.
Percentage uptake	12	26.5-58.1	49.99	∓2.53	P<0.01	79
Percentage phagocytosis	12	20.2-83.2	69.21	∓4.58	N.S.	80
Percentage kill	12	12.3-42.0	28.29	∓2.86	P<0.001	81
Peroxidase units/10^6 neutrophils	11	0.05-0.98	0.39	∓0.08	P<0.05	82
Alkaline phosphatase units/10^6 neutrophils	11	0.04-1.86	0.43	∓0.16	N.S.	83
Acid phosphatase units/10^6 neutrophils	11	2.44-4.01	3.10	∓0.13	N.S.	84

Table 3. A summary of the functional and enzymic values of MM neutrophils.

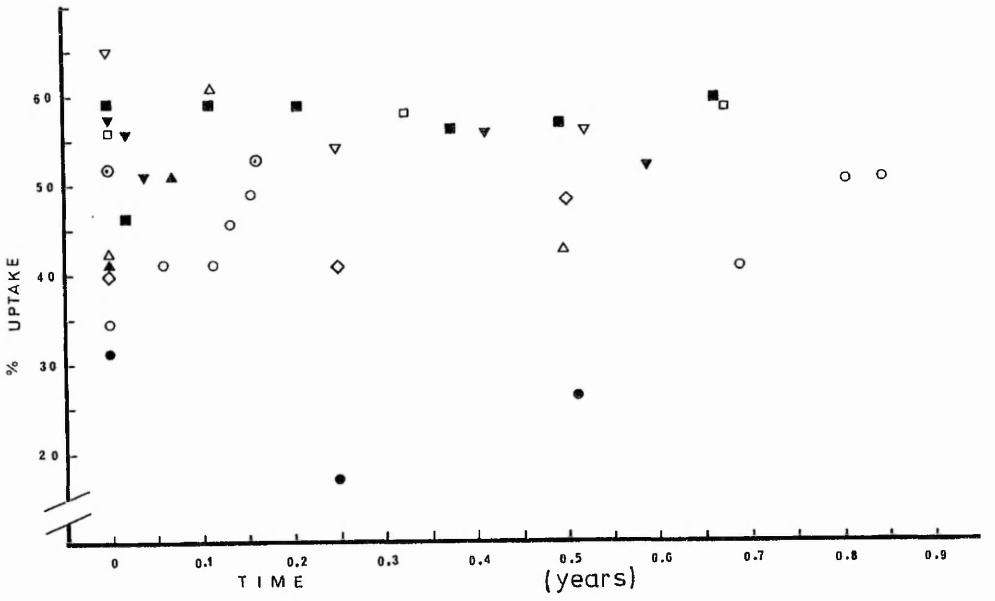


Fig. 85 The reproducability of percentage uptake by MM neutrophils of 10 individual patients when repeated over a period of time. No significant change.

There was no correlation between percentage uptake, phagocytosis or killing and time. (Figs. 85, 86 and 87).

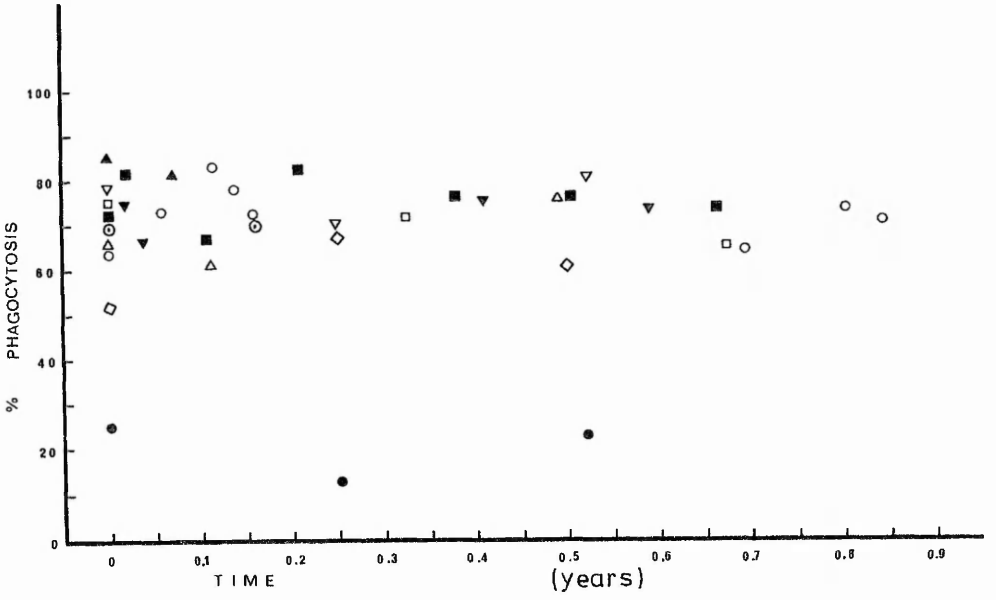


Fig. 86 The reproducability of percentage phagocytosis by MM neutrophils of 10 individual patients when repeated over a period of time. No significant change.

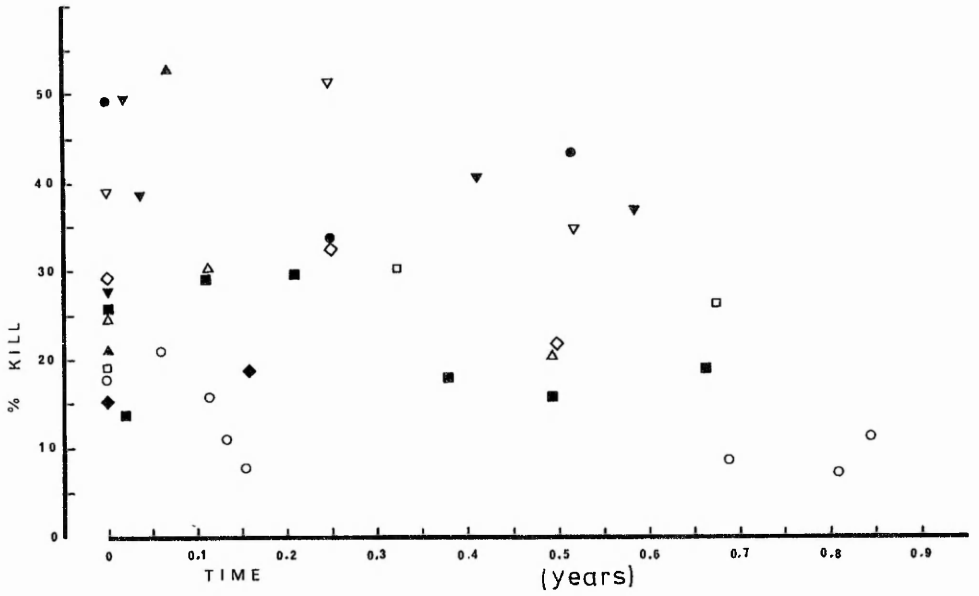


Fig. 87 The reproducability of percentage kill by MM neutrophils of 10 individual patients when repeated over a period of time. No significant change.

Also there was no correlation between percentage kill and percentage uptake. (Fig. 88).

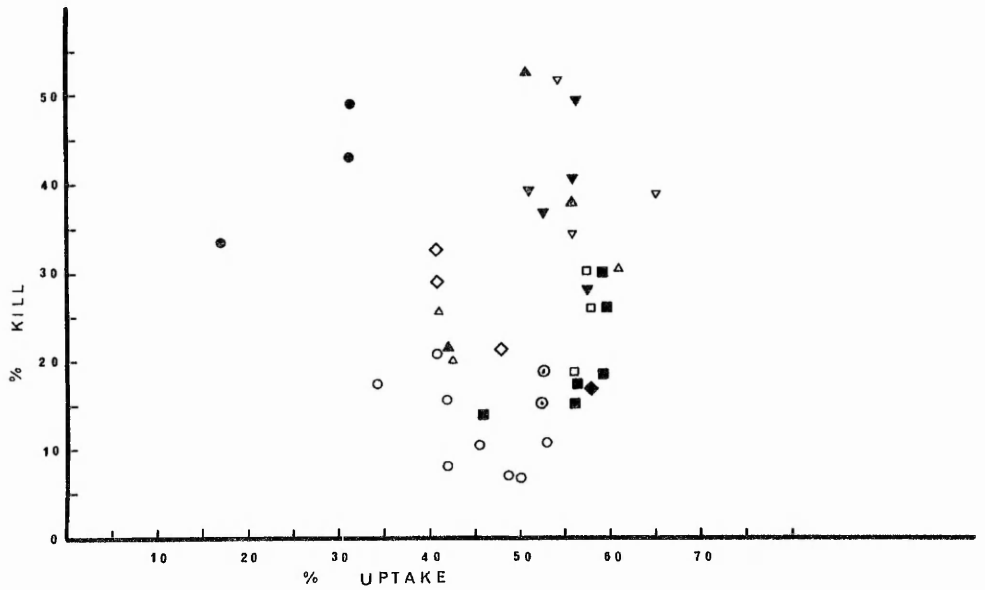


Fig. 88 Percentage kill at 90 minutes against percentage uptake at 15 minutes by neutrophils of 12 MM patients repeated 41 times. No significant correlation.

There was also no correlation between percentage uptake or phagocytosis and total leukocyte count. (Figs. 89 and 90).

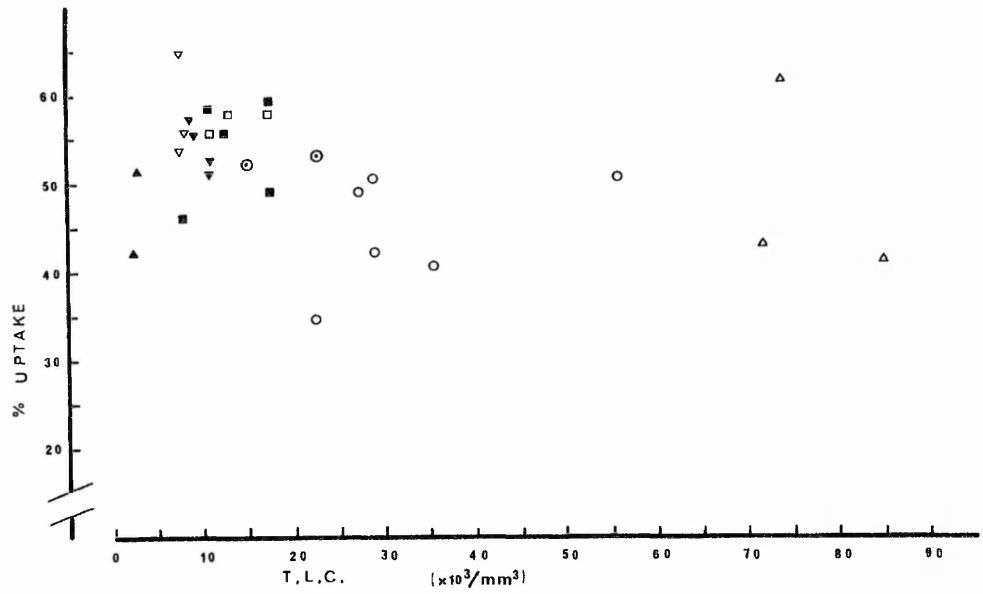


Fig. 89 Percentage uptake by MM neutrophils as a function of the total leukocyte count of the individual patients. No significant correlation.

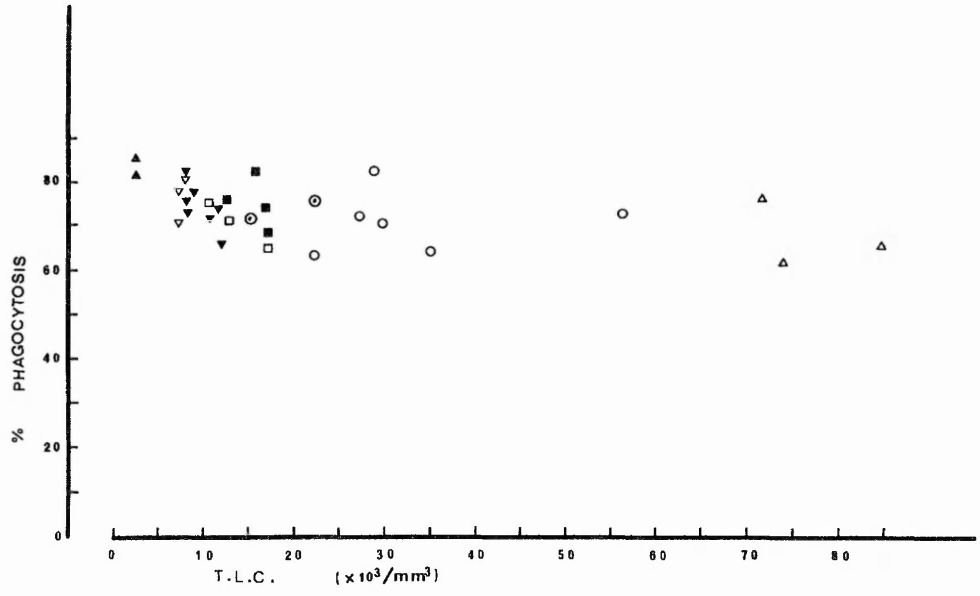


Fig. 90 Percentage phagocytosis by MM neutrophils as a function of the total leukocyte count of the individual patients. No significant correlation.

There was, however, a correlation between percentage kill and total leukocyte count in all but one of the patients. Percentage kill dropped as leukocyte count went up. (See Fig. 91).

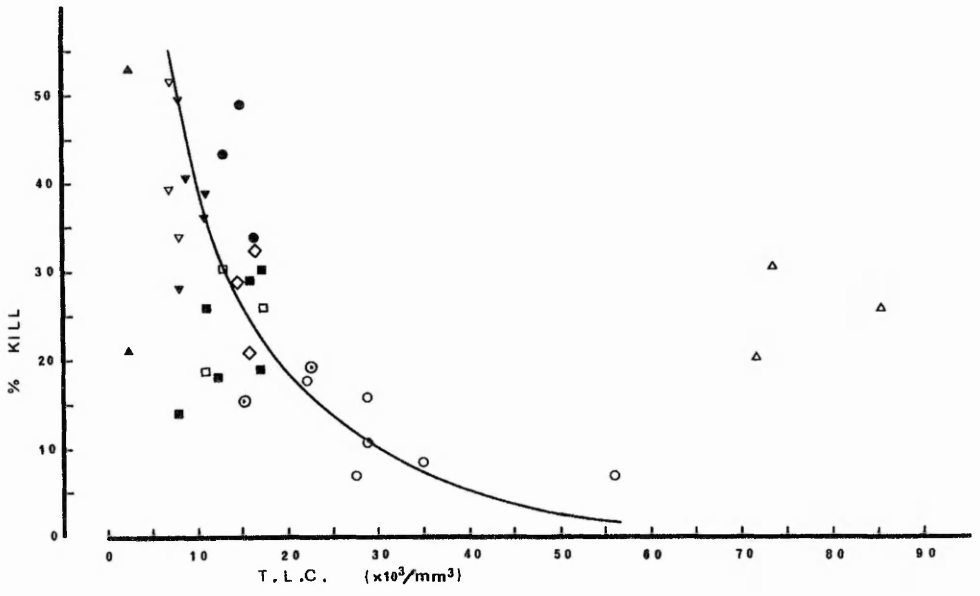


Fig. 91 Percentage kill by MM neutrophils as a function of the total leukocyte count of the individual patients. A significant correlation is apparent.

There was also a very similar correlation pattern between the peroxidase value and the total leukocyte count except for that same patient. (See Fig. 92).

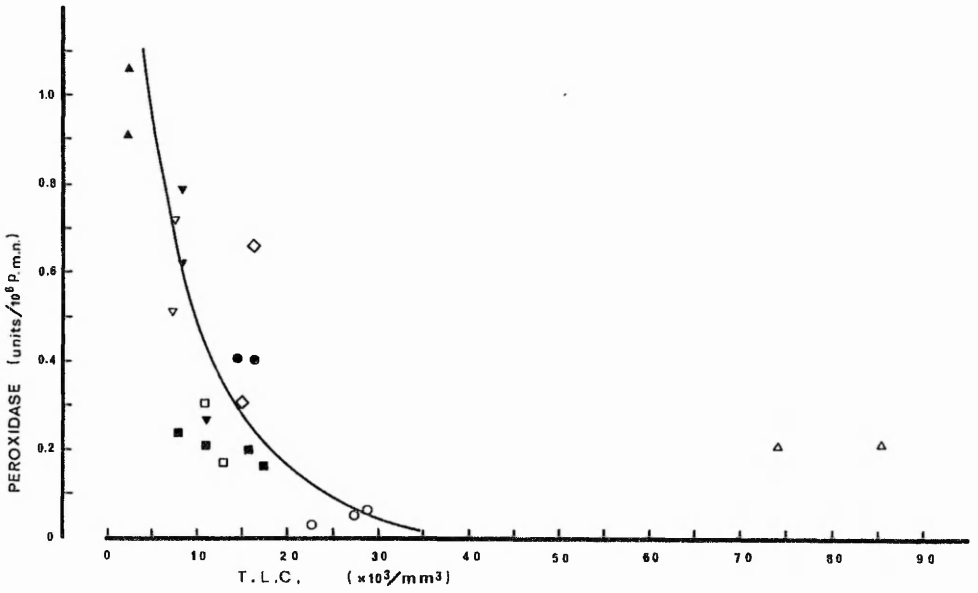


Fig. 92 Neutrophil peroxidase levels of MM patients as a function of total leukocyte count. A significant correlation is apparent.

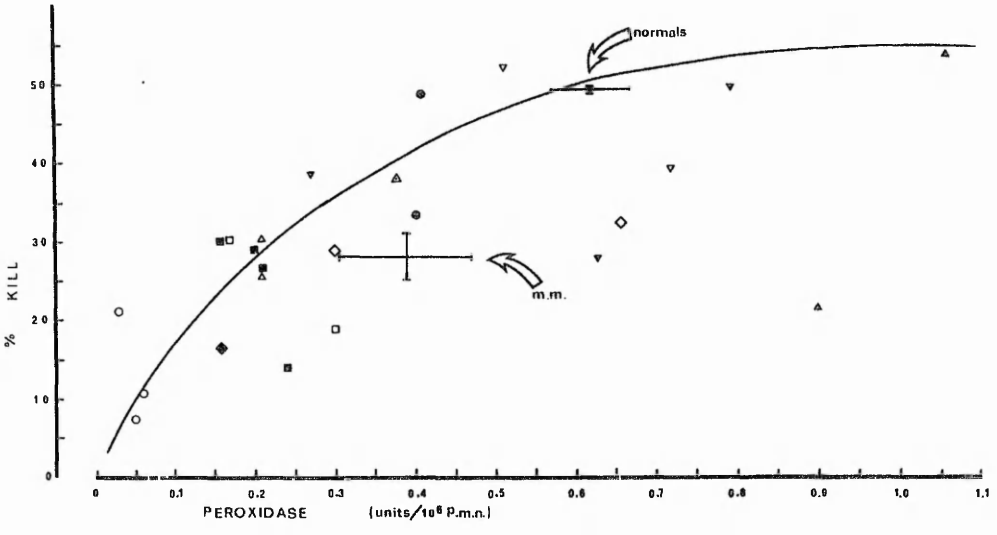


Fig. 93 Percentage kill of individual MM patients as a function of their neutrophil peroxidase level. A significant correlation is apparent.

There was no such relation between alkaline phosphatase and total leukocyte count.

There was a significant correlation between percentage kill and the peroxidase values as seen in Fig. 93.

There was no correlation, however, between percentage kill and alkaline phosphatase values (see Fig. 94).

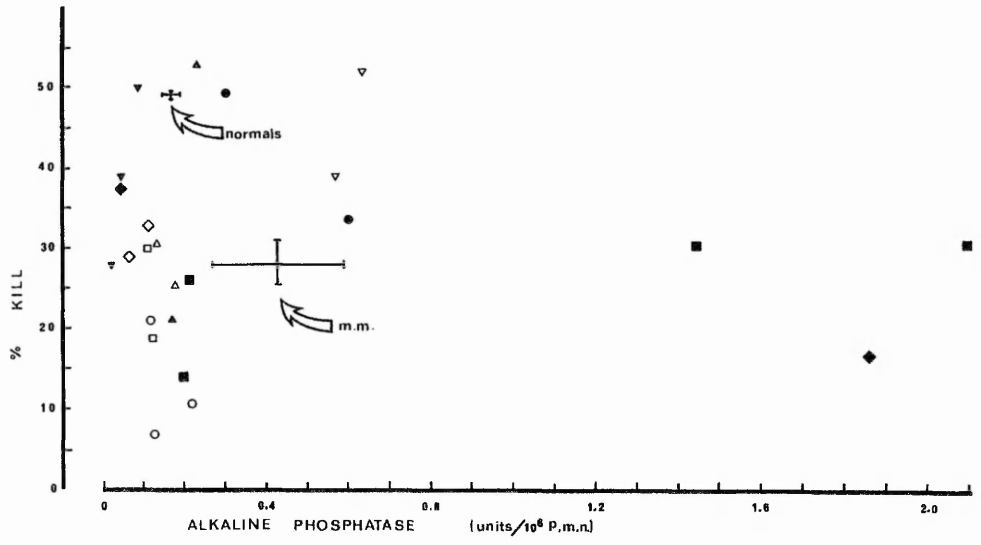


Fig. 94 Percentage kill of individual MM patients as a function of their neutrophil alkaline phosphatase level. No significant correlation.

Qualitative observation of a peroxidase stained smear of oneMM patient shows that the majority of his neutrophils failed to demonstrate any enzymic activity (Fig. 95). (Compare with normal in Fig. 47).

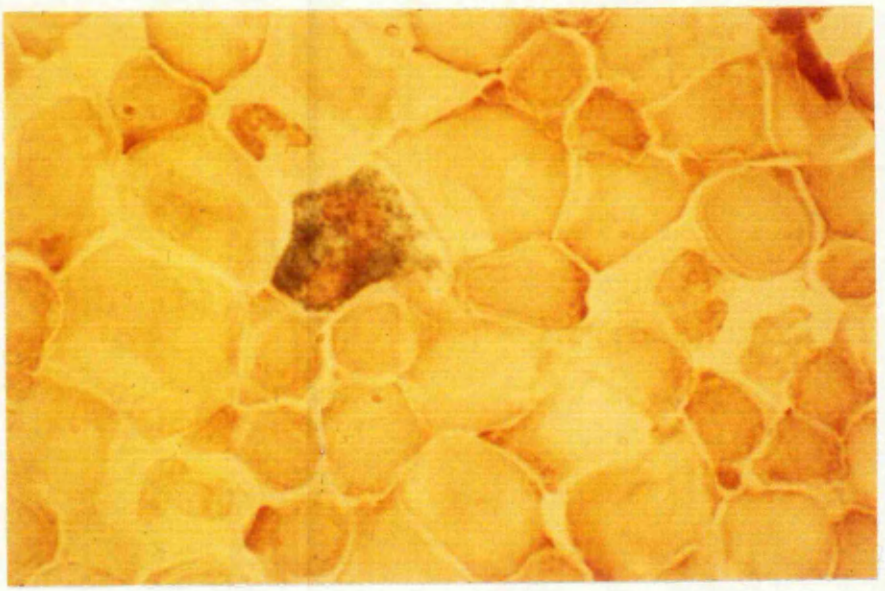


Fig. 95 A peroxidase stained leukocyte preparation of MM patient No. 1 showing complete absence of reaction products from most of his neutrophils. Compare with Fig. 47. (X1215).

Normal acid phosphatase activity, however, was present in the leukocytes of the same patient. (Fig. 96).

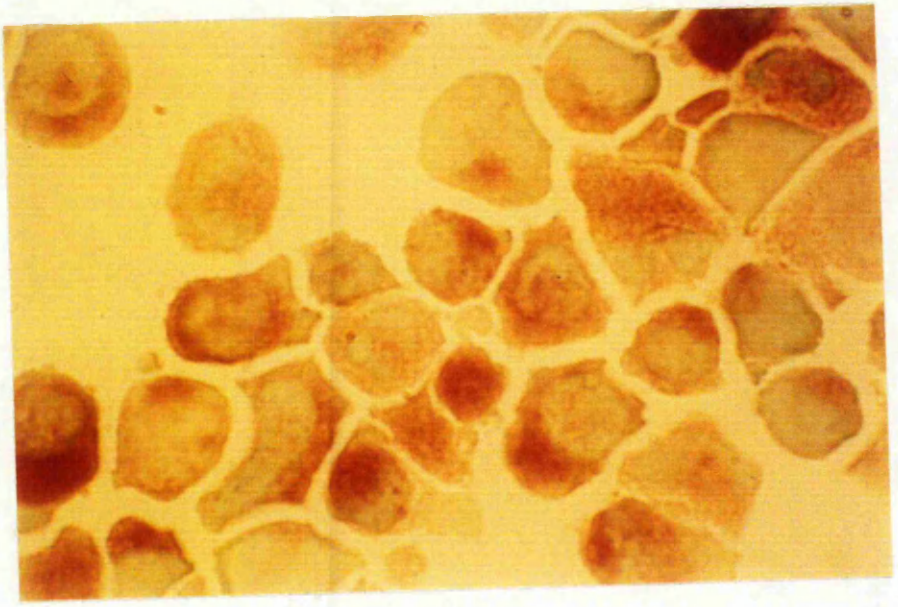


Fig. 96 An acid phosphatase stained preparation of MM patient No. 1 leukocytes showing normal deposits of the enzyme reaction products. (X1215).

When the same patient's leukocytes were stained for alkaline phosphatase, a higher than average amount can be seen. (Fig. 97). (Compare with Fig. 9).

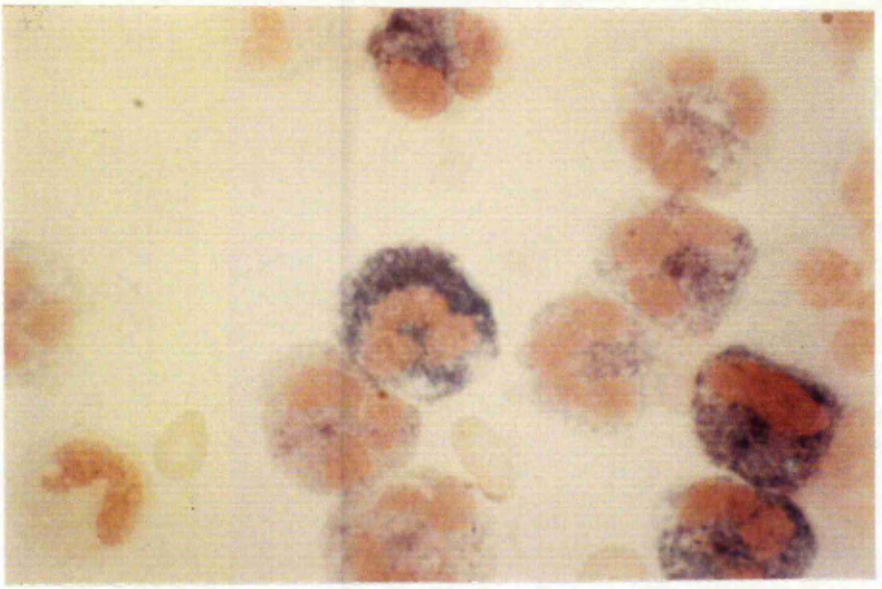


Fig. 97 An alkaline phosphatase stained leukocyte preparation of MM patient No. 1 showing higher than normal reaction products. Compare with Fig. 9. (X1215).

Electron microscope studies on peroxidase stained preparation of normal leukocytes reveals dark, electron dense areas indicating enzymic activity within the primary granules. (Fig. 98).

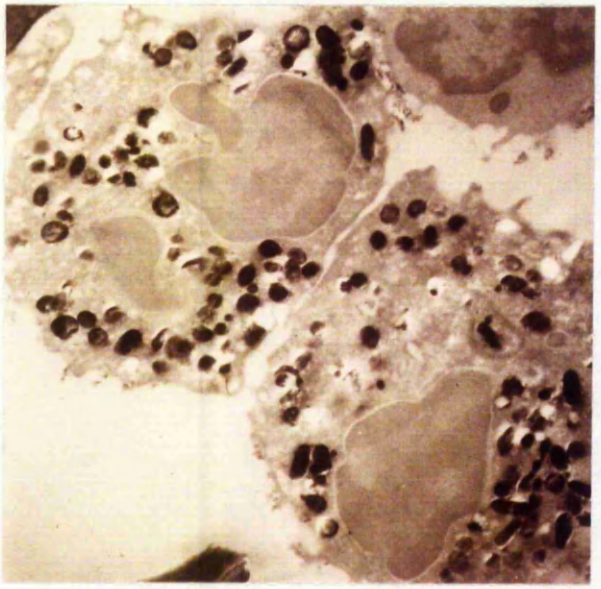


Fig. 98 An electron micrograph of peroxidase stained preparation of normal neutrophils showing electron dense reaction products within primary granules. (X7250).

Similar electron microscope studies on leukocytes from patient No. 1 reveals normal looking primary granules matching those of normal leukocytes in size and number but differ in lacking the electron dense peroxidase deposits. (Fig. 99)

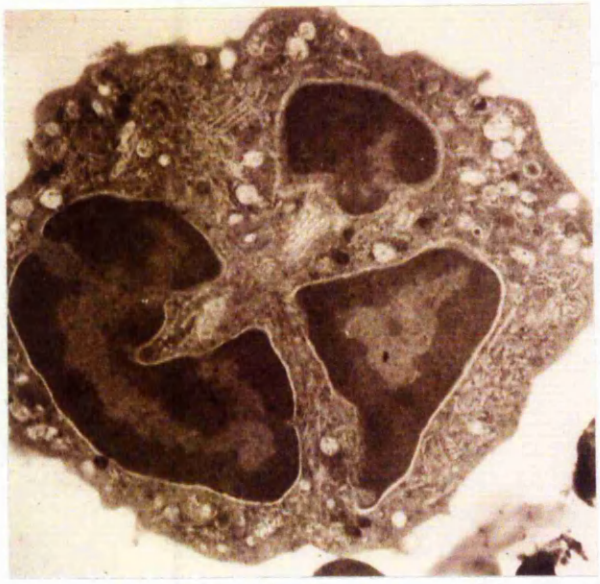


Fig. 99 An electron micrograph of peroxidase stained preparation of MM neutrophil of patient No. 1 showing lack of reaction products from the primary granules. (X11500).

The preparation and processing of the electron microscope work was done by the Department of Human Morphology at Nottingham University.

a) Discussion:

In Myeloid Metaplasia, neutrophil function has not been studied in the same detail as that of the CGL neutrophil. This apparent lack of interest is a reflection of the diversity of the diagnostic features associated with its classification. The principle abnormalities that have been demonstrated in the MM neutrophils as a result of our study were the significant reduction in the fungicidal capacity, 58% of normals, the low levels of peroxidase and the

significant correlation between peroxidase levels and the corresponding fungicidal ability. Percentage uptake was slightly but significantly reduced while percentage phagocytosis was not. This slight reduction was not the reason for the highly significant reduction in killing for the same reasons discussed earlier (see page175). A perfect example is patient number six, who had the lowest uptake and phagocytosis and the highest killing. The opposite applies in patient number ten. Although the average neutrophil alkaline phosphatase score of eleven patients was about twice that of the normals, the individual levels were variable, with normal, high and low figures being found. This is typical of the disease and as in the CGL case, did not correlate with any of the parameters investigated.

Peroxidase values, however, were on average about half those of the normals and because of the important role of the peroxidase in the cidal mechanism, a correlation between peroxidase values and the corresponding kill in the individual patients was highly likely. That is precisely what was found. This decrease in, or in one case near absence of, the peroxidase could be due to possible dilution of the peroxidase containing granules as a result of further divisions during maturation, or complete lack of formation of these primary granules, or a selective defect in peroxidase production or accumulation in the granules.

To test the first possibility, peroxidase values were plotted against the total leukocyte count and it is apparent

that there was a progressive loss of peroxidase as leukocyte count increases except in patient number three. It follows that percentage kill dropped significantly as the total leukocyte count rose except in the case of patient number three. Although this significant correlation between peroxidase and leukocyte count does seem to fit with the assumption, we will see later that this can not be the explanation.

To test the second possibility, we looked at the neutrophils of patient number one, who had near absence of the enzyme from his cells. Electron microscope studies on his neutrophils revealed the presence of normal looking granules which matched those of normal subjects in size and number but differed in lacking the electron dense peroxidase deposits. This proves that the primary granules themselves are normal.

To test the third possibility, neutrophils from patients and controls were stained for acid phosphatase and β-glucuronidase which are two primary enzymes as is peroxidase. There was no apparent difference in the amounts and distribution of these enzymes in the neutrophils of the patients and controls. Quantitative acid phosphatase measurements also revealed no difference at all.

As a result of these finding, it is concluded that peroxidase could not have disappeared as a result of further divisions during maturation as similar findings should have been revealed in the case of acid phosphatase. It is also reasonable to assume as a result of the electron microscope

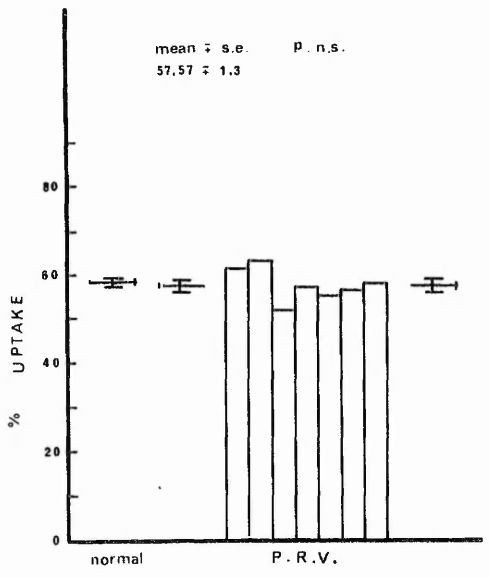
studies on the extreme case of peroxidase deficiency, that primary granules are normal in their frequency and morphology in all the patients. These findings, also, do not support our first possibility, hence we would have found diminishing number of granules. We are left with only one possibility and that is a selective defect in the production or accumulation of peroxidase. It is worth speculating on the opposing similarities between the alkaline phosphatase in CGL and peroxidase in MM neutrophils.

May be, the stimulating factor which induces the release of increasing number of leukocytes acts at the same time as an inhibiting agent on peroxidase accumulation, or induces it's selective degredation.

The decreased peroxidase values and increased alkaline phosphatase remind us of states of severe infection. Low peroxidase in MM neutrophils may be due to degranulation as a result of phagocytosis of antigen-antibody complexes. This, however, does not seem to be the case judging by the electron microscope and the acid phosphatase studies. Whether MM, however, is a special case of severe infection remains to be seen.

4. Polycythaemia Rubra Vera

Seven PRV patients were studied, all having typical clinical features. Values for percentage uptake, phagocytosis and killing, as well as peroxidase and alkaline phosphatase were estimated. (See Figs. 100, 101, 102, 103 and 104).



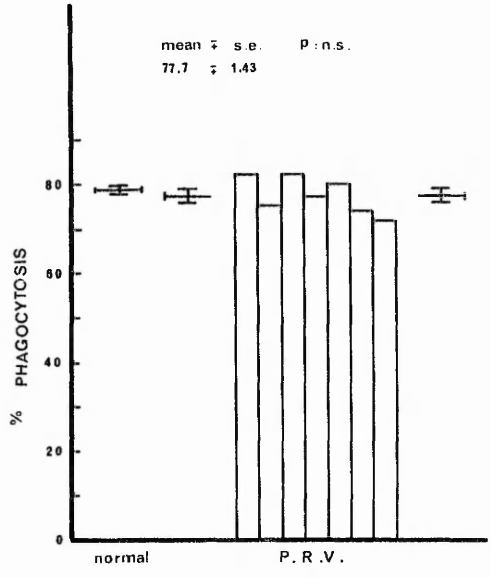


Fig. 100 Percentage uptake after 15 minutes by neutrophils of 7 PRV patients. Results as mean ∓ standard error are not significantly different from normal.

Fig. 101 Percentage phagocytosis after 15 minutes by neutrophils of 7 PRV patients. Results as mean ∓ standard error are not significantly different from normal.

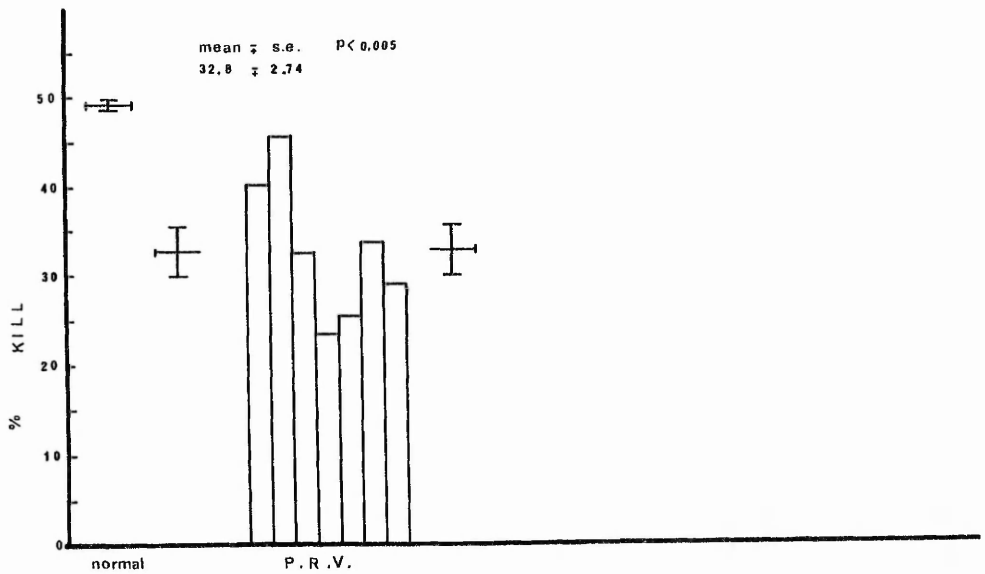


Fig. 102 Percentage kill after 90 minutes by neutrophils of 7 PRV patients. Results as mean ± standard error are significantly different from normal.

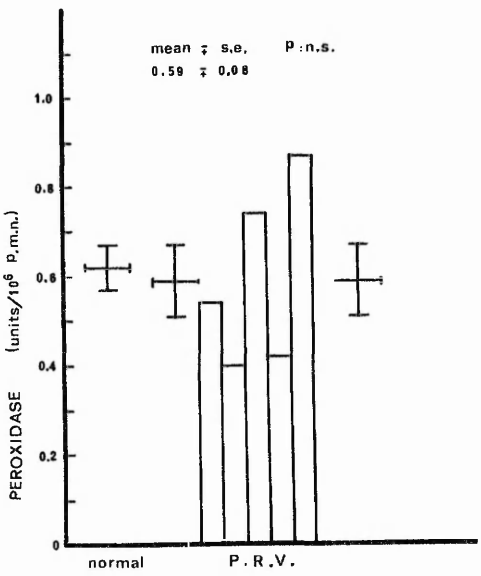


Fig. 103 Neutrophil peroxidase values of 5 PRV patients. Results as mean ∓ standard error are not significantly different from normal.

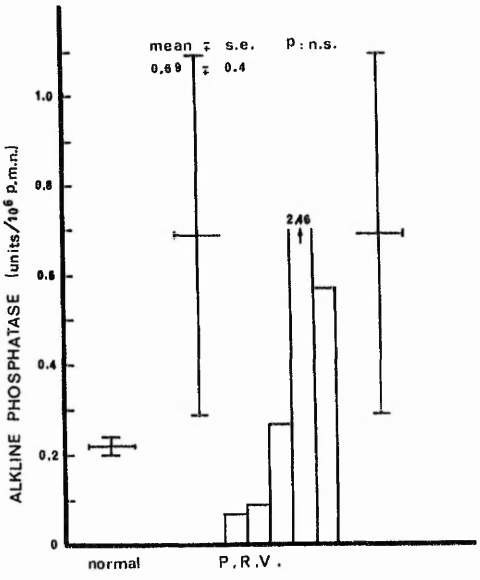


Fig. 104 Neutrophil alkaline phosphatase values of 5 PRV patients. Results as mean ∓ standard error are not significantly different from normal.

A summary of the previous results is shown in Table 4.

	No. of subjects	Range	Mean	S.E.	Signif-icance	Fig. No
Percentage uptake	7	520-629	5757	∓130	N.S.	100
Percentage phagocytosis	7	720-824	7770	∓143	N.S.	101
Percentage kill	7	235-454	3281	∓274	P<0.005	102
Peroxidase units/10^6 neutrophils	5	040-087	059	∓008	N.S.	103
Alkaline phosphatase units/10^6 neutrophils	5	007-246	069	∓040	N.S.	104

Table 4. A summary of PRV neutrophil function and enzymic values.

The only parameter which is significantly different from normal was the percentage kill. There was no correlation between percentage uptake, phagocytosis or killing and time, nor between percentage kill and total leukocyte count.

a) Discussion:

Neutrophil function in Polycythaemia Rubra Vera has been studied the least. The lack of interest may be due to the apparent minimum involvement of the neutrophil in the disease. Leukocyte alkaline phosphatase activity, however, is elevated in the majority of patients. (Anstey et al., 1963). Leukocytes metabolism is also altered, with features similar to those observed in otherwise normal subjects during severe bacterial infection. Increased resting and phagocytosing hexose monophosphate shunt activity, increased phagocytic index and increased oxygen comsumption in both resting and phagocytosing leukocytes. (Cooper et al., 1972). We have found, however, that our results do not agree entirely with all these

abnormalities. Percentage uptake as well as percentage phagocytosis were nearly identical to those of normal neutrophils and definitely not greater. Percentage kill was, however, significantly reduced and only about 67% of normal. Alkaline phosphatase was typically elevated to different levels in different patients while peroxidase levels were normal. If the neutrophils in PRV have been "turned on" then we would expect degranulation to take place and as a result much lower peroxidase values. Also a continuous autolysis of the neutrophils would occur as a result of the toxic and highly reactive end products of the stimulated metabolic activity of the neutrophil such as H_2O_2 and other oxygen metabolities as well as acids.

Judging by our findings, a defect in H_2O_2 production seems the most likely cause for the reduced candidacidal activity of the PRV neutrophils.

5. Infection

In infection leukocyte alkaline phosphatase level is raised and peroxidase level is lowered (Rosner et al., 1965; McCall et al., 1969; Solberg et al., 1972). This pattern is similar to that of MM.

Seven patients with severe infection (SI) were tested. Percentage uptake, phagocytosis and killing results as well as peroxidase and alkaline phosphatase values are shown in table 5. Percentage kill was significantly lower than that of normals. $P < 0.001$. Peroxidase was, as expected, also significantly lower than normal. $P < 0.005$, while alkaline

phosphatase, also as expected, was significantly higher than normal $P < 0.01$.

	No. of subjects	Range	Mean	S.E.	Significance
Percentage uptake	7	438-623	5527	∓227	N.S.
Percentage phagocytosis	7	690-897	7894	∓261	N.S.
Percentage kill	7	106-337	2583	∓273	P < 0.001
Peroxidase units/10^6 neutrophils	6	017-057	031	∓006	P < 0.005
Alkaline phosphatase units/10^6 neutrophils	6	045-140	095	∓013	P <0.01

Table 5. A summary of neutrophil function and enzymic levels in severe infection patients.

a) Discussion:

Neutrophil functions in severe infection have been investigated for two reasons. First to find any similarities in the impairment of function between Myeloid Metaplasia and severe infection. Patients with severe infection show the same pattern of enzyme and functional changes as MM, reduced peroxidase, elevated alkaline phosphatase and impaired killing.

Secondly, to act as an indirect control for the procedures used in the investigations. The reproduction of these anticipated abnormalities in the SI neutrophils was further justification of the procedures. Chemotaxis in neutrophils from patients with bacterial infections was found to be impaired (Mowat et al., 1971). This impairment was suggested to be due to prior phagocytosis of antibody-antigen complexes by the neutrophils. This might also explain the low peroxidase values as it could be

argued that the neutrophils have been "switched on" and degranulation might have taken place. The low peroxidase would also explain the reduced killing capacity. The high alkaline phosphatase levels could be due to increased numbers of Juvenile neutrophil released into the circulation as they tend to have a higher score. Or it could be due to activation of the enzyme, already present but dormant, as a result of increased membrane activities with which the enzyme is supposed to be associated. Further studies on oxygen uptake or H_2O_2 production in severe infection patients could reveal whether the neutrophils have been stimulated by phagocytosing antibody-antigen complexes and as a result have impaired function when challenged with actual organisms.

C H A P T E R S I X

FINAL DISCUSSION, CONCLUSIONS AND FUTURE WORK

Polymorphonuclear neutrophil function has been studied in three of the Myeloproliferative disorders, namely, Chronic Granulocytic Leukaemia, Myeloid Metaplasia and Polycythaemia Rubra Vera. The fungicidal capacity was found to be defective in all these disorders, while the mechanism responsible for the defect differed distinctly between them.

Phagocytosis is associated with profound changes in the metabolism of the granulocyte. A burst of metabolic activity is combined with an increase in oxygen consumption, hexose monophosphate shunt activity, and hydrogen peroxide formation. Following engulfment of microorganisms, the organisms are incorporated in phagocytic vacuoles into which the cytoplasmic granules discharge their contents of microbicidal substances. Among the granule consituents myeloperoxidase exerts an important bactericidal activity when in the presence of hydrogen peroxide and a halide. These studies on the metabolism and functional activity of CGL neutrophils during the phagocytic process revealed impaired degranulation as well as H_2O_2 production resulting in an abnormally low fungicidal capacity. A reduced amount of Myeloperoxidase in the MM neutrophils was responsible for their reduced fungicidal ability, while a suspected impairment in H_2O_2 production points towards the impaired killing in the PRV neutrophils.

In normal neutrophils, it has been possible to observe histochemically three stages in the cidal process when Candida guillermondii was phagocytosed for 90 minutes.

First, Candida were surrounded by peroxidase without any change in their staining properties. That is, the orangy colour of the neutral red was maintained. This stage does not indicate any activity of the cidal mechanism. Secondly, Candida were covered with the peroxidase and appeared bluish. This stage reflects that degranulation had taken place. It was also accompanied by an overall translocation of the peroxidase from the neutrophil cytoplasm to the Candida in the phagosome. Thirdly, some Candida lost their orangy colour and appeared very faint. The peroxidase had also been lost from the surrounding cytoplasm and there was an overall reduction in the peroxidase content of the neutrophil. It is possible that the faint Candida cells are those which have been acted upon by peroxidase and H_2O_2 and hence are dead. The fact that peroxidase had disappeared could be due to either H_2O_2 having a denaturing or inhibiting effect, or to a rapid deactivation of the peroxidase as soon as it is released from the granule due to attack by other hydrolytic enzymes.

On staining the preparations with Gram's stain, three states of Candida could again be seen. First, living Candida which were Gram positive. Secondly, Candida which had taken the faint orangy colour of the neutral red and were therefore completely Gram negative. Thirdly, Candida which had started to change to Gram negative but in a very localized and spotty manner. This final observation could provide some insight into the mechanism of killing. It can be visualised that peroxidase is released from the

individual granules in a very localized fashion, attacking the Candida cell wall on the spot resulting in a gradual but accumulative attack on the entire surface of the cell wall of the organism.

Visual comparisons between the peroxidase stain, Gram stain and Leishman stain of preparations of the same sample of Candida which has been phagocytosed for 90 minutes, revealed close correlations between the Candida which appeared very faint in the peroxidase stain, the Gram negative ones in the Gram stain and the pink "ghosts" in the Leishman's stain. This means that these three fractions represent the killed Candida in each of the preparations. Further evidence to support these observations was seen in the CGL neutrophils where the majority of the Candida in the peroxidase stained preparation were orange, not covered with peroxidase and the cytoplasm of the neutrophil maintained normal amounts of peroxidase. Most of the Candida maintained their Gram positivity and stained deep blue with Leishman's. This lack of kill must be due to lack of degranulation.

Regarding the fact that H_2O_2 measurements during phagocytosis in CGL neutrophils revealed impaired production, it was possible to consider the likely role of the H_2O_2 production, as well as other oxygen metabolites, in triggering the mechanism responsible for degranulation. The final step in H_2O_2 production requires an oxidase associated with the membrane of the phagocytic vacuole. It is possible that the oxygen metabolites change the nature of the vacuole membrane in such a way as to allow it's fusion with the granule to take place. Defective production of H_2O_2 and other

active oxygen metabolites would then explain impaired degranulation and killing of Candida. The impaired degranulation and H_2O_2 production of the CGL neutrophil are very similar to those abnormalities observed in neutrophils of Chronic Granulomatous disease in childhood, (CGD), where normal response to phagocytosis with increased oxygen consumption, increased hexose monophosphate shunt activity, or increased hydrogen peroxide production is impaired (Holmes et al., 1967). Although these neutrophils have been shown to phagocytose normally, they lacked normal degranulation and had defective ability to kill catalse-positive organisms (Kaplan et al., 1968). NADH oxidase has also been found to be deficient in these patients (Baehner et al., 1968).

Further work on the CGL neutrophils should be directed towards such enzymes which are responsible for the normal production of H_2O_2 as deficient degranulation might be secondary to impaired oxidative metabolism.

The microtubules and microfilaments of the neutrophil cytoplasm appear to be involved in the mechanism of degranulation (Boxer et al., 1974). Increased intracellular cyclic AMP levels impair degranulation. On the other hand, compounds that increase intracellular cyclic GMP concentrations further enhance degranulation (Zurier et al., 1974; Ignarro, 1974). These finding have been interpreted to mean that assembly of microtubules is crucial for degranulation and that the assembly might be regulated by cyclic nucleotides (Zurier et al., 1974). Further investigations regarding

these compounds in the CGL neutrophil could reveal useful findings regarding degranulation as well as killing of microorganisms.

In the MM neutrophils, however, visual observation revealed normal degranulation but despite that, there was a considerable reduction in kill. The peroxidase content of the neutrophils correlated well with the killing values in the individual patients. Under conditions of severe peroxidase deficiency or even its complete absence, we would expect some fungicidal activity due to the nonenzymic action of H_2O_2 as well as the peroxidase independent cidal substances such as acid, lysozyme, lactoferrin and cationic proteins. We would also expect H_2O_2 to accumulate in the leukocytes to a higher level due to decreased utilization by the absent peroxidase and hence offsetting in part the decreased microbicidal activity of the peroxidase system. Formate oxidation would be increased as well as the hexose monophosphate shunt pathway activity. As most of the MM patients had some peroxidase, and as we found that killing did correlate with the peroxidase value, it is possible that either the peroxidase independent cidal system was ineffective in killing Candida guillermondii or that H_2O_2 production was impaired in correspondence with the peroxidase impairment. H_2O_2 production measurements during phagocytosis would be an area to be investigated in future work on the MM neutrophil.

Finally, despite the overall finding that the fungicidal mechanism, in neutrophils of the Myeloproliferative disorders,

is impaired, these patients are not as vulnerable to fungal infection as would be expected. This apparent normal resistence may be due to the fact that most Myeloproliferative patients have abnormally high leukocyte counts which could compensate for the partial impairment in function. The fact that uptake and phagocytosis were nearly normal might also contribute towards apparent normal function in vivo. All this might be the reason why promising results have been obtained when neutropenia patients with severe septicemia have been transfused with large numbers of neutrophils derived from appropriate Chronic Granulocytic Leukaemia donors.

A P P E N D I C E S

APPENDIX 1.

The effects of ascorbic acid on bactericidal mechanisms of neutrophils

Ascorbic acid inhibits both of the H_2O_2-myeloperoxidase-halide reactions which have been implicated in the bactericidal activity of neutrophils. Both iodination of zymosan in intact neutrophils and the decarboxylation of L-alanine by neutrophil sonicates do not take place in the presence of 0.01 M ascorbic acid. Despite the inhibition of these reactions, leukocytic microbicidal activity remains unaffected (McCall et al., 1971). The mechanism by which ascorbic acid inhibits H_2O_2-myeloperoxidase-halide reactions is unknown. Inhibition of haem-containing enzymes, such as peroxidase and catalase, by ascorbic acid has been reported (Orr, 1967). A distinction should be made between ascorbic acid per se, which is a strong reducing agent and is not microbicidal, and the ascorbic acid oxidizing system. Ascorbic acid is autoxidized in the presence of copper with the formation of hydrogen peroxide which can, in turn, oxidize additional molecules of ascorbic acid. This ascorbic oxidizing system is toxic to bacteria, fungi and viruses (Ericsson et al., 1955).

The ascorbic acid oxidizing system is part of the oxygen-dependent, MPO-independent antimicrobial systems. Since the work in this thesis was concentrated on the oxygen-dependent, MPO-mediated antimicrobial system and as a result of the findings, no attempt was made to investigate the ascorbic acid oxidizing system.

APPENDIX 2.

Decimal Date System

The calendar system of years, months and days is inconvenient for many purposes of recording events and ages. The swift and accurate calculation of ages of subjects is hindered by this system, as is the easy coding and classification of this type of information.

One way round this difficulty is the use of Decimal Date System in which every day of the year is given a decimal equivalent.

For example:

Date of test 17th July 1967 becomes 67 540

Date of birth of subject 1st May 1950 becomes 50 329

Age of subject at time of examination (by subtraction) is 17 211 which is rounded off to 17 21Y.

APPENDIX 3.

Glucose Broth

"Lab-Lemco" powder	Oxoid L29	10g
Pepton Bacteriological	Oxoid L37	10g
Sodium chloride		5g
Distilled water		1000cm^3

Dissolve the chemicals in water by heating, filter hot, cool and adjust the pH to 7.5. Add 2g of Glucose, mix well, distribute in 10cm^3 portions in glass universal bottles. Sterilize by autoclaving at 115^{o}C for 10 minutes.

Oxoid Ltd., Southwark Bridge Road, London. SE1 9HF

APPENDIX 4.

TC Medium 199 Single Strength

Wellcome Tissue Culture Medium 199 is based on the formula of Morgan, Morton and Parker (1950). It is Earle's balanced salt solution with the addition of various nutrients and growth factors.

In the single strength form, the medium is a sterile working strength solution containing antibiotics and buffered with carbon dioxide. It retains its activity for at least one month if stored at $4^{o}C$.

PUBLICATIONS

El-Maallen, H. & Fletcher, J. (1975): Defective Neutrophil Function in Chronic Granulocytic Leukaemia.

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A C K N O W L E D G M E N T S

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