# INVESTIGATION OF THE IMMUNE-MODULATORY EFFECTS OF ERYTHROMYCIN

by

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# DECLARATION

This is to certify that the dissertation "INVESTIGATION OF THE IMMUNE-MODULATORY EFFECTS OF ERYTHROMYCIN" presented for the degree of Master of Science (MED) at the University of the Witwatersrand, Johannesburg, is my own work and has not been presented at any other University.

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Parts of this dissertation have been published in the following papers:-

1. Studies on the effects of ingestion of a single 500 mg. oral dose of erythromycin stearate on leucocyte motility and transformation and on release in-vitro of prostaglandin E2 by stimulated leucocytes.
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2. Enhancement of human polymorphonuclear leucocyte motility by erythromycin in-vitro and in-vivo.
A.C. Fernandes, R. Anderson, A.J. Theron, G. Joone, C.E.J. van Rensburg.
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3 An objective filter-based, enzymatic method for the *in-vivo* measurement of the migration of human polymorphonuclear leucocytes.

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## SUMMARY.

The Literature Review covers the immunosuppressive and immunopotentiating properties of antibiotics on the immune system and the effects these could have on the resolution of an infection. The possible pathogenic mechanisms of C. albicans are also reviewed in this section.

The experimental section shows that pre-treatment of mice with erythromycin increases the mean survival time following intraperitoneal inoculation of C. albicans. It was shown that erythromycin enhanced lymphocyte transformation and PMNL migration in both in-vivo and in-vitro situations. These enhanced immunological components probably caused improved survival times in the aforementioned animal experiments.

To investigate the effects of oral administration of erythromycin on in-vivo PMNL migration in adult volunteers a new quantitative test which could only be applied to humans was developed and is described in detail. Using this method preliminary data were obtained which show that erythromycin increases PMNL migration in-vivo.

Erythromycin in addition to well documented antimicrobial activity also possess immunostimulatory properties which, although of secondary consideration, may contribute to the eradication of microbial pathogens during chemotherapy with this antibiotic.

# LITERATURE REVIEW.

## CHAPTER I.

# LITERATURE REVIEW

The successful antibiotic-mediated resolution of a bacterial infection is not merely dependent upon the effect of the antibiotic on the causative organism(s) but rather is the result of an interplay of host, antibiotic and pathogenic factors. Some relevant considerations which determine the outcome of antibiotic therapy are:-

1 The direct antibiotic effects on the infecting bacteria.

2 Indirect antibiotic effects on the infecting bacteria.

3 The achievement of adequate serum antibiotic levels.

4 The failure of the immune system to neutralize intracellular pathogens.

5 The ability of the antibiotic to cross biological membranes.

6 Antibiotic-immune system interaction.

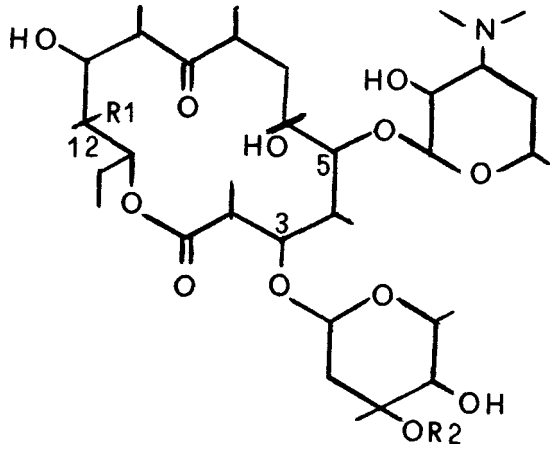
- 6.1 Antibiotic effects on chemotaxis.
- 6.2 Antibiotic effects on phagocytosis.
- 6.3 Antibiotic effects on lymphocyte transformation.
- 6.4 Antagonistic antibiotic effects on antibodies.
- 6.5 Enhancing antibiotic effects on antibodies.

6.6 Antagonistic antibiotic effects on complement.

6.7 Antibiotic effectiveness associated with a general suppressed immune system.

## THE DIRECT ANTIBIOTIC EFFECTS OF ERYTHROMYCIN ON THE MICROBIAL PATHOGEN.

Erythromycin:- Erythromycin belongs to a group of antibiotics called macrolides and is a metabolic product of Streptomyces erythreus which was isolated from a soil sample originating in the Phillipines in 1952 (Smith 1977). There are four erythromycins and their structures are shown in FIGURE 1 (Garrod and O'Grady 1971).




| | R1 | R2 |
|---|---|---|
| Erythromycin A | OH | Me |
| Erythromycin B | H | Me |
| Erythromycin C | OH | H |
| Erythromycin D | H | H |

FIGURE 1: Structures of the 4 erythromycins.

Mode of action:- The two suggested mechanisms of action of erythromycin are, a) that it is an inhibitor of peptide bond formation (Mao and Robinshaw 1972 and Mao and Robinshaw 1971) and b) that erythromycin inhibits translocation (Tanaka et al. 1973, Igarashi et al. 1969 and Cundliffe and McQuillen 1967).

Otaka and Kaji (1982), postulated that erythromycin, binds to the 50S ribosome sub-units in growing bacteria, this is supported by Wilhelm and Corcaran (1967), and Fernandez-Munoz et al. (1971).

Once the nascent oligopeptides reach a certain size, erythromycin can not stop further elongation of the chain and protein synthesis goes on until that ribosome completes translation of one cistron. In a sense, erythromycin acts as if it is an initiation inhibitor because it only binds to ribosomes with either no peptidal tRNA or short peptidal tRNA. This is consistent with the evidence put forward by Tai et al. (1974).

Administered orally or parenterally erythromycin diffuses well into body fluids and tissue with the exception of brain. It also enters into pleural and peritoneal cavities (Smith 1977).

The intravenous dosage for man is 500 mgm. of erythromycin lactobionate administered 6 hourly, which develops immediate blood levels of 11,5 to 30,0 microgram/ml.

Erythromycin has a half life of 1-2 hours (Garrod and O'Grady 1971). Erythromycin is excreted both in urine

and in bile, but only a fraction of the dose can be accounted for in this way (Garrod and O'Grady 1971). With so little recoverable from the urine and bile, the greater part of the drug must be broken down in the body (Garrod and O'Grady 1971). Mao and Tardrew (1965), have shown that an enzyme capable of demethylating erythromycin is widely distributed in rabbit tissue, the greatest activity being found in the liver.

The action of erythromycin is predominantly bacteriostatic in low concentrations. Somewhat higher concentrations are distinctly bactericidal (Garrod and O'Grady 1971). Balbirsingh et al. (1978) reported that when using erythromycin stearate dosages within the usual recommended range, there was lethal in-vivo action. The lethal in-vivo action could be observed by means of scanning and transmission electron microscopy, of secretions or tissue samples or both from patients. This does not however exclude a contribution by the immune system.

Toxicity:- Untoward effects resulting from the use of erythromycin are rare. However, occasionally hypersensitivity and jaundice can occur after the patient has taken the drug for 1Ø days (Smith 1977). The above clnically observable toxic effects are taken as non-applicable to this dissertation as the

erythromycin regimen applied to the mouse experimental model (to be described) consisted of only one dose of erythromycin within therapeutic levels. Further the experiments were concluded well within 7 days.

## INDIRECT ANTIBIOTIC EFFECTS ON THE INFECTIVE MICRO-ORGANISMS DUE TO ANTIBIOTIC MEDIATED CHANGES.

Antibiotic mediated changes of bacteria:- Some antimicrobial agents indirectly enhance the host phagocytic response. A number of studies have shown that even at subminimal inhibitory concentrations, certain antimicrobial agents indirectly decide the fate of micro-organisms by rendering them more susceptible to host immune-defence systems (Lorian 1980). Enhancement of phagocytosis by this mechanism usually occurs as a consequence of antibiotic mediated changes in the cell wall or capsular integrity of the target bacteria, the consequence being disruption of anti-phagocytic surface components and increased complement fixation, opsonisation and phagocytosis. Antibiotic-induced increased phagocytosis of bacteria by this mechanism at sub-minimum inhibitory concentrations has been reported for carbenicillin-treated Pseudomonas aeruginosa (Nishidi et al. 1976), naficillin-treated Staphylococcus aureus (Friedman and Warren 1974), Streptococcus pyogenes exposed to clindamycin (Gemmell et al. 1981 and

Gemmell et al. 1982), lincomycin (Gemmell and Abdul-Amir 1980), erythromycin and penicillin (Gemmell et al. 1982).

## BACTERIAL INHIBITION OF THE HOST'S IMMUNOLOGICAL DEFENCE MECHANISMS.

Once the bacteria have breached the host's mechanical defence mechanisms (skin, epithelium etc.), an infection can ensue. This is dependent on the ability of the bacteria to protect themselves against the host's cellular/humoral immunological defence mechanisms. The bacterial anti-host defence mechanisms include anti-phagocytic capsules as well as toxins such as leucocidin etc. Of particular interest is the ability of certain bacteria to invade cells, and thereby gain protection from the host's specific immunologic defence mechanisms. Legionella pneumophila, that once phagocytosed, can resist being killed by the phagocyte's killing mechanisms, and can even multiply within the human phagocyte. Thus phagocytes can serve as a haven where intracellular parasites such as Legionella can thrive and multiply, protected from the body's normal antimicrobial mechanisms, (Miller et al. 1984). The privileged position occupied by these intracellular micro-organisms can produce prolonged or recurrent infections, (World Health Organisation 1973).

The optimal therapy for infections due to facultative intracellular micro-organisms requires the use of antibiotics that can penetrate cells, including phagocytes, and which are bioactive intracellularly (Prokesch and Hand 1982).

## THE ACHIEVEMENT OF ADEQUATE SERUM ANTIBIOTIC LEVELS.

Effective in-vivo antibiotic action, can only be achieved if an adequate concentration of active antibiotic reaches a sensitive micro-organism. Factors governing this are, absorbtion, protein-binding, metabolism of antibiotic by the host, excretion by kidneys, action on antibiotic by bacterial enzymes (e.g. betalactamase) and the transport of antibiotic to the infected sites by blood supply and or cells such as polymorphonuclear leucocytes (PMNLs).

## THE FAILURE OF THE IMMUNE SYSTEM TO NEUTRALIZE INTRACELLULAR PATHOGENS.

An intracellular micro-organism is one that has the ability to establish a privileged position within a cell. The class of cell that offers the greatest interest is the phagocyte.

The functions of phagocytes are to migrate to the site of the invasive antigen, phagocytose, and mediate the intracellular destruction of the antigen. The

repertoire of killing mechanisms available to the phagocyte consists of agents amongst the most reactive known to chemistry, viz. the superoxide anion, hydrogen peroxide, the hydroxyl radical and possibly singlet oxygen (Babior 1978). Oxygen independent killing mechanisms which also participate include lysosomal hydrolysis, lysozyme and rapid acidification of phagocytic vacuoles. There are pathogens which parasitize macrophages and replicate intracellularly by avoiding the above destructive processes. Such organisms include Listeria, Salmonella, Brucella, Mycobacteria, Chlamydia and Rickettsia species. Bacterial intraphagocytic survival can be explained by mechanisms that avoid triggering of the above killing mechanisms. In the case of M. tuberculosis the defensive mechanisms of the bacteria consist of the production of catalase and sulpholipids that interfere with the fusion of lysosomes and phagosomes. Leishmania and M. lepraemurium survive within phagosomes despite fusion with lysosomes. The latter bacterium is ensconced in a peptoglycolipid that resists lysosomal hydrolysis (Nathan et al. 1980). Legionella pneumophila also resists killing by both cellular and humoral factors (Horowitz et al. 1981a and Horowitz et al. 1981b). When antibiotic therapy is being considered for the treatment of the above bacteria, of prime importance is whether the selected antibiotic can traverse the cell membrane of the host cells.

## THE ABILITY OF ANTIBIOTICS TO CROSS BIOLOGICAL MEMBRANES.

Legionella spp., which are facultative intracellular bacteria, and Chlamydia spp., which are obligate intracellular parasites, are organisms known to parasitize a large number of cell types (Martin et al. 1985).

The intracellular location of these micro-organisms protect them from antibiotics that are unable to cross biological membranes and exert their action (Martin et al. 1985). This is supported by Prokesch and Hand (1982), who have shown that these micro-organisms survive intracellularly even when incubated with certain antibiotics at a level well above the lethal concentration for that particular organism. The possible explanation for this phenomenon is that these antibiotics penetrate phagocytes poorly. Penicillin G and cephalosporin antibiotics fail to penetrate either alveolar macrophages (AMs) or PMNLs efficiently. This probably reflects the limited lipid solubility of these drugs and the inability of these drugs to cross the biological membranes. Previous studies have suggested that antibiotics with good lipid solubility penetrate biological membranes, and thus are accumulated by mammalian cells (Kornguth and Kunin 1976, Mandell 1973 and McLeod et al. 1977). These observations are supported by the observation that

chloramphenicol, rifampin and lincomycin, drugs which are lipid soluble, achieve intracellular concentrations in both PMNLs and AMs twice that of the extracellular medium (Prokesch and Hand 1982). These findings are consistent with the knowledge that one of these drugs, rifampin, is able to kill intraphagocytic bacteria (Ezer and Soothill 1974). Uptake of rifampin and chloramphenicol was independent of cell viability, suggesting that entry of these drugs into cells is energy independent (Prokesch and Hand 1982).

Trans-membrane passage of antibiotics is essential for effective eradication of intracellular organisms. If this trans-membrane crossing is not achieved, the protected intracellular organisms will be the cause of persistent or relapsed infections.

Erythromycin, belonging to the lipid soluble group of antibiotics, has the ability to cross biological membranes and undergo concentration within the eukaryotic cells. This can occur even after cessation of antibiotic therapy (Prokesch and Hand 1982). Distribution studies on tissue samples excised from dogs and rats have shown that erythromycin is concentrated well above blood levels in organs such as the liver, spleen, kidney, lung, pancreas and in submaxillary and adrenal glands (Lee et al. 1953 and Lee et al. 1956). Recent studies (Hand et al. 1983 and Johnson et al. 1980) have shown that both human PMNLs and AMs accumulate erythromycin at intracellular

concentrations 15 to 24 times of that found in the extracellular compartment. For this intracellular concentration of erythromycin to take place, viable cells, a physiological environmental temperature and metabolic energy are required (Prokesch and Hand 1982).

Erythromycin is not simply adsorbed on to the surface of PMNLs, but is randomly dispersed throughout the cytoplasm of cells. The drug does not appear tightly bound to any specific cell organelles since it rapidly diffuses out of cells upon lowering of the extracellular drug concentration (Miller et al. 1984). Martin et al. (1985) suggest that this accumulation may be responsible for the aberrant levels in plasma usually observed after erythromycin administration, and that when this intracellular erythromycin is released it is in a form still effective against bacteria. Martin et al. (1985) propose that the intracellular localisation of erythromycin may be more relevant when treating micro-organisms known to be capable of causing an intracellular infection.

The effectiveness of erythromycin in the treatment of Legionella infections can be related to, Legionella spp. being sensitive to erythromycin, erythromycin being able to traverse the biological membranes, and the concentration of erythromycin within phagocytes (Miller et al. 1984).

Miller et al. (1984) comment that because blood

contains many PMNLs, each of which can concentrate erythromycin and release the drug in an unaltered active form, and because PMNLs are drawn to the sites of infection by chemotactic factors, it is possible that the PMNLs provide a unique drug delivery system that transports erythromycin to the various infected tissues. In this way erythromycin, through the use of a cellular delivery system, may achieve higher levels in infected tissues than would otherwise be expected from simple blood levels.

## ANTIBIOTIC/IMMUNE SYSTEM INTERACTION.

The different mechanisms by which an antibiotic exerts a direct bactericidal or bacteriostatic effect on bacteria will not be discussed. What is pertinent to this dissertation is that in the in-vivo situation the cure of an infection is not achieved solely by the action of the antibiotic on the bacteria, "in isolation". The action of an antibiotic on a micro-organism "in isolation", is a necessary feature of the in-vitro sensitivity test method for the determination of the bacteria's antibiogram. The cure of an infection by an antibiotic is due to the action of an effective level of antibiotic on a sensitive micro-organism in an in-vivo micro-environment. This in-vivo micro-environment is influenced by many dynamic immunological and non-immunological systems. It might

be that differences in the in-vitro/in-vivo micro-environments are occasionally responsible for patients not responding to antibiotic therapy as indicated by antibiograms.

Since antibiotics were not developed by micro-organisms specifically as a medication for the treatment of bacterial infections in man, it is not surprising that coincident with the desired antibacterial effects achieved there can be unpleasant side effects. In fact the ratio of antibiotics discovered, that are systemically non-toxic or tolerably toxic : to non-usable antibiotics because of their prohibitive toxicity, is extremely small.

One could define a useful antibiotic as a substance produced by micro-organisms that has a systemic anti-microbial action in the complex environment of the body without being prohibitively toxic to the host.

Side effects can be categorised into those that are clinically obvious (blindness, loss of balance etc.), and those that are not clinically obvious. With the development of improved immunological techniques, many antimicrobial agents at attainable in-vivo concentrations have recently been found to be immunosuppressive (Finch 1980, and Miller and North 1981). Neftel et al. (1983) observed that penicillin-G rapidly degrades in an aqueous solution and that the

resulting products exert a direct toxic effect on the myeloid series in the bone marrow. Further this report describes that the addition of fresh or degraded penicillin-G to bone marrow cultures is associated with inhibition of in-vitro granulopoiesis. The inhibitory effect of penicillin-G on in-vitro granulopoiesis is seen only when the drug is present during the proliferative phase. These covert actions on the part of the above antimicrobial agents have remained unnoticed due to the lack of obvious clinical symptoms. The suppression of the immune system by antibiotics may seem of questionable value since short courses of treatment with antimicrobial agents are invariably successful. According to Miller and North (1981) the principal beneficiaries of advances in antimicrobial chemotherapy have been healthy (i.e. immunocompetent) people with acute infections. Occasionally the results of antibiotic therapy, as indicated by in-vitro antibiograms, are disappointing, which might be ascribed to antagonism between the antimicrobial agent and the immune system. Conversely it has been shown that under certain in-vitro conditions some antibiotics can have an enhancing effect on the immunological system and consequently might have an in-vivo advantage over the immuno-suppressive antibiotics.

The absence of immunosuppressive properties may be an important factor in the selection of antimicrobial

agents for prophylactic use or in treatment of infections requiring long term chemotherapy.

Antibiotic effects on chemotaxis:- Chemotaxis is of primary importance in both the specific and non-specific immune responses, since a protective response is dependent on rapid and adequate accumulation of leucocytes at the site of infection. This phenomenon is initiated and sustained by a variety of humoral factors termed leuco-attractants. The most biologically active of these are the complement-derived fragment C5a and the low molecular weight lipids leukotriene B4 and 5-hydroxyeicosatetraenoic acid, both of which are products of 5'-lipoxygenase mediated oxidation of arachidonic acid (Ford-Hutchinson et al. 1980 and Goetzl et al. 1980). Tetracycline (Forsgren and Schmeling 1977), doxycycline and lymecycline (Forsgren and Schmeling 1977 and Belsheim et al. 1979), have been reported to inhibit PMNL migration at therapeutic concentrations in-vitro. It is likely that these observations have clinical significance since they have been confirmed in two independent in-vivo studies. Using a skin chamber technique, one group of investigators described inhibition of PMNL chemotaxis in-vivo, in each of eight healthy volunteer subjects, within 48 hours after initiating a course of doxycycline (200 mg. once orally, then 100 mg. orally per day)

(Forsgren et al. 1980). Likewise Martin et al. (1974), found that systemic administration of tetracycline was associated with reduced PMNL chemotaxis. Fusidic acid, tobramycin and chloramphenicol have also been reported to inhibit human PMNL migration at therapeutic concentrations in-vitro (Forsgren and Schmeling 1977). In-vitro tests have shown that therapeutic concentrations of gentamicin and amikacin inhibit the migration of PMNLs (Goodhart 1977 and Seklecki et al. 1978). These findings have been confirmed by in-vivo studies in which gentamicin and amikacin were administered in therapeutic doses to normal healthy adults (Kahn et al. 1979). The authors state that their findings may be clinically significant, especially with patients whose immune system might be already impaired and require aminoglycoside therapy. Commonly used antmicrobial agents which apparently do not influence PMNL migration are ampicillin, benzylpenicillin, carboxypenicillin, cephalothin, and clindamycin (Forsgren and Schmeling 1977). Erythromycin (van Rensburg et al. 1982 and Anderson et al. 1982) and dapsone (Anderson et al. 1981) have been found to increase the migration of human PMNLs in-vitro at greater than therapeutic concentrations. This enhanced PMNL migration to serum derived leucoattractants was related to the anti-oxidant properties of the antimicrobial agents (Anderson et al.

1982 and Anderson et al. 1981). These observations were made from in-vitro studies and may not be entirely valid in the in-vivo context.

Antibiotic effects on phagocytosis:- Certain antimicrobial agents have been found to inhibit phagocytosis and antimicrobial activity. Lehrer (1971) and Miller and North (1981) reported that sulphonamides decreased the ability of human PMNLs to kill C. albicans and C. tropicalis in-vitro. Anderson et al. (1980) found that this effect was not due to the inhibition of phagocytosis of these micro-organisms, but that sulphadiazine, sulphamethoxazole and trimethoprim individually and in combination inhibited activity of the PMNL peroxidase: $H_2O_2$:halide system in-vitro. Other antimicrobial agents which have been reported to inhibit PMNL oxygen-dependent antimicrobial activity are tetracycline, cephalothin (Welch et al. 1981), cefotaxime (van Rensburg, et al., 1983) and amphotericin B (Bjorksten et al. 1976).

Forsgren et al. (1974) found that doxycycline and tetracycline at therapeutic levels inhibit the phagocytosis of yeast by human PMNLs. They also observed that PMNLs from healthy adult volunteers who had ingested one gram of tetracycline 2-4 hours earlier had decreased phagocytic capacity for yeast. The inhibitory mechanism was not investigated.

There are some antimicrobial agents which enhance host

responses to infection by increasing phagocyte and lymphocyte functions. Erythromycin, penicillin, cephalothin, chloramphenicol and tetracycline at therapeutic concentrations have been reported to enhance the phagocytosis of non-microbial particles by human PMNLs in-vitro (Mandell and Ehsen 1981). Since inert non-bacterial particles were used, the stimulatory effect observed is likely to be related to a direct interaction of the antimicrobial agent with the PMNLs.

Antibiotic effects on lymphocyte transformation:-

Banck and Forsgren (1979) tested the effects of 20 different antimicrobial agents on lymphocyte transformation in-vitro and observed significant impairment of the mitogenic responses of both B and T-lymphocytes with therapeutic concentrations of fusidic acid, nitrofurantoin and doxycycline. Rifampicin has been found to inhibit antigen and mitogen-activated lymphocyte transformation in-vitro (Banck and Forsgren 1979, Ruben et al. 1974, Grassi and Pozzi 1972 and Goldstein et al. 1976). In studies using human subjects ingestion of rifampicin was associated with depressed lymphocyte transformation which was only evident 3-4 months after the commencement of antimicrobial chemotherapy (Ruben et

al. 1974 and Goldstein et al. 1976). The antibacterial agents erythromycin (Anderson et al. 1982), dapsone and cefotaxime (Anderson et al. 1981), influence lymphocyte transformation. Ingestion or injection of therapeutic doses of these agents is associated with increased mitogen-induced lymphocyte transformation (Anderson et al. 1981 and Anderson et al. 1982).

Antagonistic antibiotic effects on antibodies:-
Chloramphenicol has been reported to inhibit the antibody response in humans. Daniel et al (1965) found that production of specific antibodies in response to tetanus toxoid was reduced in patients receiving 2-4 grams of chloramphenicol daily.

Enhancing antibiotic effects on antibodies:- Howie (1975) observed an increase in serum IgM antibodies in 11 of 23 patients with acute otitis media treated with eryhthromycin and sulfonamides which compared with only 4 out of 22 patients treated with ampicillin.
These observations suggest the existence of a more effective production of antibodies during erythromycin treatment than during penicillin treatment. It was suggested that this difference may be an example of a more effective antigenic stimulation during therapy with bacteriostatic antibiotics. It is noteworthy however that erythromycin has been reported to possess

both bactericidal (Balbirsingh et al. 1978) and immunostimulatory properties (van Rensburg et al. 1982, and Anderson et al. 1982).

Cefotaxime treatment of mice experimentally infected with E. cloacae or C. albicans (for which cefotaxime has no antimicrobial activity at the concentrations used) is associated with increased survival of the mice in comparison to controls (Gillisen 1982). These results were paralleled with a stimulation of antibody production.

Amphotericin B potentiates the antibody response of mice to antigens (Blanke et al. 1977), an effect which may be related to potentiation of macrophage activity or inhibition of T-suppressor lymphocytes (Little et al. 1980).

Antagonistic antibiotic effects on complement:- It has been shown that ingestion of ampicillin by normal adult volunteers is associated with reduced complement activation (Chakrabarty 1981).

Antibiotic effectiveness associated with a general suppression of the immune system:- Clinical studies in humans with neutropenia, agammaglobulinemia (Miller and North 1981) and acquired defects of cell mediated immunity (CMI) associated with different types of cancer (Middleman et al. 1972,

Johnston and Colley 1969, Nieman and Lorber 1980, Nirmal et al. 1971 and Schroter and Weil 1977) have shown that the responses to antimicrobial therapy are frequently poor despite the acquisition of antimicrobial concentrations of the antibiotic. These findings clearly demonstrate that the optimal activity of antimicrobial agents in-vivo is dependent upon an intact host immune system. This conclusion has been confirmed by results obtained in experimental animals.
Watson et al. (1978) showed that the efficacy of ampicillin against infections with L. monocytogenes is dependent upon intact host defence mechanisms
Using a model of optimal but delayed aminoglycoside antibiotic therapy in immunologically normal mice similar observations were made by Greisman et al. (1979). In these studies, infection was induced in outbred Swiss mice by one of three Gram-negative bacterial species (Esch. coli, Pr. mirabilis or Kl. pneumoniae) and the antibiotics used were the aminoglycosides gentamicin and kanamycin to which these micro-organisms were found to be sensitive in-vitro. An inoculum of Gram-negative bacteria was selected in each case that resulted in 90-100% lethality without antibiotic therapy and in no mortality with prompt antibiotic administration. With each species a delayed period was selected before starting antibiotic therapy such that despite optimal dosage schedules of

antibiotics a 50-70% mortality ensued. Using such a model of optimal but delayed aminoglycoside antibiotic therapy the synergistic interactions between the antimicrobial agents and specific antisera were assessed. Specific antisera when co-administered with optimal aminoglycoside antibiotics after the standard delay period prevented mortality not preventable by the antibiotics alone. In an extension of these experiments it was observed that pre-treatment of the mice with the immunosuppressive agent cyclophosphamide abrogated the protective effects of specific antiserum and antibiotic. Although these studies were undertaken primarily to study protective mechanisms against Gram-negative endotoxemia, they further emphasize the beneficial interactions which should occur between the host immune system and antimicrobial agents. According to Miller and North (1981) the principal beneficiaries of advances in antimicrobial chemotherapy are normal individuals. Immunocompromised individuals often have unsatisfactory responses.

## PATHOGENICITY OF C.albicans.

C. albicans is considered an opportunistic pathogen affecting persons whose host defences are in some way or other suppressed (Odds 1979). These immunosuppressed groups being, congenital or acquired immunological deficiencies, the infant group whose

immunological defence mechanisms have not fully matured and the old, whose immunological defence mechanisms are failing. It is still poorly understood which mechanisms in fact are responsible for the pathological process.

Views presently falling out of favour, are that C. albicans is capable of producing an endotoxin and the ability of C. albicans to produce pseudomycelia with the ability to physically disrupt the phagocytic cell (Louria and Brayton 1964, Stanley and Hurley 1969, and Odds 1979). Also, two C. albicans cell surface enzymes have been described which are similar to substances responsible for the pathogenicity of many bacteria. These are acid phosphatemonoesterase (Odds & Hierholzer 1973) and phopholipase (Pugh and Cawsen 1975 and Price and Cawsen 1977).

An enzyme first described by Staib (1965) as an inducible secreted proteinase has recently attracted increasing attention as a possible virulence factor. It has been shown by Macdonald and Odds (1983) that there is a correlation between the ability of C. albicans to produce this inducible proteinase and virulence. For comparison a non-pathogenic proteinase deficient mutant prepared from the former virulent strain was shown to be incapable of producing the inducible proteinase to the extent of the parent pathogenic strain. Factors supporting this latter hypothesis are that patients suffering from systemic

Candidiasis frequently have antibodies to this enzyme (Macdonald & Odds 1980). Further investigations by Macdonald and Odds (1980) showed that the mortality rate of mice which had been intravenously inoculated with $1x10^6$ viable cells of the proteinase producing C. albicans was 100% in 7 days, whereas the mortality of mice inoculated with the same number of non-proteinase producing strain was 50% at 34 days. The way in which the proteinase exerts its pathological effects in vivo remain unknown. Most probably the enzyme mediates tissue degradation, assisting the fungus to invade tissue. An alternative is that the enzyme might be involved in defending the fungus against host defence mechanisms. This concept is supported by experiments showing that phagocytosis and killing of C. albicans by PMNLs was markedly reduced in the case of proteinase producing C. albicans. The addition of proteinase to the non-proteinase producing strains did not influence phagocytosis nor the killing ability of the PMNLs, indicating that this proteinase is an effective defence against phagocytosis and intracellular killing, only when it is being actively produced by C. albicans (Macdonald & Odds 1983).

It seems that C. albicans rarely causes infections in persons with intact immune defences and possesses no single virulence factor as potent or as easily demonstrable in vivo as, for example, bacterial toxins (Macdonald & Odds 1983).

## RATIONALE FOR THIS STUDY.

Successful antimicrobial chemotherapy depends ultimately on beneficial co-operative interactions which occur between antibiotics and host immune defence mechanisms (Miller and North 1981). Antibiotic sensitivity tests performed in vitro are therefore not necessarily predictive of the full potential of some antimicrobial agents since they exclude the enormous contribution made by antibiotic/host immune defence interactions in the eventual eradication of microbial pathogens. The ability to co-operate with host immune defence mechanisms is probably a common but variable property of antibiotics. Some antibiotics are better equipped than others to improve the anti-bacterial defences of the infected host. Erythromycin has emerged as an antimicrobial agent which interacts beneficially with host immune defence mechanisms (Prokesh and Hand 1982). In this dissertation interactions which occur between erythromycin and certain host immune defence mechanisms have been investigated.

# REAGENTS and METHODS.

## CHAPTER II.

## REAGENTS

### Ammonium chloride solution.

Ø,83% ammonium chloride (NH4Cl), was dissolved in triple glass distilled water, containing Ø,1% sodium bicarbonate (NaHCO3) and Ø,Ø74% EDTA.

### Ficoll hypaque solution.

1) Sodium metrizoate solution 75% w/v (Nyeggard and Co., As. Oslo). One ampoule contains 2Ø ml.
2) Ficoll powder, molecular wt. of 4ØØØØ.

Method.

1) Ficoll powder 27Ø gm.
2) Double distilled water 3ØØØ ml.
3) Heat and stir at 5Ø deg C.
4) Cool to room temperature.
5) Sodium metrizoate solution 48Ø ml.
6) Adjust the specific gravity (SG) to 1,1 for mouse spleen cells and Ø,7 for human blood.

### 3% Gelatin phosphate buffered saline (PBS).

3,Ø% gelatin in PBS (Ø,15 M, pH 7,2) dissolved in a 56 deg. C. water bath and then cooled to 37 deg. C.

## Gey's fluid.

| | |
|---|---|
| 1) PBS | 200 ml. |
| 2) Calcium chloride (CaCl2.2H2O) | 8 mgm. |
| 3) Magnesium chloride (MgCl2.6H2O) | 20 mgm. |
| 4) Glucose | 400 mgm. |

## Heparin solution.

0,3% heparin in triple glass distilled water. The solution was filter sterilised.

## Nutrient broth No.2 (NB2) AGAR.

NB2 agar consisted of 1% agar (Oxoid Agar No.1) in NB2.

## Nutrient broth No.2.

NB2 (Oxoid CM 67) described as being comparable to Nutrient Broth but richer in nutrients. Made up as prescribed by the manufacturer.

## Medium M199.

1) Medium M199 (Green Island Biological Co., Paisley, Renfrewshire, Scotland (Gibco)), containing Hanks salt and L-Glutamine, without Sodium carbonate.

2) Streptomycin/Penicillin 10000 units/ml.

Method.

| | |
|---|---|
| 1) M199 medium | 11 gm. |
| 2) N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid (Hepes) | 4 gm. |

3) Sodium bicarbonate (NaHCO3) 700 mgm.

4) De-ionised water 2 L.

5) Adjust pH with 0,1 N Sodium Hydroxide to 7,1.

6) Streptomycin/Penicillin (10000 units) 20 ml.

Sabouraud dextrose agar (SDA) (Merck Chemicals (PTY) Ltd., Federal Republic of Germany).

Sabouraud dextrose Agar. Made up as prescribed by the manufacturer.

Sabouraud dextrose broth medium (SDB) (Merck).

Sabouraud dextrose broth media. Made up as prescribed by the manufacturer.

Concanavalin A (CON-A) solution, 800 µg./ml.

Concanavalin A (Sigma Chemical Company, St. Louis, USA).

Method.

1) CON-A 4 mgm.

2) M199 medium 5 ml.

3) Sterilise by filtraton

4) Dispense into test tubes 0,4 ml.

Phytohaemagglutinin (PHA) solution, 800 µg./ml.

Reagent:- Purified Phytohaemagglutinin (Wellcome Diagnostics, Temple Hill, Dartford, U.K.) 2 mgm./phial.

Method:- As for CON-A solution with the exception that CON-A is substituted by PHA.

Decomplemented serum.

Fresh human serum was decomplemented at 56 deg. C. for 30 min.

Tritiated thymidine.

Thymidine-methyl-3H (New England Nuclear Corporation; Boston; Mass.; USA).

Method.

Stock solution:- H/3 thymidine of 1 mi/Ci per ml. dispensed in 50 /u/L. volumes per tube and stored at -70 deg. C.

White cell counting fluid.

| | |
|---|---|
| 1) Gentian violet 1% | 1 ml. |
| 2) Acetic acid | 2 ml. |
| 3) Distilled water | 100 ml. |

Erythromycin.

For in-vitro studies erythromycin base was used, erythromycin stearate (500 mg. tablets) was used for human oral studies and erythromycin lactobionate used for animal inoculations.

All forms of erythromycin were obtained from Abbott Laboratories Ltd., Abbott Park, North Chicago, Illinois, U.S.A.

## INSTRUMENTATION.

### Beta counter.

All beta counts were determined on a Searle Isocap/300 Liquid Scintillation Counter.

### Spectrophotometer.

The spectrophotometer used was the Pye Unicam SP1700 Ultraviolet Spectrophotometer.

## FORMULA.

Formula to determine the centrifugal force in Gs.

$$G = 11{,}2 \left(\frac{RPM}{1000}\right) X R$$

G = gravity.

RPM = revolutions per second.

R = radius in cm. from the centre of the boss to the end of the centrifuge tube.

## EXPERIMENTAL ANIMALS.

The experimental animals were NMRI mice, as described by Festing (1973). Origin:- Non-inbred by Poiley. To US Naval Med. Res. Inst. at F51, known as NMRI. The mice were supplied by the H.F. Grove, Animal Research Centre, Pretoria. For test purposes, groups of 20 mice, excepting where otherwise stated, were matched in sex, age (2 months) and weight (20 gm.).

## TEST ORGANISMS.

Candida albicans (C. albicans) Y240:CBS5736, was supplied by Dr. van der Walt, Council for Industrial and Scientific Research, Pretoria. Enterobacter cloacae (E. cloacae) ATCC 13047, was obtained from Merck Chemicals (PTY) Ltd.

## DETERMINATION OF THE MINIMUM INHIBITORY CONCENTRATION (MIC) AND MINIMUM CIDAL CONCENTRATION (MCC) OF *E. cloacae* AND *C. albicans*.

The standard laboratory methods for determining the MIC and MCC as described by Cruickshank et al. (1975), were used. The serial dilutions of erythromycin, ranged from 6 mgm./ml. to Ø,Ø1 micro gm./ml. in NB2. The results obtained are shown in TABLE 1 (page 58).

## DETERMINATION OF COLONY FORMING UNITS (CFU) RELATIVE TO OPTICAL DENSITY (OD) OF *E. cloacae* AND *C. albicans*.

*E. cloacae* and *C. albicans* were grown in NB2. After washing the micro-organisms three times in PBS, the ODs of various dilutions of the concentrated micro-organisms were read on a spectrophotometer at a wavelength of 54Ø nm. (all OD readings of micro-organism cell suspensions relating to CFU were read at the wavelength of 54Ø nm.). Ø,1 ml. aliquot of each of the micro-organism dilutions were added to a petri dish into which was poured 3Ø,Ø ml. of NB2 agar. Colony counts were performed after incubation periods of 48 hrs. for *E. cloacae* and 72 hrs. for *C. albicans*. Graphs relating OD to CFU/ml. are shown in FIGURES 4 and 5 (pages 59 and 6Ø).

## PREPARATION OF THE *E. cloacae* INOCULUM.

*E. cloacae* was grown in NB2 for 24 hrs. The organisms were then harvested, washed three times in PBS and resuspended in PBS. The OD540 was determined on a 1/15 dilution of the inoculating suspension in PBS, using a spectrophotometer. The OD was adjusted to Ø,15 equivalent to 115 x $10^6$ CFU/ml. Conversion from OD to CFU was by means of the graph in Figure 4 (page 59).

## PREPARATION OF THE *C. albicans* INOCULUM.

*C. albicans* was grown in Sabouraud's dextrose agar for 48 hrs. The organisms were then harvested, washed three times in PBS and resuspended in PBS. The OD540 was determined on a 1/15 dilution of the inoculating suspension in PBS, using a spectrophotometer. The OD was adjusted to 1,5 equivalent to 16 x $10^6$ CFU/ml. Conversion from OD to CFU was by means of the graph in Figure 5 (page 60).

## INOCULUM CONCENTRATION OF MICRO-ORGANISMS USED.

*E. cloacae*:- It was established that a Ø,5 ml. inoculum of *E. cloacae* suspension at an OD540 of Ø,15 after a 1/15 dilution in PBS, when inoculated intraperitoneally into mice, killed 5/5 mice within 24 hours. This concentration of bacteria was used throughout this dissertation, unless otherwise stated.

C. albicans:- It was established that a Ø,5 ml. inoculum of C. albicans at OD54Ø of 1,5 after a 1/15 dilution in PBS, inoculated intraperitoneally into mice, killed 5/5 mice within 48 hours. This concentration of C. albicans was used throughout this dissertation, unless otherwise stated.

DETERMINATION OF THE EFFECTS OF ERYTHROMYCIN ON THE IN-VITRO GROWTH RATE OF C. albicans AND E. cloacae USING A TURBIDOMETRIC METHOD.

C. albicans:- A 2Ø ml. volume of NB2 was inoculated with a colony of C. albicans and incubated for 18 hrs. The organisms were then washed once and resuspended in 4 ml. of PBS. 3ØØ ml. of NB2 was inoculated with 6Ø micro L. of the above suspension of micro-organism. The 3ØØ ml. of yeast suspension was dispensed into 3 X 1ØØ ml. volumes. Erythromycin was added to 2 volumes of inoculated broth, to arrive at concentrations of 7,5 and 3Ø,Ø micro gm./ml. of erythromycin. The third 1ØØ ml. volume, without erythromycin acted as a control. Each 1ØØ ml. volume of broth was dispensed into 3 X 2Ø ml. volumes which were then incubated at 37 deg. C. At time intervals of 16, 24 and 42 hours, 5 ml. samples were taken. The samples were washed twice and resuspended in two ml. of

PBS. The OD540 of these undiluted suspensions of micro-organisms were determined, Results are presented in TABLE 3 page 62.

E. cloacae:- This method was similar to the method used to determine the effect of erythromycin on the growth of C. albicans, with the exception that the cultures were sampled at 18 hrs. only. No further samples were taken as there was an obvious indicaton that erythromycin had an effect on the growth rate of E. cloacae.
Results are presented in TABLE 2, page 61.

## ASSESSMENT OF THE SUITABILITY OF E. cloacae.

Initial justification for the use of E. cloacae in this investigation was based on the MIC and MCC as previously described. However it was observed that erythromycin retarded the growth rate of E. cloacae. This is illustrated in Table 2. From this point on no further experiments using E. cloacae were performed.

## MEASUREMENT OF ERYTHROMYCIN LACTOBIONATE IN THE PERITONEUM OF MICE, AT VARIOUS TIME INTERVALS AFTER INTRAPERITONEAL INOCULATION.

A 0,5 ml. volume of a 3 mgm./ml. solution of erythromycin lactobionate was inoculated into the peritoneum of four

mice. At intervals of Ø, 15, 3Ø and 6Ø minutes a mouse was sacrificed. Immediately after sacrifice, 2 ml. of PBS was introduced into the peritoneum and the abdomen massaged for two minutes. The peritoneal cavity was then opened and the peritoneal wash harvested. The peritoneal fluids were assayed for erythromycin lactobionate. Results are shown in TABLE 4 (page 64).

DETERMINATION OF THE TOXICITY OF ERYTHROMYCIN LACTOBIONATE INOCULATED INTRAPERITONEALLY INTO MICE.

3 groups of six mice, were inoculated with 1,Ø ml. of a 3,Ø, 2,Ø and 1,Ø mg./ml. solutions of erythromycin lactobionate in PBS, intraperitoneally. The result of this experiment is presented on page 63.

INVESTIGATION OF THE EFFECT OF BENZYL ALCOHOL (PRESENT AS A PRESERVATIVE IN ERYTHROMYCIN LACTOBIONATE) ON THE SURVIVAL RATE OF MICE INOCULATED WITH C. albicans.

A group of mice were inoculated with Ø,5 ml. of a Ø.9 mg./ml. suspension of benzyl alcohol in PBS (the effective dose being Ø,45 mg. per mouse) this amount being contained in 1 mg. of erythromycin lactobionate. The control group was inoculated with Ø,5 ml. of PBS. One hour after the above inoculation the two groups of mice were inoculated with C. albicans intraperitoneally. Results are shown in FIGURE 6 (page 66).

## EFFECTS OF ERYTHROMYCIN ADMINISTRATION ON THE SURVIVAL RATE OF MICE INFECTED WITH A LETHAL INTRAPERITONEAL INOCULUM OF C. albicans.

Groups of twenty, two month old , sex-matched NMRI mice weighing approximately 20 gm. were used. The animals in the experimental group were inoculated as follows.

1) LD 50 experiment (C. albicans OD 1,3); intraperitoneal erythromycin inoculation with 1 mg. of erythromycin lactobionate in 0,5 ml of PBS. Results shown in FIGURE 7 (page 67).

2) LD 100 experiment; intraperitoneal erythromycin inoculation with 1 mg. of erythromycin lactobionate in 0,5 ml of PBS. Results shown in FIGURE 8 (page 68).

3) LD 100 experiment; intraperitoneal erythromycin inoculation showing a dose response curve; inoculums consisted of 1 mg. and 0,5 mg, of erythromycin lactobionate respectively in 0,5 ml. of PBS. Results shown in FIGURE 9 (page 69).

4) LD 100 experiment; a series of four experiments; inoculations were by the intravenous route with 0,1 mg. of erythromycin lactobionate in 0,2 ml. of PBS. Results are shown in FIGURES 10a, 10b, 10c and 10d (page 71).

Control mice were inoculated with an equivalent amount of lactobionic acid, in the same volume of inoculum as the test, via the same route as the test.

After a 60 min. delay period the mice were lethally infected intraperitoneally with $2x10^7$ CFU (OD 1,5) of C. albicans.

Prior to each series of experiments the C. albicans were passaged twice in mice, re-isolated, cultured in-vitro, harvested, washed and the OD540 determined and adjusted as previously described prior to injection. The results were recorded as accumulated deaths against time after the lethal inoculation with C. albicans. Solutions were coded and decoded only on completion of the experiment.

Statistical analysis of the results were performed using the Student's t-test for two means.

## INVESTIGATION OF THE EFFECTS OF PRE-INOCULATION WITH ERYTHROMYCIN LACTOBIONATE ON MITOGEN INDUCED MOUSE SPLEEN LYMPHOCYTE TRANSFORMATIONS.

Mice were pre-inoculated with 1 mgm. of erythromycin lactobionate in a volume of 0,5 ml PBS intraperitoneally or 0,1 mgm. of erythromycin lactobionate in 0,2 ml. of PBS intravenously 2 hours before sacrifice. Control mice were inoculated as above, excepting that lactobionic acid was used instead of erythromycin lactobionate.

Spleens were removed aseptically from the sacrificed mice and placed into M199 medium.

Lymphocytes were harvested from the spleens by firstly slicing the spleens, suspending the sliced spleens in about Ø,5 ml of M199 and then grinding the sliced spleens gently in a Thompson Tissue Grinder.
The ground spleen suspensions were made up to a volume of 5ml in M199, layered onto 5 ml of ficoll-hypaque (S.G. 1,1) and centrifuged at 12ØØ RPM for 3Ø min. (242xG). Lymphocytes concentrated at the M199/ficoll-hypaque interface were harvested, washed twice with M199 and suspended in 1 ml of M199.
A lymphocyte count was then performed and the lymphocyte concentration adjusted to $4X1Ø^{6}$ lymphocytes per ml.
Into round bottomed micro-titre trays Ø,1 ml of M199, Ø,Ø2 ml of decomplemented serum and Ø,Ø5 ml. of the lymphocyte cell suspension was added. To the above, Ø,Ø2 ml of either phytohaemagglutinin or concanavalin-A in the following concentrations was added, 6, 12,5, 25, 5Ø, 1ØØ, 2ØØ, 4ØØ and 8ØØ micrograms/ml. A back ground (BG) control consisted of one series of triplicate wells containing the above reagents with the exception that Ø,Ø2 ml of M199 instead of mitogen was added. The trays were then incubated for 36 hours, after which the cells were pulsed with Ø.Ø2 ml of tritiated thymidine (Ø,5 $\mu$ Ci) and incubated for exactly 18 hours, harvested and the radioactivity determined in counts per

minute (CPM) in a beta counter. Triplicate wells were included for each mitogen concentration.

Intraperitoneal results are expressed as the means of four series of tests and are shown in TABLES 6 and 7 (pages 73 and 74).

Intravenous results are shown as above in TABLES 8 and 9 (pages 76 and 77).

## MOUSE AIR BLEB METHOD TO EVALUATE THE IN-VIVO MIGRATION RATE OF PMNLs.

Lawman et al. (1984) described the following technique which is a modification of the techniques of Higginbotham (1968) and Clark et al. (1979).

The procedure consisted of:-

1) A connective tissue air bleb was generated by injecting Ø.9 ml. of air subdermally on the back of a mouse to one side of the midline and away from the neck region.

2) Ø,1 ml of a C. albicans suspension (OD 1,5) was inoculated into the air bleb. C. albicans was used as a leucoattractant instead of FMLP. This was justified as C. albicans is a recognised leucoattractant and was the predominant micro-organism used in this dissertation.

3) At two and four hour intervals after the injection

of the C. albicans suspension, mice were sacrificed and the skin was surgically removed exposing the thin membranous lining of the bleb.

4) The membraneous lining of the bleb was excised and gently stretched onto a coverslip.

5) The membranous lining was fixed in ethanol and stained with Harris's haematoxylin stain.

6) Evaluation was done microscopically in terms of PMNLs per high power field.

Results are dicussed on page 75.

PREPARATON OF HUMAN PMNL SUSPENSION.

PMNLs were isolated from heparinised human venous blood. The blood was diluted 1/2 with PBS, and layered onto ficoll/hypaque. The layered blood-ficoll/hypaque tube was centrifuged at 1200 RPM (290xG) for twenty minutes. The supernatant of the ficoll/hypaque tube was discarded. The RBC/PMNL deposit was resuspended in 20 ml. of a 3% gelatin in PBS solution, and incubated at 37 deg. C for 20 min. The PMNL rich top layer was transferred into a round bottomed 25 ml. container and centrifuged at 1800 RPM (363xG) for 10 min. The supernatant was discarded, the container filled with ammonium chloride solution and the cells resuspended. The cell suspension was incubated at 4 deg. C for 10 min. to lyse the

contaminating RBC and then centrifuged at 1800 RPM (653xG) for 10 min. This deposit consisted of PMNLs. The PMNLs were resuspended in 0,9 ml of Gey's fluid and the final PMNL concentration adjusted as required. This method routinely yielded PMNL suspensions of high purity ( > 95% ) and viability ( > 90% ).

## THE EFFECT ON THE RATE OF % PHAGOCYTIC UPTAKE BY PMNLs OF C. albicans PREVIOUSLY EXPOSED TO ERYTHROMYCIN.

C. albicans was inoculated into NB2 containing 20 $\mu$/Ci of tritiated thymidine (thymidine-methyl-3H, New England Nuclear Corp., Boston, Mass.) per 10 ml. of broth. The above culture was divided into 3 X 20 ml. aliquots, to which was added erythromycin in the following concentrations, 0,0, 5,0 and 30 micro gm./ml. The cultures were incubated for 72 hrs. at 37 degrees C. After incubation the yeasts were washed three times (2000xG for 15 min.) with sterile glucose PBS and the yeast suspension then adjusted to a concentration of $5x10^8$ CFUs/ml. in glucose PBS, by means of a haemacytometer. The yeasts were then pre-opsonized by incubating 0,3 ml. of the yeast suspension with 1,2 ml. of 5% fresh human serum in PBS for thirty minutes at 37 degrees C on a rotator. The yeasts were washed three times (2000xG for 15 min.) with PBS and resuspended to a concentration of $1x10^8$ yeasts/ml. in PBS. A PMNL

suspension was prepared from normal human volunteers as described previously and adjusted to a concentration of $1x10^{7}$ PMNLs/ml. in PBS. The phagocytosis assay consisted of adding $5X10^{5}$ PMNLs to $5X10^{6}$ radiolabelled opsonized yeasts in a volume of 2 ml. PBS. This PMNL/yeast suspension was incubated on a rotator at 37 deg. C. A tube consisting only of $5X10^{7}$ radiolabelled yeasts in 2,0 ml. was processed in parallel with PMNL/organism suspension, to determine the counts per min. (CPM) of the total yeasts used. Aliquots of 0,5 ml. PMNL/organism suspensions were transferred into 2,5 ml. ice cold PBS after 2, 5 and 15 min. incubation, centrifuged (500xG. for 5 min.) to deposit the PMNLs and washed twice to remove non-phagocytosed yeasts. The radioactivity associated with the PMNLs and phagocytosed yeasts were measured in a beta counter. The results are expressed as the percentage of uptake of radiolabelled C. albicans by PMNLs.

$$\% \text{ phagocytosis} = \frac{\text{CPM in PMNL pellet}}{\text{CPM in total yeast pellet}} \times 100$$

The results are presented in TABLE 10 (page 79).

## THE EFFECT OF ERYTHROMYCIN BASE ON INTRA-CELLULAR KILLING OF YEASTS BY HUMAN PMNLs IN-VITRO.

In the study of the intracellular bioactivity of erythromycin, PMNLs from normal individuals were incubated with opsonized C. albicans in the ratio of 1 PMNL:10 CFUs of C. albicans for 30 min at 37 deg. C in a final volume of 1 ml. of Gey's fluid containing $5 \times 10^6$ PMNLs and erythromycin from 0,3 to 20 µgm./ml. The PMNL/yeast/erythromycin mixtures were rotated on a turntable for 30 min. at 37 deg. C. The PMNLs and phagocytosed yeasts were then pelleted and washed by centrifugation at 250 G for 10 min., and the extracellular yeasts were removed. The PMNL pellet containing the phagocytosed C. albicans was suspended in the same erythromycin concentrations after removal of extracellular yeasts. The PMNL/intracellular yeast/antibiotic mixtures were then incubated for 4 hrs. at 37 deg. C, after which the PMNLs were washed three times in PBS to remove the antibiotic. The intracellular yeasts were released by distilled water lysis of PMNLs and 50 µl. of the lysate was inoculated into nutrient broth containing radiolabelled amino acids and incubated for 8 hrs. at 37 deg. C. The yeasts were then washed three times in PBS, and the extent of the incorporation of radiolabelled amino acids into the yeast cells was measured in a liquid scintillation counter. These results are

expressed as radioactive CPM. PMNLs were omitted in identically processed control systems containing yeasts and erythromycin only. The result is discussed on page 78.

## MITOGEN-INDUCED TRANSFORMATION OF HUMAN BLOOD LYMPHOCYTES, AFTER THE ADMINISTRATION OF A 500 mg. DOSE OF ERYTHROMYCIN STEARATE, USING A WHOLE-BLOOD METHOD.

To exclude the "washing-off" effect of cell-preparative procedures a whole blood method for lymphocyte transformation was developed to eliminate the loss of leucocyte-associated erythromycin. The method is identical to that described for pure mouse spleen lymphocytes with the exception that 20 µl. of whole blood was added to each well instead of purified lymphocyte suspension. The method was applied to investigate the effect of oral administration of erythromycin stearate on leucocyte transformation. Blood specimens were taken before and 2 hours after the administration of a single 500 mg. oral dose of erythromycin stearate to 6 adult volunteer subjects (informed consent was obtained and administration of the antibiotic was clinically supervised). Mitogen-induced transformation was performed using 4 concentrations of PHA and CON-A (3, 25, 50, and 100 µg./ml.). Results are shown in TABLES 11 and 12 (pages 81 and 82).

## THE EFFECTS OF THERAPEUTIC CONCENTRATIONS OF ERYTHROMYCIN ON THE MIGRATION OF HUMAN PMNLs IN-VITRO.

PMNLs were purified from human venous blood as described previously and resuspended to a concentration of $6X10^6$ PMNLs per ml. of Hanks balanced salt solution (HBSS) pH 7,4, supplemented with 0,05% bovine serum albumin (BSA). One ml. aliquots of PMNL suspension were incubated at 37 deg. C. for 15 min. followed by the addition of an equal volume of erythromycin base to give final concentrations of 1,75, 3,5, 7,0, 17,5 and 35 µgm./ml. and a final PMNL concentration of $3X10^6$ /ml. Control (antibiotic-free) systems received 1 ml. of HBBS. PMNLs with and without erythromycin were incubated for a further 15 min. at 37 deg. C. PMNL migration was then measured using modified Boyden chambers (Anderson et al. 1981). The upper cell compartment of the chamber was separated from the lower leucoattractant chamber by a 5 µm. pore size nitrocellulose filter (Sartorius Membranfilter, Göttingen FRG). Two hundred µl. of control or erythromycin-treated PMNL suspension were delivered into the upper compartment of each chamber. Into the lower compartments of each chamber 1 ml. of HBSS

containing the leucoattractant endotoxin-activated serum (EAS) was added. Erythromycin was also present in the lower chambers to maintain a constant antibiotic concentration. EAS was generated by incubating 1 ml. of fresh autologous serum with 500 µgm. of bacterial endotoxin for 30 min at 37 deg. C. followed by dilution with HBSS. EAS was used at a final optimum concentration of 10%. The chambers were incubated for 45 min. at 37 deg. C., after which the chambers were removed and immersed in methanol to fix the PMNLs to the filter and to detach the filters. The filters were then stained with haematoxylin, dehydrated in ethanol and cleared in xylene. The filters were then placed on glass microscope slides and the numbers of PMNLs which had traversed from top to bottom in response to the gradient of EAS were enumerated microscopically and the results expressed as PMNLs per microscope high-powered field (PMNL/HPF). EAS was excluded from random migration (background) systems and these values +- erythromycin were subtracted from corresponding EAS systems to give the real values for EAS.
The results are shown in TABLE 13 (page 83).

AN OBJECTIVE, FILTER BASED ENZYMATIC METHOD FOR THE IN-VIVO MEASUREMENT OF THE MIGRATION OF HUMAN POLYMORPHONUCLEAR LEUCOCYTES.

Adult volunteers:- Informed consent was obtained from the volunteers and the study was approved by the Human Investigations and Research Committees of the Faculty of Medicine of the University of Pretoria. Adult male volunteers were used with a mean age of 28 +- 2 years (range 22 - 52).

Blister apparatus:- This is shown in Figure 2. The device was based on that described by Hellum and Solberg (1977) and consists of a suction block with eight outlet pipes, leading into a manifold with individual control valves for each outlet pipe, connected by a Y-tube to a suction pump (Model 13156 pressure/vacuum pump, Gelman Instruments Co, Ann Arbor, Michigan, U.S.A). The transparent plexiglass suction block (9,5cm. x 4,5cm. x 3cm.) contained 8 cylindrical, vertical, polished bores in a parallel, longitudinal arrangement of two rows of 4 bores. Each bore terminated in a shallow circular groove on the lower surface of the suction block. The short airtight outlet pipes were inserted into each bore and firmly attached to plastic connecting tubes leading into the manifold (10cm. x 5cm. x 2,5cm.) which contained 8 control valves to regulate individual blister formation.

Blister-induction:- The volar surface of the forearm was shaved and cleaned with 90% ethanol. The suction block was placed on the mid-volar area and fixed in position with 3 strips of adhesive tape (FIGURE 2). All the control valves on the manifold were fully opened and a suction pressure of 400 mm. of mercury applied and maintained throughout the blistering period. The blistering process was monitored visually through the transparent suction block. As described by Hellum and Solberg (1977) blister formation started at about 75 minutes, as minute blisters which coalesced to form a half spherical bulla as the dermo-epidermal separation reached the circumference of the bore, within 105 minutes. Closure of the appropriate valves slowly released the suction on the formed blisters preventing the first formed blisters from bursting while waiting for slower-forming blisters to develop. When all 8 blisters were formed the suction device was detached. A typical completely-formed blister is shown in FIGURE 3a. Using sterile forceps applied to the base of the blister, the blister fluid, epidermal blister roof and the basement membrane were torn-off at the blister circumference, leaving circular areas of denuded dermis approximately 0,8 cm squared (FIGURE 3b). Attempts were made to obtain bulla on animals with the following results:- pigs, sheep, dogs and rabbits developed haematomas instead of bulla, the baboon was the only animal other than human that produced a bulla

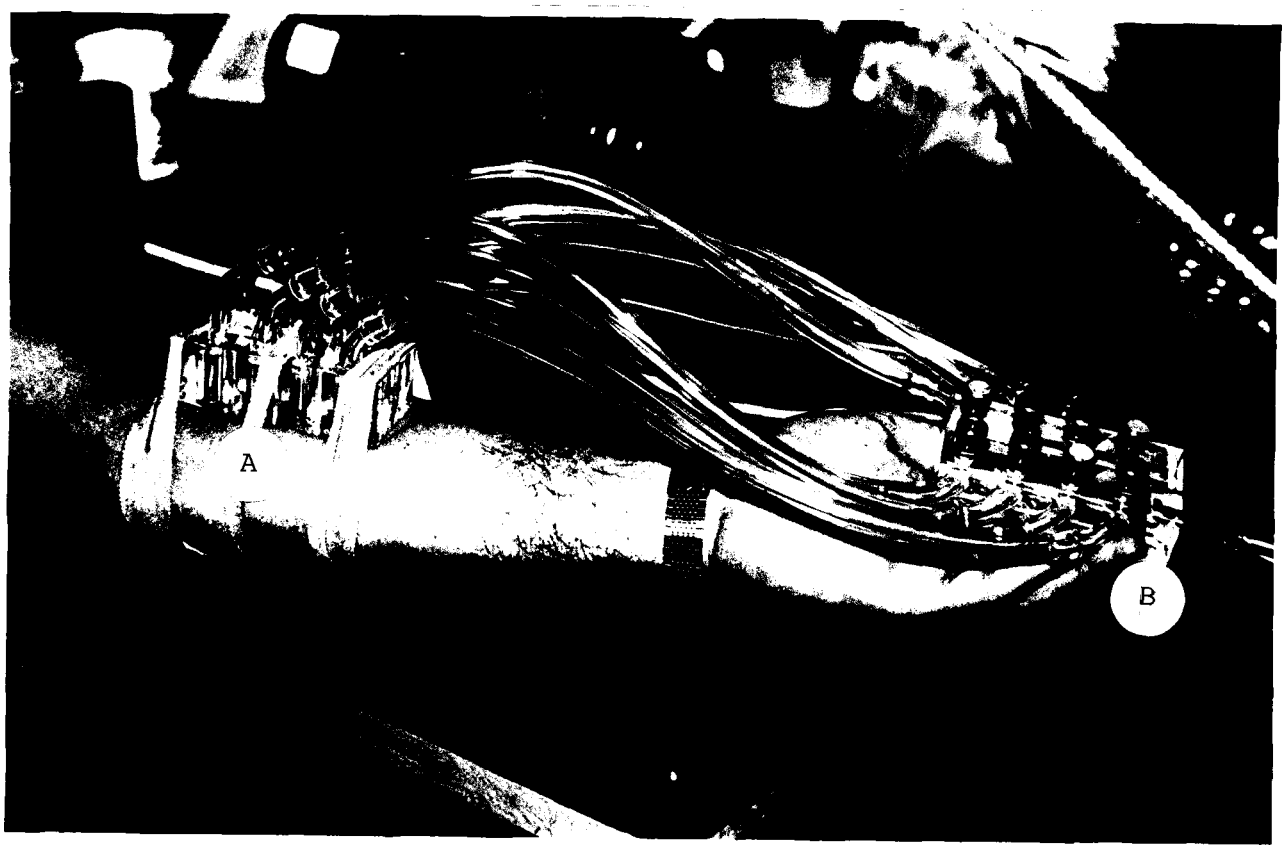



FIGURE 2: Photograph of the suction blister apparatus applied to the upper forearm of an adult volunteer subject. The suction block (A) and manifold (B) with control valves can be easily identified.

when the suction apparatus was placed on the abdomen. The baboon model had to be abandoned as it was necessary to immobilise the baboon with a general anaesthetic. It has been well documented that anaesthetics have an inhibiting effect on PMNL motility.

Measurement of PMNL accumulation:- To standardise the area of denuded dermis adhesive ring reinforcers with a central circular hollow zone of diameter 5 mm. were placed onto each region of exposed dermis as shown in FIGURE 3c. The central exposed areas of dermis were moistened with 50 micro litres of Hanks' balanced salt solution without phenol red (HBSS, Flow Laboratories Ltd., Irvine, Scotland) as shown in FIGURE 3d. A single nitrocellulose filter of porosity 0,2 microns or a double filter combination with pore sizes of 5 microns and 0,2 microns respectively were placed onto the moistened exposed dermis as shown in FIGURE 3e. The filters (13 mm. diameter; 150 microns depth) were obtained from Sartorius Membranfilter Gottingen, FRG. The single 0,2 micron pore size filter was chosen to permit PMNL accumulation on the lower surface of the filter in contact with the dermis with minimal intra-filter penetration of PMNLs. The double filter system was used to increase the surface area available to PMNLs. Filter-adherent PMNLs could thus migrate onto and through the 5 micron pore size filter and be collected on the PMNL impregnable 0,2 micron upper filter.

The synthetic chemotactic tripeptide N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP; Bio-Yeda Ltd, Kiryat Weizman, Rehovot, Israel) was incorporated into 1% agarose (FMC Corporation, Marine Colloids Div., Rockland ,USA) containing Ø,1% bovine serum albumin, to permit slow release of the leucoattractant and maintenance of a positive FMLP gradient through the filters and dermis. FMLP was used at final concentrations of $10^{-6}$ M, $10^{-7}$ M and $10^{-8}$ M. Using <u>in-vitro</u> systems $10^{-8}$ M - $10^{-7}$ M concentrations were found to be the optimum for PMNL migration and $10^{-6}$ M FMLP was supra-optimal. In control systems FMLP was omitted from the agarose. Cylindrical agarose Blocks +- FMLP (15 mm. diameter; 10 mm. depth) were cut and placed onto the nitrocellulose filters (FIGURE 3f). For each FMLP-exposed system a corresponding, laterally-positioned FMLP-free control system was included to give a final arrangement of 1 row of 4 control dermal zones and a parallel row of 4 FMLP-exposed dermal zones. Individual areas of denuded dermis with superimposed filters and agarose blocks +- FMLP were then encased in transparent plexiglass cups (16 mm x 12 mm ) glued firmly to the skin. The filters and agarose blocks were replaced at 2 hour intervals up to 10 hours.

Filter processing for microscopic enumeration of PMNLs:- The nitrocellulose filters were removed and replaced at 2 hour intervals and placed in methanol for

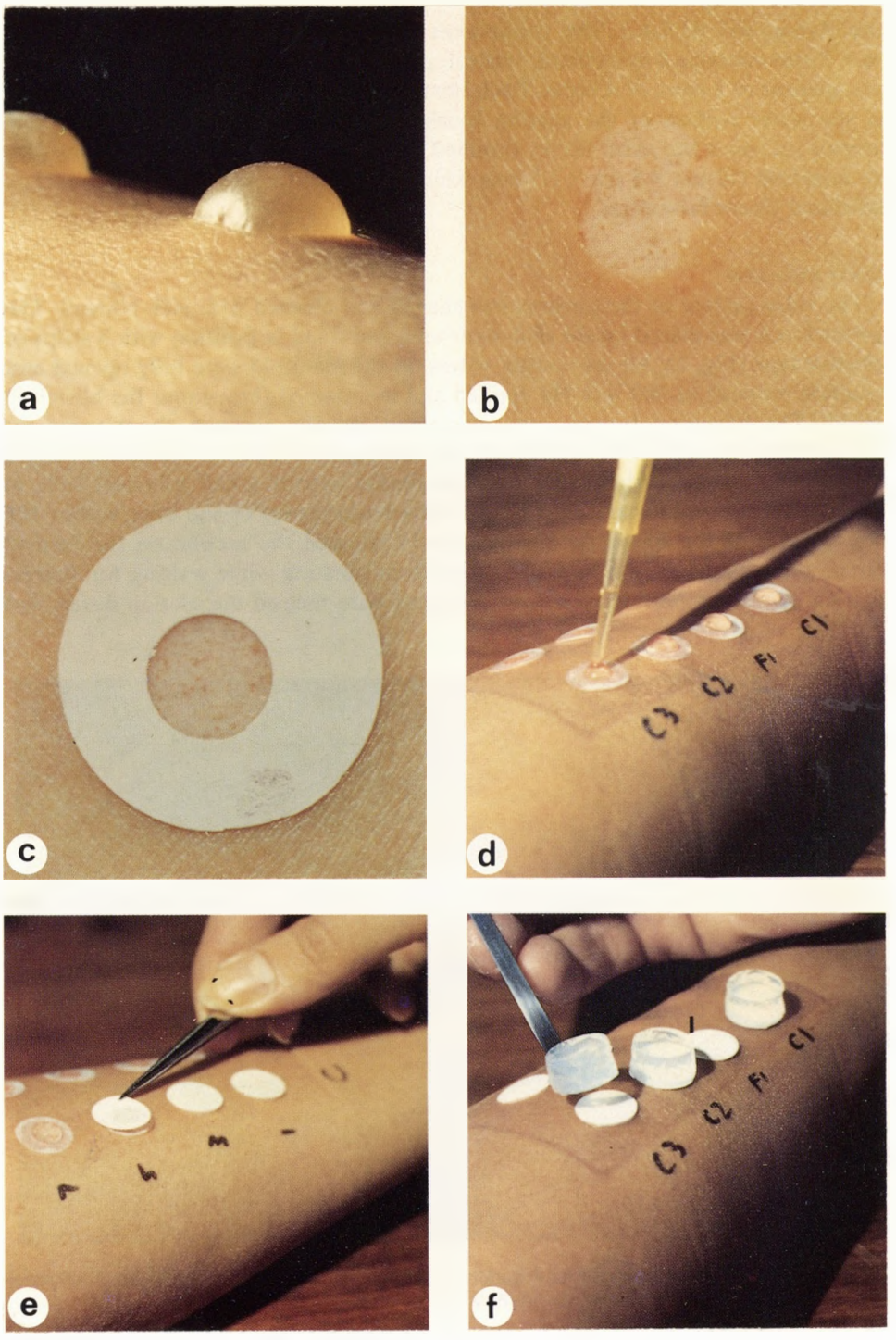



FIGURE 3: (a) A typical blister; (b) the zone of exposed denuded dermis following the removal of epidermis, basement membrane and blister fluid; (c) the placement of the hollow adhesive ring reinforcer onto the dermis; (d) moistening of the dermis with HBSS; (e) the placement of the nitrocellulose filters onto the dermis; and (f) placement of agarose blocks +- FMLP onto the filters.

30 seconds to fix the PMNLs, rinsed in distilled $H_2O_2$ and stained in 1% haematoxylin for 1 minute, rinsed, dehydrated in ethanol and cleared in xylene and examined microscopically for PMNL accumulation.

Enzymatic method for the measurement of PMNL accumulation:- Using this method the filters were placed into 0,8 ml of HBSS only (background control systems), or into 0,8 ml of HBSS containing 0,1 % triton-X 100, in 4,8 cm. x 1 cm. polypropylene test tubes. After 10 minutes the tubes were centrifuged at 500XG for 10 minutes and the control supernatants and triton-X 100 lysates assayed for myeloperoxidase (MPO) and lysozyme content.

MPO assay:- Volumes of 200 micro litres of control supernatant or lysate were transferred to the wells of a microtitre tray and 50 micro litres of MPO substrate added to each well. The substrate was 0,3 % guaiacol (Sigma Chemical Co., St. Louis, Mo., USA) and 0,3 % $H_2O_2$ in HBSS. Enzyme activity was measured spectrophotometrically in a Titertek R multiscan apparatus (Flow Laboratories Ltd.) at OD450 at 1 minute intervals and the OD450/minute calculated.

Lysozyme assay:- 50 micro litre of a 0,8% lysozyme substrate Micrococcus lysodeikticus (Sigma chemical Co.) in HBSS was added to 200 micro litre volume of filter supernatant or lysate in the wells of microtitre trays. The turbidity of the M. lysodeikticus was measured at OD450. Spectrophotometric readings at

OD450 were recorded at time 0 and thereafter at 30 minutes and 60 minutes. The lysozyme activity was measured as the decrease in turbidity of the bacterial suspension.

For both enzymes calibration curves were constructed using horseradish peroxidase type VI (Sigma Chemical Co.) and chicken egg white lysozyme grade 1. To relate MPO and lysozyme activities directly to the PMNL concentration on the filters, pure PMNLs were prepared from blood specimens from normal adult volunteers as previously described (Anderson and Van Rensburg, 1979). The PMNLs at concentrations varying from $5 \times 10^6$ - $1 \times 10^8$ /ml. were spotted onto pre-moistened nitrocellulose filters in 20 micro litre volumes ($1x10^5$ - $2 \times 10^6$ PMNLs and transferred into HBSS +- triton-X 100 and the MPO and lysozyme concentrations in the lysates measured as above. Calibration curves were drawn of enzyme activity vs PMNL concentration.

Other investigations:- Histological examination of the detached epidermal blister roof and basement membrane from different volunteers and total and differential blood leucocyte counts were performed by the Departments of Histopathology and Haematology of the Institute for Pathology, University of Pretoria, respectively.

Results are presented on page 84.

## THE EFFECTS OF ORAL ADMINISTRATION OF ERYTHROMYCIN ON THE HUMAN IN-VIVO MIGRATION OF PMNLs USING THE ABOVE METHOD.

A single 500 mg. oral dose of erythromycin stearate or a matched placebo were administered to 4 pairs of age and sex-matched healthy adult human volunteers. The erythromycin and placebo tablets were coded and administered by an attendant clinician. Following the completion of blister formation as described previously (page 48) PMNL migration was then measured in erythromycin and placebo treated volunteers. Results are shown in TABLE 15 (page 93).

## EXPRESSION AND STATISTICAL ANALYSIS OF RESULTS.

The results are expressed as the mean value and standard error of the mean for each series of experiments. Statistical analyses were performed using the paired Students t test and Students t test for two means.

# RESULTS.

# CHAPTER III.

## THE DETERMINATION OF MIC AND MCC OF ERYTHROMYCIN FOR *E. cloacae* AND *C. albicans*.

As shown in TABLE 1 the MIC's and MCC's for *E. cloacae* and *C. albicans* were 3,Ø mgm./ml. and >6,Ø mgm./ml. respectively.

The method was as described on page 32.

## RELATIONSHIP OF OD TO CFU.

FIGURES 4 and 5 show a linear OD/CFU relationship for *E. cloacae* and *C. albicans* within the CFU range used.

The above graphs were used to convert the OD of *E. cloacae* and *C. albicans* to CFUs.

The method was as described on page 32.

## THE EFFECTS OF ERYTHROMYCIN ON THE IN-VITRO GROWTH RATE OF *E. cloacae* AND *C. albicans* IN THE PRESENCE OF ERYTHROMYCIN USING A TURBIDOMETRIC METHOD.

TABLE 2 shows that there is no effect on *C. albicans* when co-incubated with erythromycin up to a concentration of 3Ø µg./ml.

TABLE 3 shows that although *E. cloacae* was resistant to erythromycin, in a clinical sense, co-incubating *E. cloacae* with erythromycin at a concentration of 7,5 µg./ml. retarded the growth rate of *E. cloacae*.

The method was as described on page 34.

TABLE 1

THE DETERMINATION OF MIC AND MCC OF ERYTHROMYCIN FOR *E. cloacae* AND *C. albicans*.

| | MIC | MCC |
|---|---|---|
| *E. cloacae* | 3,Ø mgm./ml. | 3,Ø mgm./ml. |
| *C. albicans* | > 6,Ø mgm./ml. | > 6,Ø mgm./ml. |

The above results indicate that *E. cloacae* and especially *C. albicans* are resistant to erythromycin.

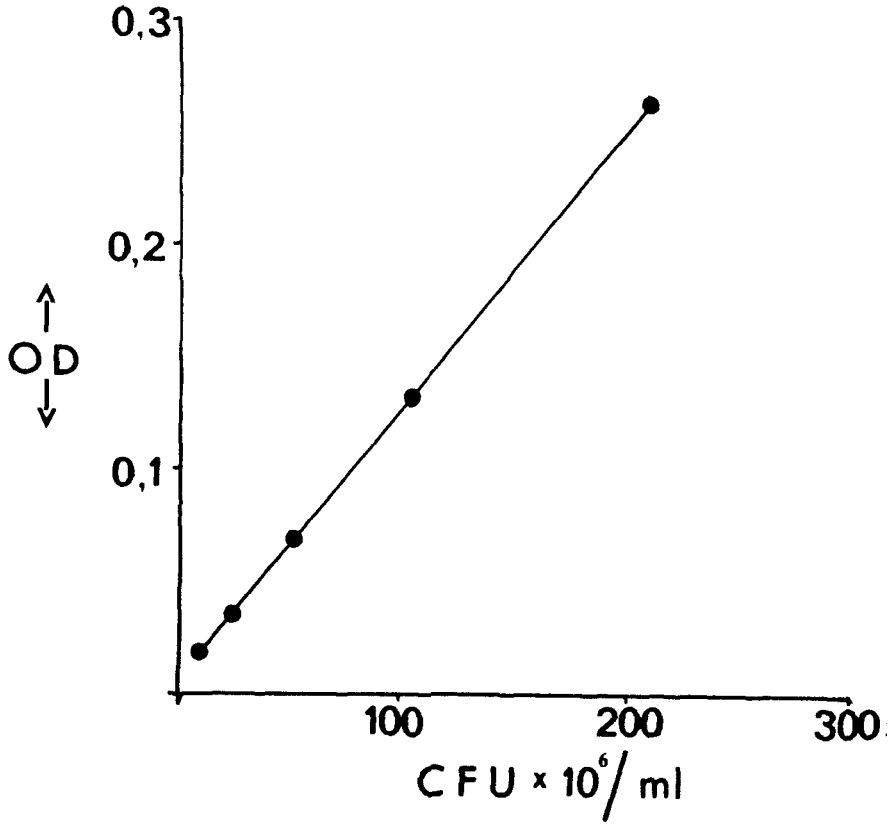



FIGURE 4.

Demonstration of the linear CFU/OD relationship of E. cloacae.

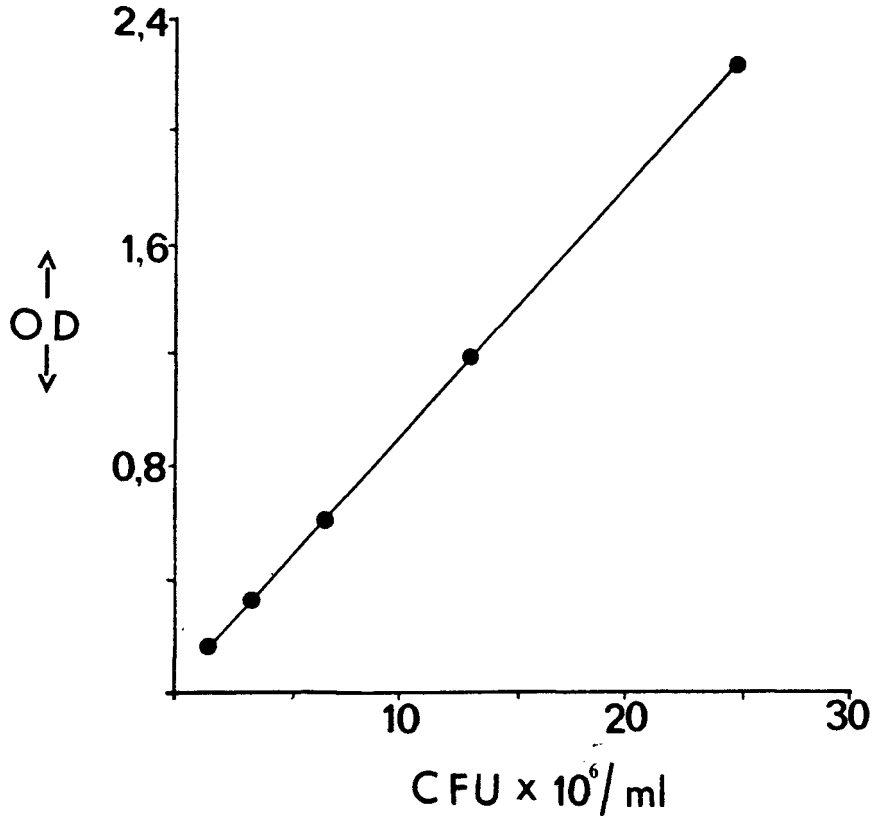



FIGURE 5.

Demonstration of the linear CFU/OD relationship of C. albicans.

TABLE 2

THE EFFECT OF ERYTHROMYCIN ON THE IN-VITRO GROWTH RATE OF *E. cloacae* IN THE PRESENCE OF ERYTHROMYCIN USING A TURBIDOMETRIC METHOD.

| Time interval | OD No erythromycin | OD 7,5 µg/ml. erythromycin | OD 30 µg./ml. erythromycin |
|---|---|---|---|
| 18 hrs. | 0,301+-(.005)* | 0,177+-(.002) | 0,154+-(.006) |

* Results are the mean value +- SEM of 3 assays.

TABLE3

THE EFFECT OF ERYTHROMYCIN ON THE IN-VITRO GROWTH RATE OF C. albicans IN THE PRESENCE OF ERYTHROMYCIN USING A TURBIDOMETRIC METHOD.

| Time intervals | OD No erythromycin | OD 7,5 µg/ml. erythromycin | OD 30 µg./ml. erythromycin |
|---|---|---|---|
| 16 hrs. | 0,047 +-.006 * | 0,050 +-.001 | 0,056 +-.002 |
| 24 hrs. | 0,072 +-.016 | 0,072 +-.005 | 0,084 +-.002 |
| 42 hrs. | 0,333 +-.020 | 0,333 +-.021 | 0,331 +-.025 |

* Results are the mean values +- standard error of the mean (SEM) of three assays.

## DETERMINATION OF THE QUANTITY OF ERYTHROMYCIN LACTOBIONATE IN THE PERITONEUM OF MICE AFTER INTRAPERITONEAL INOCULATION WITH ERYTHROMYCIN LACTOBIONATE.

TABLE 4 shows that the residual amount of erythromycin lactobionate present in the peritoneal wash (2 ml.) 60 min. after a mouse has been inoculated with 0,5 ml. of a 3 mg./ml. of erythromycin lactobionate solution intraperitoneally, was less than 0,002 mg./ml. (volume of wash was taken into account).

Taking into consideration the results obtained in the investigation of the effects on the in-vitro growth of C. albicans in the presence of erythromycin (TABLE 3), one can assume that the erythromycin dose given to the mice prior to inoculation with C. albicans did not affect the C. albicans inoculum in a direct manner.

The method was as described on page 35.

## DETERMINATION OF THE TOXICITY TO MICE OF ERYTHROMYCIN LACTOBIONATE WHEN INOCULATED INTRAPERITONEALLY.

Intraperitoneal administration of erythromycin lactobionate at a concentration of up to 3 mgm. was not associated with toxic effects.

The method was as described on page 36.

TABLE 4

DETERMINATION OF THE QUANTITY OF ERYTHROMYCIN LACTOBIONATE IN THE PERITONEUM OF MICE AT DIFFERENT TIME INTERVALS AFTER INTRAPERITONEAL INOCULATION OF ERYTHROMYCIN LACTOBIONATE.

| Peritoneal wash at stated times | Erythromycin lactobionate in the peritonial wash. mg./ml. |
|---|---|
| 0 min. | 0,425 +- 0,013 * |
| 15 min. | 0,01 +- 0,007 |
| 30 min. | <0,001 |
| 60 min. | <0,001 |

* Results are the mean values +- SEM of three determinations.

## DETERMINATION OF THE EFFECT OF BENZYL ALCOHOL ON THE SURVIVAL RATE OF MICE INOCULATED WITH *C. albicans*.

FIGURE 6 shows that the presence of benzyl alcohol in erythromycin lactobionate did not influence the survival rate of mice inoculated with a lethal dose of *C. albicans*.

The method was as described on page 36.

## THE EFFECTS OF INTRAPERITONEAL ADMINISTRATION OF ERYTHROMYCIN LACTOBIONATE ON THE SURVIVAL RATE OF MICE INOCULATED WITH A LETHAL INTRAPERITONEAL INOCULUM OF *C. albicans*.

FIGURES 7, 8 and 9 show a significant increase of the mean survival rate of mice pre-inoculated with erythromycin lactobionate relative to their controls inoculated with lactobionic acid.

Furthermore, FIGURE 9 shows a dose response curve to erythromycin lactobionate.

The method was that as described on page 37.

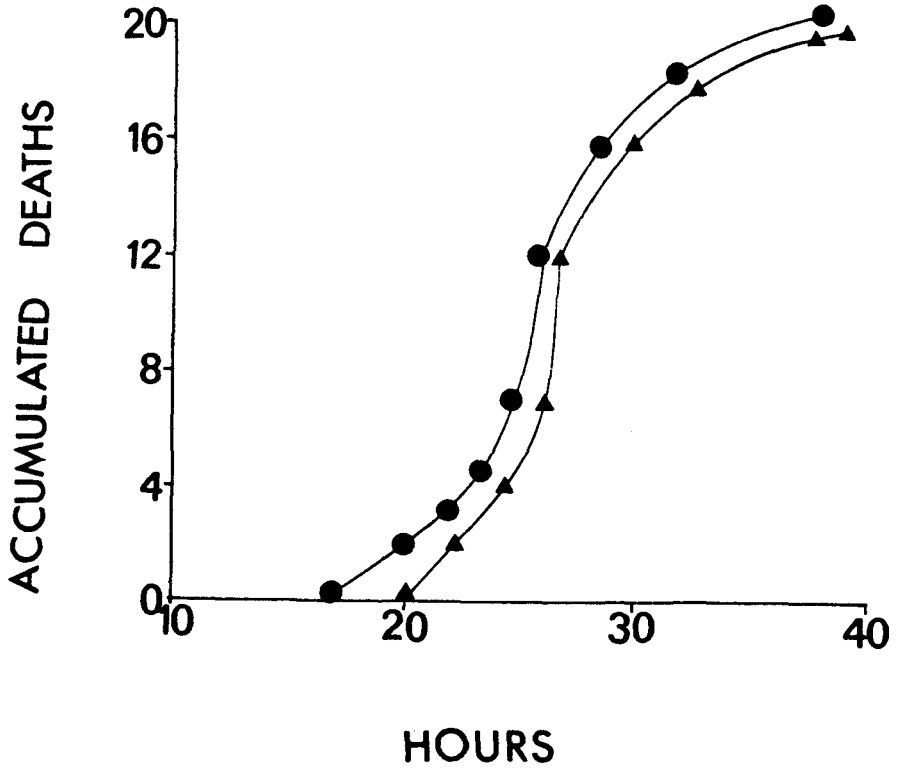



FIGURE 6: Determination of the effect of benzyl alcohol on the survival rate of mice inoculated with a lethal dose of C. albicans.

Benzyl alcohol (▲——▲). PBS (●——●).

Using students t Test for two means t = Ø,33

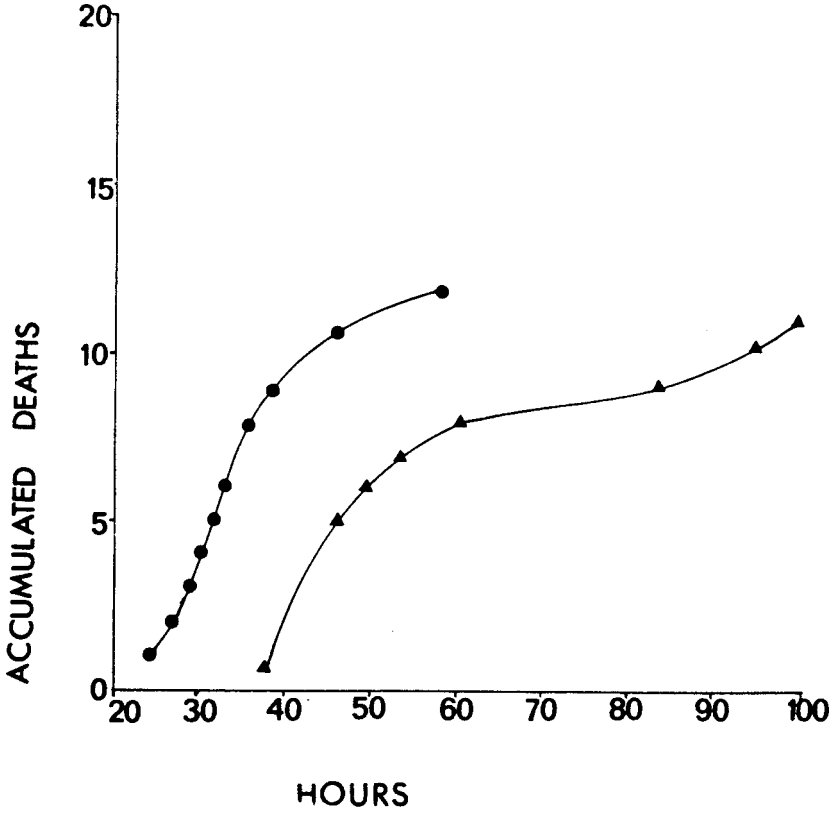



FIGURE 7: Mice inoculated intraperitoneally with 1 mg. of erythromycin and challenged with a LD50 inoculum of C. albicans.

(▲——▲) erythromycin lactobionate.

(●——●) lactobionic acid.

Using Students t Test for two means t = 3,37

P < 0,005.

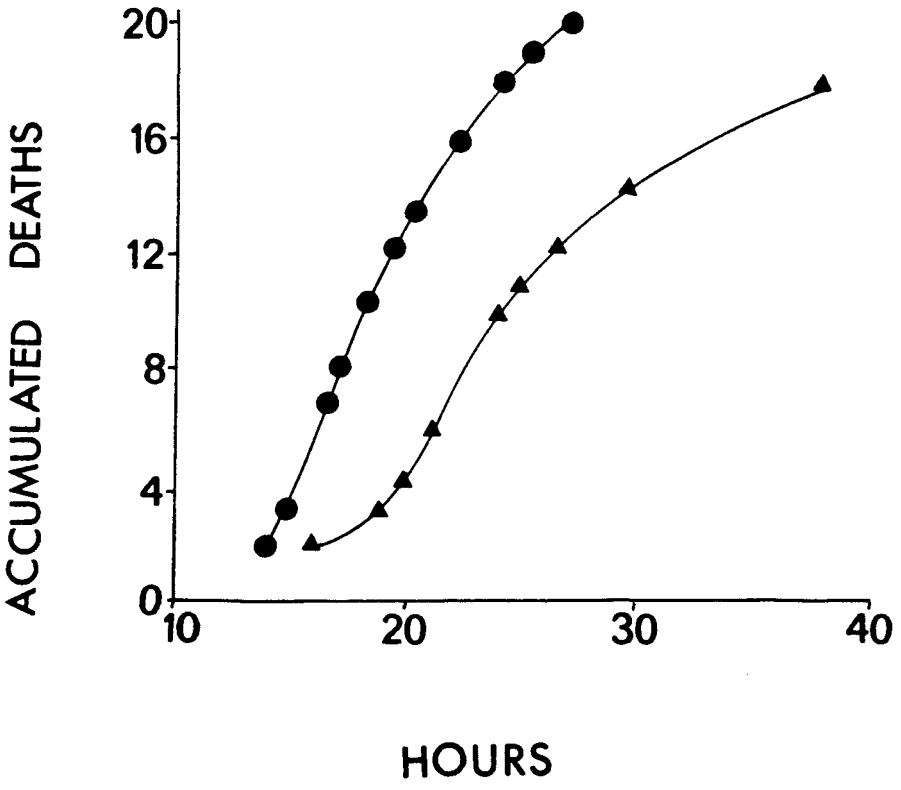



FIGURE 8: Mice inoculated intraperitoneally with 1 mg. of erythromycin and challenged with a LD100 inoculum of C. albicans.

(▲——▲) erythromycin lactobionate.

(●——●) lactobionic acid.

Using Students t Test for two means t = 3,37

P < 0,005.

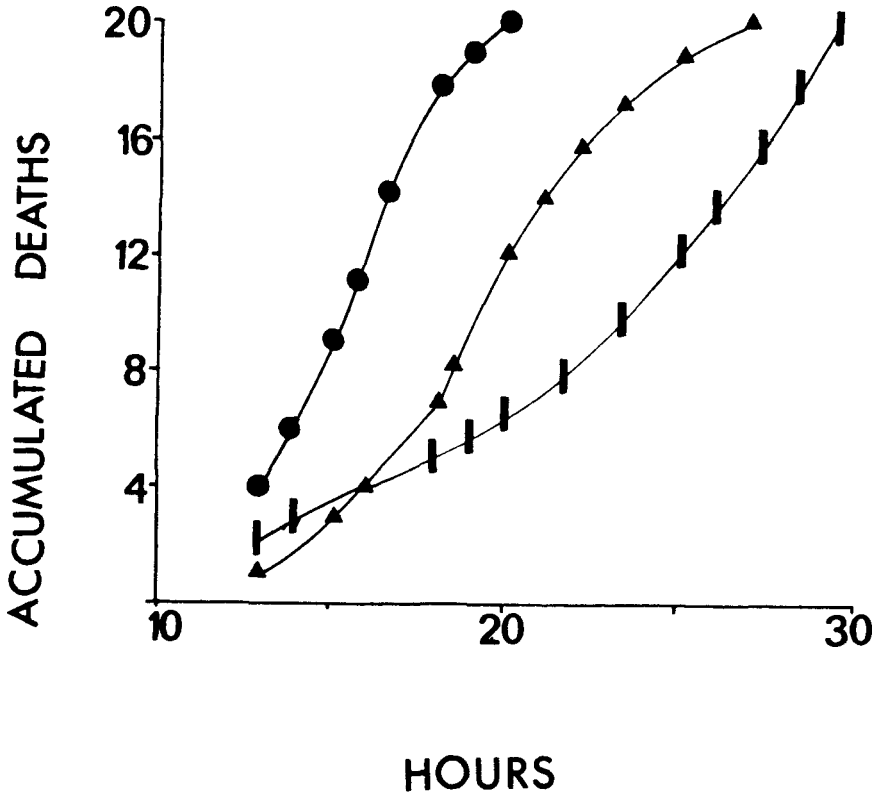



FIGURE 9: Mice inoculated intraperitoneally with 1 mg. and Ø,5 mg. of erythromycin and challenged with a LDIØØ inoculum of C. albicans.

(▌——▌) 1,Ø mg. erythromycin lactobionate.

(▲——▲) Ø,5 mg. erythromycin lactobionate.

(●——●) lactobionic acid.

Using Students t Test for two means:-

1,Ø mg. erythromycin lactobionate t = 5,3; P < Ø,ØØ5

Ø,5 mg. erythromycin lactobionate t = 4,Ø; P < Ø,ØØ5

THE EFFECTS OF INTRAVENOUS ADMINISTRATION OF ERYTHROMYCIN LACTOBIONATE ON THE SURVIVAL RATE OF MICE INOCULATED WITH A LETHAL INTRAPERITONEAL INOCULUM OF C. ALBICANS.

FIGURES 10a, 10b, 10c, 10d and their statistical analysis shown in TABLE 5 show that the intravenous inoculation of 100 µgm. erythromycin lactobionate causes a significant increase in the mean survival time of mice inoculated with a lethal intraperitoneal inoculum of C. albicans.

The method was as described on page 37.

MITOGEN-INDUCED TRANSFORMATIONS OF MOUSE SPLEEN LYMPHOCYTES; MICE WERE PRE-INOCULATED INTRAPERITONEALLY WITH 1 MG. OF ERYTHROMYCIN LACTOBIONATE 2 HOURS PRIOR TO SACRIFICE.

TABLES 6 and 7 show that neither mitogens PHA nor CON-A enhanced the lymphocyte transformation of mouse spleen lymphocytes when erythromycin lactobionate was inoculated intraperitoneally.

The method was that as described on page 38.

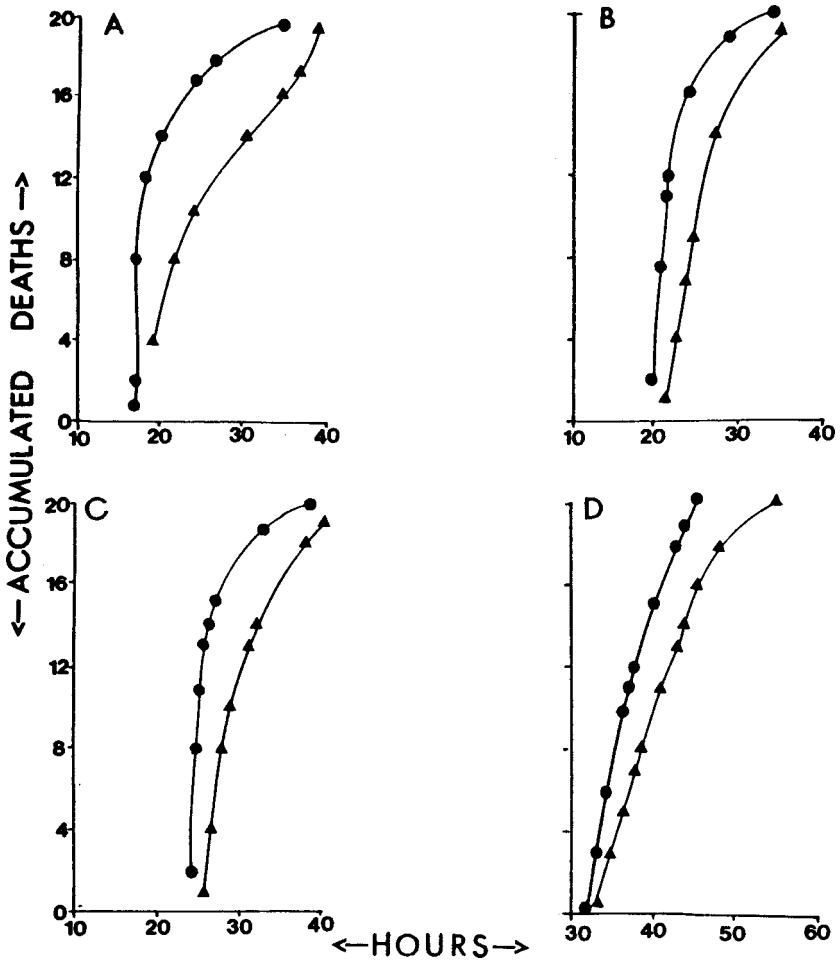



FIGURE 10a, 10b, 10c and 10d.

The effects of intravenous administration of 100/ugm. of erythromycin lactobionate on the survival rate of mice inoculated with a lethal intraperitoneal inoculum of C. albicans.

(▲----▲) erythromycin lactobionate.

(●----●) lactobionic acid.

TABLE 5

STATISTICAL ANALYSIS OF

FIGURES 10a, 10b, 10c and 10d.

| FIGURE | 10a | 10b | 10c | 10d |
|---|---|---|---|---|
| Students t Test for two means | 1,9 | 2,5 | 2,99 | 2,46 |
| P values | <0,05 | <0,025 | <0,005 | <0,01 |

TABLE 6

PHA-INDUCED TRANSFORMATIONS OF MOUSE SPLEEN LYMPHOCYTES; MICE WERE PRE-INOCULATED INTRAPERITONEALLY WITH 1 MG. OF ERYTHROMYCIN LACTOBIONATE 2 HOURS PRIOR TO SACRIFICE.

| PHA concentration, micro gm./ml. | Lactobionic acid. | Erythromycin lactobionate. | t value. | P value. |
|---|---|---|---|---|
| 6 | 3998 +- 839 * | 11230 +- 5596 | 1,629 | >0,05 |
| 12 | 11381 +- 5397 | 18876 +- 1165 | 0,613 | >0,05 |
| 25 | 23438 +- 10633 | 33180 +- 10804 | 0,841 | >0,05 |
| 50 | 13487 +- 9244 | 21760 +- 11148 | 0,371 | >0,05 |
| 100 | 6310 +- 5386 | 14862 +- 7171 | 0,962 | >0,05 |
| 200 | 4850 +- 4186 | 8890 +- 5264 | 0,629 | >0,05 |
| 400 | 2766 +- 2356 | 2892 +- 1057 | 0,056 | >0,05 |
| 800 | 797 +- 387 | 628 +- 169 | 0,574 | >0,05 |
| BG | 452 +- 135 | 420 +- 122 | - | - |

BG = background.

* Results of TABLE 6 are shown as the means +- SEM of four sets of experiments.

t Value was obtained by using Students t Test for matched groups.

TABLE 7

CON-A INDUCED TRANSFORMATIONS OF MOUSE SPLEEN LYMPHOCYTES; MICE WERE PRE-INOCULATED INTRAPERITONEALLY WITH 1 MG. OF ERYTHROMYCIN LACTOBIONATE 2 HOURS PRIOR TO SACRIFICE.

| CON-A concentration micro gm./ml. | Lactobionic acid | Erythromycin lactobionate | t value. | P value. |
|---|---|---|---|---|
| 6 | 399 +- 57 | 277 +- 20 | 0,592 | >0,05 |
| 12 | 509 +- 215 | 750 +- 443 | 1,01 | >0,05 |
| 25 | 522 +- 98 | 1160 +- 636 | 1,093 | >0,05 |
| 50 | 830 +- 658 | 2914 +- 1558 | 1,355 | >0,05 |
| 100 | 9258 +- 3923 | 6396 +- 3612 | 0,378 | >0,05 |
| 200 | 16897 +- 7868 | 16918 +- 9221 | 0,002 | >0,05 |
| 400 | 26246 +- 10905 | 29761 +- 11907 | 0,234 | >0,05 |
| 800 | 3610 +- 1351 | 7137 +- 3609 | 0,810 | >0,05 |
| BG | 400 +- 182 | 389 +- 172 | | |

BG = background.

* Results of TABLE 7 are shown as the means +- SEM of four sets of experiments.

t Value was obtained by using Students t Test for matched groups.

MITOGEN-INDUCED TRANSFORMATION OF MOUSE SPLEEN LYMPHOCYTES; MICE WERE PRE-INOCULATED INTRAVENOUSLY WITH 100 /UGM OF ERYTHROMYCIN LACTOBIONATE 2 HOURS PRIOR TO SACRIFICE.

TABLES 8 and 9 show that there is a significant difference between the spleen lymphocyte transformations of mice pre-inoculated intravenously with erythromycin lactobionate and controls inoculated with lactobionic acid at the following mitogen concentrations.

PHA 6, 12, and 25 /ugm./ml.

CON-A 100 /ugm./ml.

Method was as described on page 38.

EVALUATION OF THE AIR BLEB METHOD TO DETERMINE THE IN-VIVO MIGRATION RATE OF PMNLs GENERATED BY C. albicans.

Microscopic observations showed that the C. albicans inoculum did elicit a chemotactic infiltration of leucocytes into the membraneous lining of the bleb, the predominating infiltrating cells were microscopically determined as being PMNLs. It was observed however that the distribution of the leucocytes in the membranous lining of the bleb was irregular and the

TABLE 8

PHA-INDUCED TRANSFORMATION OF MOUSE SPLEEN LYMPHOCYTES; MICE WERE PRE-INOCULATED INTRAVENOUSLY WITH 100/UGM. OF ERYTHROMYCIN LACTOBIONATE 2 HOURS PRIOR TO SACRIFICE.

| PHA concentration, micro gm./ml. | Lactobionic acid. | Erythromycin lactobionate. | t value. | P value. |
|---|---|---|---|---|
| 6 | 606 +- 130 | 1413 +- 434 | 3,080 | <0,05 |
| 12 | 6533 +- 4732 | 12304 +- 3712 | 2,581 | <0,05 |
| 25 | 8304 +- 5296 | 19631 +- 6373 | 5,782 | <0,01 |
| 150 | 4957 +- 3415 | 13366 +- 7869 | 1,885 | >0,05 |
| 100 | 1615 +- 1032 | 7726 +- 6431 | 1,131 | >0,05 |
| 200 | 610 +- 71 | 2617 +- 2273 | 0,888 | >0,05 |
| 400 | 354 +- 45 | 436 +- 147 | 0,456 | >0,05 |
| 800 | 321 +- 37 | 335 +- 68 | - | - |
| BG | 319 +- 64 | 336 +- 130 | | |

BG = Background.

Results are expressed as the means +- SEM of four sets of experiments.

t Value was obtained by using Students t Test for matched groups.

TABLE 9

CON-A INDUCED TRANSFORMATION OF MOUSE SPLEEN LYMPHOCYTES; MICE WERE PRE-INOCULATED INTRAVENOUSLY WITH 100/UGM. OF ERYTHROMYCIN LACTOBIONATE 2 HOURS PRIOR TO SACRIFICE.

| CON-A concentration micro gm./ml. | Lactobionic acid. | Erythromycin lactobionate. | t value. | P value. |
|---|---|---|---|---|
| 6 | 408 +- 98 | - | - | |
| 12 | 740 +- 81 | 1171 +- 674 | 1,325 | >0,05 |
| 25 | 2722 +- 265 | 2952 +- 1517 | 1,712 | >0,05 |
| 50 | 5725 +- 1336 | 9564 +- 3864 | 2,092 | >0,05 |
| 100 | 6709 +- 3880 | 11570 +- 5733 | 3,057 | <0,05 |
| 200 | 1601 +- 4018 | 9504 +- 5265 | 2,183 | >0,05 |
| 400 | 1136 +- 615 | 1343 +- 377 | 1,103 | >0,05 |
| 800 | - | 429 +- 156 | - | |
| BG | 298 +- 78 | 339 +- 136 | | |

BG = background.

Results are expressed as the means +- SEM of four sets of experiments.

t Values were obtained by using Students t Test for matched groups.

concentrated areas appeared to be associated with adjacent arterioles or veinioles. The insurmountable difficulties encountered in trying to modify this method from a qualitative to a quantitative method caused its abandonment.

The method was as described on page 40.

## THE EFFECT ON THE RATE OF % PHAGOCYTIC UPTAKE BY PMNLs OF C. albicans PREVIOUSLY EXPOSED TO ERYTHROMYCIN.

TABLE 1Ø shows that growing C. albicans in the presence of erythromycin does not influence the % phagocytic uptake by PMNLs.

The method as described on page 42.

## THE EFFECT OF ERYTHROMYCIN BASE ON INTRA-CELLULAR KILLING OF YEASTS BY HUMAN PMNLs IN-VITRO.

Using the method as described on page 44, it was found that erythromycin base had no significant effect on the intracellular killing of yeasts by PMNLs.

TABLE 10

THE EFFECT ON THE RATE OF % PHAGOCYTIC UPTAKE BY PMNL OF *C. albicans* PREVIOUSLY EXPOSED TO ERYTHROMYCIN.

| Times harvested | No eryth. | Eryth. 5,0 micro. gm./ml. | Eryth. 30,0 micro. gm./ml. |
|---|---|---|---|
| | % uptake | % uptake | % uptake |
| 2 min. | 48 +- 4* | 41 +- 5 | 41 +- 6 |
| 5 min. | 50 +- 5 | 48 +- 5 | 46 +- 5 |
| 15 min. | 48 +- 4 | 48 +- 6 | 44 +- 4 |

* Results are expressed as the means +- SEM of six determinations.

THE EFFECT OF THE ADMINISTRATION OF A 500 MG. DOSE OF ERYTHROMYCIN STEARATE ON MITOGEN INDUCED TRANSFORMATION OF HUMAN LYMPHOCYTES CONTAINED IN WHOLE BLOOD.

TABLE 11 shows a significant increase in the PHA induced transformation between controls and tests of human peripheral lymphocytes at concentrations of 50 and 25 µgm.

TABLE 12 shows no significant increase in the CON-A induced transformations between controls and tests.

The method was as described on page 45.

THE EFFECT OF THERAPEUTIC CONCENTRATIONS OF ERYTHROMYCIN ON THE MIGRATION OF HUMAN PMNLs IN VITRO.

TABLE 13 shows that erythromycin base at concentrations of 3,5 µgm./ml. and greater caused a significant enhancement of PMNL migration to EAS.

The method was as described on page 46.

TABLE 11

THE EFFECT OF THE ADMINISTRATION OF A 500 MG. DOSE OF ERYTHROMYCIN STEARATE ON PHA INDUCED TRANSFORMATION OF HUMAN LYMPHOCYTES CONTAINED IN WHOLE BLOOD.

| PHA conc. | Before administration of erythromycin stearate. | After administration of erythromycin stearate. | t value. | P value |
|---|---|---|---|---|
| BG | 2180,0 +- 802,4 | 871,8 +- 245,0 | 1,91 | >,05 |
| 100 | 27310,0 +- 3098,4 | 34121,3 +- 15259,4 | 1,58 | >,05 |
| 50 | 34064,2 +- 15233,9 | 46728,3 +- 20897,5 | 2,17 | <.05 |
| 25 | 35889,0 +- 6831,2 | 47126,8 +- 21075,7 | 3,40 | <.01 |
| 3,1 | 18724,0 +- 8373,6 | 28931,8 +- 12938,7 | 1,46 | >.05 |

PHA conc. = PHA Concentration in micro gm./ml.

BG = Background

The above results are the means, +- SEM, Students t test for matched groups and P values of 6 sets of experiments.

TABLE 12

THE EFFECT OF THE ADMINISTRATION OF A 500 MG. DOSE OF ERYTHROMYCIN STEARATE ON CON-A INDUCED TRANSFORMATION OF HUMAN LYMPHOCYTES CONTAINED IN WHOLE BLOOD.

| CON-A conc. | Before administration of erythromycin stearate. | After administration of erythromycin stearate. | t value. | P value. |
|---|---|---|---|---|
| BG | 615,0 +- 355,1 | 716,3 +- 413,5 | 0.80 | >,05 |
| 100 | 24429,0 +- 10925,0 | 19265,7 +- 8616,9 | 0,91 | >,05 |
| 50 | 15097,7 +- 6752.0 | 11989,3 +- 5361,8 | 0,92 | >.05 |
| 25 | 6833,7 +- 3056,1 | 5874,0 +- 2627,0 | 0,68 | >,05 |
| 3,1 | 1157,0 +- 517,4 | 1059,7 +- 474,0 | 0,47 | >.05 |

CON-A conc. = CON-A concentration in micro gm./ml.

BG = Background

The above results are the means, +- SEM, Student t test for matched groups and P values of 6 sets of experiments.

TABLE 13

THE EFFECT OF THERAPEUTIC CONCENTRATONS OF ERYTHROMYCIN ON THE MIGRATION OF HUMAN PMNLS IN VITRO.

Percentage enhancement of PMNL chemotaxis to the leucoattractant (endotoxin-activated serum) by erythromycin.

| Erythromycin Concentration µgm./ml. | Percentage stimulation of PMNL migration.* | P value.+ |
|---|---|---|
| 3,5 | 22% | <Ø,Ø1 |
| 7,Ø | 34% | <Ø,Ø1 |
| 17,5 | 52% | <Ø.ØØ5 |
| 35,Ø | 45% | <Ø,Ø25 |

* By comparison with the untreated control value.

\+ Paired Students *t*-statistics.

## THE FILTER BASED, ENZYMATIC METHOD FOR THE IN-VIVO MEASUREMENT OF THE MIGRATION OF HUMAN POLYMORPHONUCLEAR LEUCOCYTES.

Skin blistering:- The initial attempts to form suitable blisters without using the manifold containing the valves to regulate blister formation, were unsuccessful because of asynchronous blister formation. The first blisters formed, either burst or formed intradermal haemorrhage while awaiting completion of the slower forming blisters. With the development and use of the manifold containing the valves, standardised blister formation could be attained.

Measurement of PMNL migration:- PMNL accumulation on the nitrocellulose filters was measured microscopically and enzymatically after 2, 4, 6, 8, and 10 hrs. Microscopic enumeration of stained PMNLs on processed filters was found to be quite unsuitable for quantitative studies due to the cell density on the lower side of the filter (in contact with the skin) and the irregular distribution of PMNLs on the upper and lower surfaces of the 5 /um. and 0,2 /um. pore size filters respectively when the double filter system was used. Enzymatic measurement of MPO and lysozyme activities was however found to be objective and reproducible.

Filter requirement:- In initial studies PMNL accumulation on single Ø,2 µm. pore size filters was compared with the double filter system using a lower 5 µm. (in contact with the dermis) and an upper Ø,2 µm. pore size filter. These studies were performed on 3 different individuals with duplicate control and FMLP-exposed blisters for both the single and double filter systems per person. A single FMLP concentration of $-10^{-6}$ M was used for these studies. Measurable PMNL accumulation occurred after 4 hrs. and was maximal at 6-1Ø hrs. Significantly greater PMNL accumulation was achieved using the double filter system especially with FMLP. This probably reflects the greater surface area available to the PMNL. These results for MPO are shown in FIGURE 11. Lysozyme results (not shown) were similar.

FMLP requirement:- The optimal FMLP concentration was established using 3 different volunteers. Duplicate areas of denuded dermis was exposed to agarose blocks only (control system) or agarose blocks containing $-10^{-8}$ M, $-10^{-7}$ M and $-10^{-6}$ M FMLP respectively. The double filter system was used and PMNL accumulation on the filters measured after 2, 4, and 6 hrs. These results for MPO after 6 hrs. are shown in FIGURE 12. FMLP exposure promoted a dose dependent increase in PMNL accumulation on the filters. $-10^{-6}$ M was found to be the optimal concentration of the leucoattractant.

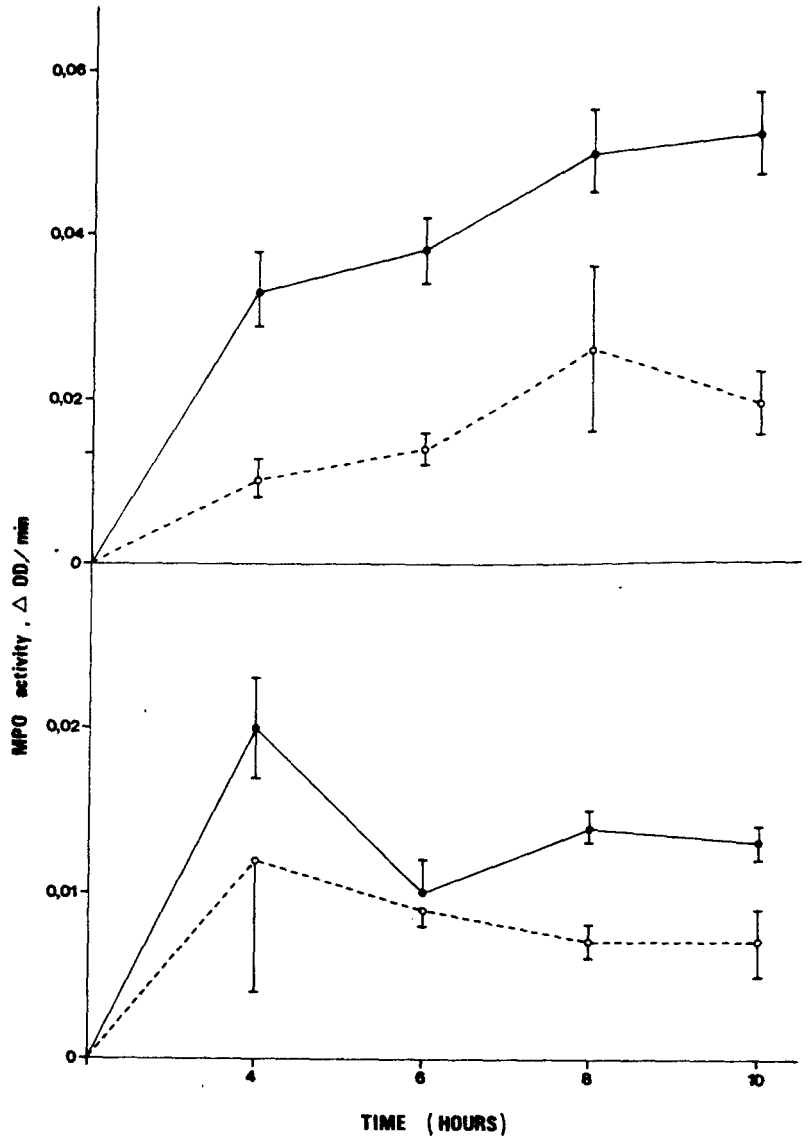



FIGURE 11. In the lower graph PMNL accumulation in control systems onto a single Ø,2 µm. pore size nitrocellulose filter (○----○) or a combination of Ø,2 µm. and 5 µm. pore size filters (●——●). In the upper graph the corresponding results are shown for FMLP-exposed systems using single (○----○) and double filters (●——●). Results are expressed as the mean value +- SEM for 3 different individuals.

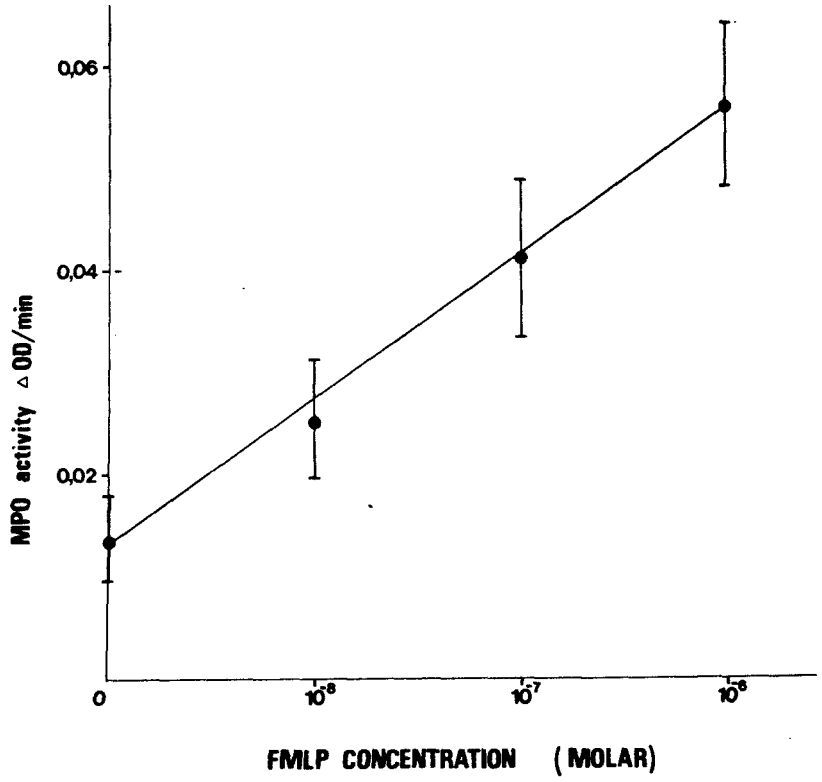



FIGURE 12. PMNL accumulation, measured by MPO release, on double filters with various FMLP concentrations. These results were obtained 6 hrs. after blister induction and are expressed as the mean values +- SEM for 3 different individuals.

Triton-X 100 requirement:- This was determined using the double filter system and $10^{-6}$ M FMLP. Duplicate filters from control and FMLP-exposed areas of dermis were placed in HBSS only or HBSS containing 0,1% triton-X 100. After 15 min. incubation followed by centrifugation the supernatants were tested for MPO and lysozyme activities. These studies were performed on 3 different individuals. The filters and agarose blocks were replaced after 2, 4, 6, 8 and 10 hrs. The results at 6 hrs. for MPO are shown in TABLE 14. Inclusion of triton-X 100 in HBSS markedly increased the activity of MPO and lysozyme (not shown). These findings demonstrate that the measurable enzyme activity in the triton-X 100 lysates is derived from PMNL on the filters of MPO and lysozyme released during cell migration.

Kinetics of PMNL migration:- Using the standardised method with double filters, $10^{-6}$ M FMLP and 1% triton-X 100, PMNL accumulation was measured in 5 different individuals after 2, 4, 6, 8 and 10 hrs. using 4 control and 4 FMLP-exposed areas of denuded dermis per person. The results are shown in FIGURE 13. Measurable PMNL migration occurred at 4 hrs. and was maximal at 6-10 hrs. Significantly greater PMNL accumulation was observed at each time interval in the FMLP-exposed systems ( $P<0,05$-$P<0,005$ ). Staining of

TABLE 14.

INVESTIGATION OF THE REQUIREMENT FOR TRITON-X 100 TO DETECT PMNL ACCUMULATION.

| | MPO activity in | | | |
|---|---|---|---|---|
| | Control filters | | FMLP-exposed filters | |
| Time hrs. | Without triton-X 100 | With triton-X 100 | Without triton-X 100 | With triton-X 100 |
| 4 | 0 | 0,021+-0.005* | 0.011+-0,004 | 0,054+-0,007 |
| 6 | 0 | 0,031+-0.006 | 0,012+-0,004 | 0,061+-0,006 |

* Results are the mean values +- SEM for 3 different individuals expressed as OD/min.

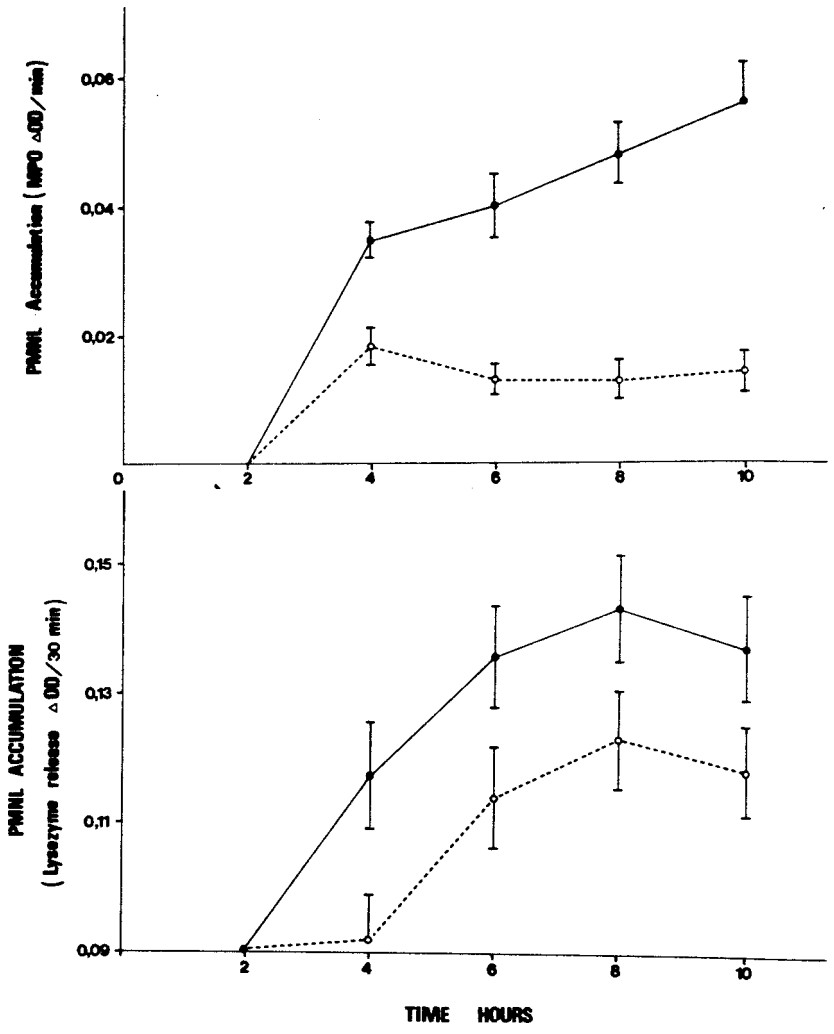



FIGURE 13. Kinetics of PMNL accumulation on double filters according to MPO (upper graph) or lysozyme (lower graph) release for control (○----○) and $10^{-6}$ M FMLP-exposed systems (●----●). Results are expressed as the mean value +- SEM for 5 different individuals.

the filters revealed a predominantly PMNL infiltrate which was greater than 90% at each time interval for both control and FMLP-exposed systems.

The mean variability +- SEM between quadruplicate blisters was 26 +- 3% and 32 +- 3% with MPO and 22 +- 2% and 32 +- 3% with lysozyme for FMLP-exposed and control systems respectively at 6 hrs. for 7 different individuals.

Conversion of enzyme activities to cell numbers:- Using the standard curve the enzymatic activities in FIGURE 13 correspond to $3,3 \times 10^6$, $4,2 \times 10^6$, $4,7 \times 10^6$ and $5,1 \times 10^6$ PMNLs at 4, 6, 8 and 10 hrs. respectively for FMLP systems. The corresponding figures for control systems are $2,8 \times 10^6$, $3,7 \times 10^6$ and $3,6 \times 10^6$ PMNLs respectively.

Variability of PMNL MPO and lysozyme content:- The mean MPO and lysozyme contents of PMNLs from 8 different individuals was 112,5 +- 4,2 units and 368,1 +- 12,8 units/$10^7$ PMNLs respectively. These results indicate very slight variability in MPO and lysozyme contents of PMNLs from different individuals.

Other investigations:- All of the volunteers used in this study had normal white blood cell counts, 6,6 +- 1,0 $\times 10^6$ leucocytes per ml. of blood with 63 +- 3% polymorphonuclear leucocytes. Histopathological analysis of the blisters showed that blistering occurred

at the epidermal-dermal junction. The blisters healed quickly without scarring leaving only faint dyspigmentation as described by Hellum and Solberg (1977).

## THE EFFECTS OF ORAL ADMINISTRATION OF ERYTHROMYCIN ON THE IN-VIVO MIGRATION OF HUMAN PMNLs.

TABLE 15 shows that the oral administration of erythromycin causes a significant enhancement of PMNL accumulation, however these studies should be extended to include more volunteers.

The method was as described on page 56.

TABLE 15

THE EFFECTS OF ORAL ADMINISTRATION OF ERYTHROMYCIN ON THE IN-VIVO MIGRATION OF PMNLs.

| Matched pairs of human volunteers. | Myeloperoxidase | | Lysozyme | |
|---|---|---|---|---|
| | Placebo | Test | Placebo | Test |
| 1st set | Ø,262 | Ø,346 | Ø,Ø18 | Ø,111 |
| 2nd set | Ø,316 | Ø,444 | Ø,Ø95 | Ø,132 |
| 3rd set | Ø,381 | Ø,4Ø2 | Ø,Ø27 | Ø,Ø68 |
| 4th set | Ø,362 | Ø,39Ø | Ø,162 | Ø,16Ø |

Results are expressed as OD45Ø for MPO and lysozyme.

Using Students t Test for matched groups:

MPO t = 2,587 P = <,Ø5

Lysozyme t = 2,2Ø1 P = <,Ø5

# DISCUSSION.

## CHAPTER IV.

# DISCUSSION

Using a mouse model of non-specific immunostimulation as described by Gillisen (1982), erythromycin administration was found to increase the mean survival time of mice experimentally infected with C. albicans. These results were obtained irrespective of the route of administration of erythromycin (either intraperitoneally or intravenously). Stringent control systems excluded false positive results due to other components of the erythromycin preparation such as benzyl alcohol or lactobionic acid. Likewise the C. albicans strain used was absolutely insensitive to direct antimicrobial effects of erythromycin. Since similar results were obtained with intraperitoneal and intravenous administration of erythromycin, it is unlikely that the results of the intraperitoneal studies are due to a non-specific irritant effect leading to activation of peritoneal macrophages.

To identify possible targets of erythromycin mediated immunostimulation the effects of erythromycin administration on mitogen-induced lymphocyte proliferation and PMNL migration were investigated in-vivo. In-vitro investigation on the effects of erythromycin on migration and antimicrobial activity of PMNLs were also performed.

Pretreatment of PMNLs with erythromycin did not

influence either phagocytic or antimicrobial activities in-vitro using C. albicans as the test micro-organism. Erythromycin does not therefore influence PMNL phagocytosis or intrinsic PMNL antimicrobial systems. The results of the mouse spleen lymphocyte transformations show that a 1 mg. dose of erythromycin lactobionate administered intraperitoneally caused a slight but statistically insignificant increase in mitogen induced lymphocyte transformation. However 100 /ugm. of erythromycin lactobionate administered intravenously resulted in a statistically significant increase in lymphocyte proliferation. In an attempt to explain this phenomenon it is suggested that although the absorbtion of erythromycin from the peritoneal cavity is rapid erythromycin lactobionate has a more direct influence on lymphocytes when introduced intravenously. Enhancement of mitogen induced lymphocyte transformation during administration of erythromycin was also observed in human volunteers following administration of a single 500 mg. oral dose of the antibiotic. Roche et al. (1986) however reported that erythromycin inhibits mitogen (PHA and pokeweed mitogen) induced transformation in-vitro. These results are in accordance with the observations of Anderson et al. (1982), who reported that erythromycin at concentrations of up to 35 /ugm./ml. had no significant effect on lymphocyte transformation to

CON-A and PHA in-vitro, but a progressive dose dependent inhibition was observed with concentrations of erythromycin greater than 35 µgm./ml. These in-vitro/in-vivo discrepancies are not without precedence, since acetylspiramycin has been shown to depress macrophage phagocytosis in-vitro but enhances macrophage phagocytosis in-vivo (Si-Ying Li and Nelson 1985). Likewise ketoconazole chemotherapy improves mitogen induced lymphocyte proliferation but causes inhibition in-vitro (van Rensburg et al. 1983). Erythromycin mediated potentiation of lymphocyte transformation could be due to inhibition of prostaglandin (PG) E2 synthesis. PG E2 a naturally occurring prostaglandin is a product of arachidonic acid a cell membrane phospholipid. Inhibition of PG E2 release from stimulated lymphocytes by erythromycin possibly acting as an inhibitor of PG synthetase may be the mechanism responsible for the erythromycin-mediated increased lymphocyte transformation (Anderson et al. 1984). Fox et al. (1982) suggest that erythromycin possesses anti-inflammatory properties. This may be due to the inhibitory effects of erythromycin on PG E2 release by leucocytes (Anderson et al. 1984).
An alternative mechanism has been described by Becker et al. (1974) who suggest that erythromycin protects leucocytes from oxidative damage

mediated by the MPO/$H_2O_2$/halide system which is activated within monocytes and macrophages during exposure to leucoattractants (Anderson et al. 1982). To study the effect of erythromycin on PMNL migration both in-vitro and in-vivo methods were used. The mouse air bleb method was discarded because of insurmountable difficulties associated in converting this test into a quantitative test. Since no suitable mouse methods were available to measure leucocyte migration, the investigation for the cause of the increased survival rate of mice pretreated with erythromycin and subsequently challenged with a lethal dose of C. albicans was directed to the effects of erythromycin on human PMNLs in both in-vitro and in-vivo conditions. In-vitro investigations showed that human PMNLs co-incubated with therapeutic concentrations of erythromycin in-vivo migrated in significantly greater numbers towards the chemotactic stimulus, endotoxin-activated autologous serum (EAS), relative to untreated control PMNLs. To measure leucocyte migration in-vivo, a modified suction-blistering technique (Hellum and Solberg 1977) was developed. It was found that the insertion of control valves into the suction device facilitated the synchronisation and standardisation of blister formation. When the blisters had formed, usually after 105 minutes, the raised epidermis, basement membrane

and blister fluid were removed to expose the 8 different areas of denuded dermis. Subsequent modifications were aimed at the development of an objective, sensitive and reproducible method of quantitating PMNL migration into the zones of denuded dermis. A method was devised using moistened nitrocellulose filters placed onto the areas of denuded dermis and overlaid with cylindrical blocks of 1% agarose containing the leucoattractant FMLP. In control systems FMLP-free agarose blocks were used. Incorporation of the FMLP into agarose had a number of probable advantages such as the maintenance of a positive FMLP gradient and protection of the FMLP from tissue and leucocyte proteases. Furthermore the pressure of the blocks and moisturising properties of the agarose promoted contact between the nitrocellulose filters and dermis.

As expected microscopic enumeration of PMNLs on the filters was found to be unsuitable for quantitative studies due to the density and irregular distribution of PMNLs on the filters. However enzymatic methods were found to be ideally suited to the measurement of PMNL accumulation on the filters. Filter-adherent PMNLs were disrupted by a 10 minute exposure to triton-X 100 and MPO and lysozyme activities in the lysates measured colourimetrically and turbidometrically respectively. Conversion of enzyme

activities to absolute cell numbers was accomplished using appropriate calibration curves. The results of standardisation studies revealed that maximal PMNL accumulation was detected using a double filter system and an FMLP concentration of $10^{-6}$ M. The double filter system with lower and upper filters of 5 microns and 0,2 micron porosities respectively presents a larger surface area for PMNL accumulation than the single 0,2 micron pore size, PMNL impregnable filter. The optimal $10^{-6}$ M FMLP concentration is 10 - 100 fold greater than the corresponding in-vitro optimal concentrations. This probably reflects the requirement of a high concentration of FMLP due to the slow diffusion out of the agarose and through the filters.

Using this standardised method it was observed that measureable PMNL accumulation occurred at 4 hours and was maximal at 6 - 10 hours. These studies were not extended beyond 10 hours. Significantly greater PMNL accumulation occurred in FMLP exposed systems at 4 - 10 hours. It must be emphasized that easily detectable PMNL accumulation was also observed in control systems, probably, due to the release of a variety of different leucoattractants derived from arachidonic acid metabolism and proteolytic cleavage of the third and fifth complement components during the induction of tissue injury by the suction blistering process. The

inclusion of FMLP increased the intensity of the leucotactic stimulus and amplified PMNL migration.
To date this method has only been applied to the measurement of PMNL migration in healthy adult volunteers. However it is likely that it could be modified to measure monocyte accumulation by extending the time (10 hours and beyond) and using a monocyte-specific marker to quantitate these cells on the filters. This could be achieved using a monocyte-specific extractable enzyme such as an esterase detectable by colourimetric analyses or by using radiolabelled or enzyme-linked monoclonal antibodies to monocyte specific antigens.
There are many possible immunological and immunopharmacological applications of this method. It could be used to evaluate the in-vivo significance of abnormalities of PMNL migration detected in-vitro as well as the in-vivo response of abnormal PMNL migration to the administration of agents which stimulate PMNL migration in-vitro. At present no suitable human model exists to investigate the effects of antibiotics and other pharmacological agents on PMNL movement in-vivo.
The improved, objective method for the in-vivo measurement of PMNL migration is uncomplicated by the removal of PMNLs from their natural environment and by the trauma of cell processing procedures.
This filter based in-vivo method was applied to

determine the effect of administration of erythromycin on PMNL migration and showed that therapeutic doses of erythromycin increase the in-vivo migration of PMNLs in adult humans. These investigations should be extended to include more volunteers and to confirm the role PMNLs play in the active transport of erythromycin to sites of bacterial infection. Mechanisms ascribed to potentiation of PMNL migration are the same as those causing potentiation of lymphocyte transformation, namely erythromycin inhibition of PG E2 release and inhibition of auto-oxidation mediated by the MPO/$H_2O_2$/halide system activated during exposure to leucoattractants as described previously.

In conclusion it has been shown that erythromycin is responsible for the potentiation of both lymphocyte transformation and PMNL migration in-vivo and in-vitro, resulting in an enhanced immune defence mechanism. It is suggested that this augmentation of the immune defence mechanism by erythromycin increased the mean survival time of mice inoculated with a lethal dose of C. albicans. The enhancement of host immune-defences by erythromycin during treatment of an infection may contribute to the ultimate eradicatiion of target microbial pathogens.

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