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DIAGNOSTIC, EPIDEMIOLOGICAL AND PATHOLOGICAL ASPECTS OF OPPORTUNISTIC MYCOSES

By

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Submitted in fullfilment of the requirements for the degree of Doctor of Philosophy in the Department of Dermatology, University of Glasgow.

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SUMMARY

Laboratory tests can help to confirm or establish the diagnosis of a fungal infection. They can provide an assessment of the response to therapy and monitor the resolution of infection. Histopathological recognition of fungal elements in biopsy or post-mortem material provides the definitive diagnosis of fungal infection. To improve the performance and specificity of histopathological procedures, a peroxidase anti-peroxidase (PAP) staining method for the specific recognition of *Aspergillus*, *Candida* and *Rhizopus* was developed. To verify the specificity of the primary fungal antisera employed in this method, they were tested for cross-reactivity by CIE, double diffusion and ELISA. The antisera were also tested for their cross-reactivity in PAP staining using sections cut from agar blocks inoculated with fungi. This novel technique was developed to simulate fungi growing in three dimensions through tissue. The sera could be tested against a wide range of fungi for which no control material was available. The agar block method conferred the additional advantage of having no background staining to complicate the results.

The PAP technique was shown to be extremely sensitive and able to detect fungi in tissue sections. It was able to distinguish fungi by genus and showed little cross-reactivity among the genera tested. However, none of the

antisera were completely specific and cross-reactivity was evident among species within the same genus.

The various manifestations of *Candida* infection present a range of diagnostic problems. One presentation of candidosis is *Candida* endophthalmitis. The epidemiology of this disease, in a cluster of heroin addicts from the Castlemilk area of Glasgow, was studied. Heroin samples, diluent, injection paraphernalia and clinical samples were cultured. Isolates of *Candida albicans* from these samples were further differentiated by a biotyping system. It was found that there was a preponderance of one biotype. The source of the *Candida* was unlikely to be the street drug as diamorphine hydrochloride was shown to have antifungal properties. The "Jif" lemon juice used by the addicts as a diluent proved to be the probable source of infection. The SO_2 content of lemons purchased in the Castlemilk area or from new "Jif" lemons heated to drive off the preservative, was lower than the recommended standard. Juice from lemons with reduced levels of SO_2 was shown to be able to support the growth of *C. albicans*.

An experimental murine model of haematogenous *C. albicans* endophthalmitis was investigated to study the pathology of the disease. The model demonstrated the presence of yeast in murine retinae five minutes after infection. Over several days the yeasts were seen to multiply and produce pseudomycelium. Local areas of inflammation developed around the yeasts and these

resulted in protrusions of the retinae. The presence of infection was confirmed by culture of the eyes and by tests for the detection of *Candida* antigenaemia.

1. INTRODUCTION

1.1. Systemic Fungal Infection in the United Kingdom

The systemic fungal diseases most often found in the United Kingdom are opportunistic infections caused by fungi which are common as commensals or saprophytes in the environment. This is unlike other areas of the world, notably the Americas and Africa where the true primary pathogenic fungi *Histoplasma capsulatum* and *H. duboisii*, *Coccidioides immitis*, *Blastomyces dermatitidis* and *Paracoccidioides brasiliensis* are endemic.

Primary systemic fungal infections are restricted in their geographical distribution. They are mainly respiratory and cases of self limiting infection are only recognised by a positive skin test reaction, as a result of hypersensitivity. However, severe and fatal generalised infection can occur (Chandler, Kaplan and Ajello, 1980; Rippon, 1974; Emmons, *et al.*, 1977).

The majority of systemic fungal diseases found in Britain are secondary or opportunistic infections, that is, the fungi are able to invade a host which has a diminished ability to overcome the organism. The fungi which are implicated infiltrate following depression of the host's immune system and infection is rare in patients with intact immunological systems.

However, with the increasing number of passengers travelling internationally some patients with primary systemic mycoses are now being diagnosed in Britain. These

are people who have contracted the diseases in the endemic areas before visiting or returning to the United Kingdom (Warren, 1985; Gentles, personal communication).

Although bacteria still account for the vast majority of infections in compromised patients, the number of fungal infections in these subjects is increasing. This is undoubtably due to the expanding number of such patients with underlying predisposing factors and the eventuality that, more of these patients survive episodes of bacterial infection as a result of improved antibacterial therapy. This means there are increasing numbers of potential hosts susceptible to fungal infection. Opportunistic fungal infections are now recognised more often and are being sought more actively.

The most common systemic fungal infections in Britain are :- Candidosis, Aspergillosis, Mucormycosis and Cryptococcosis.

1.2. Factors Predisposing to Fungal infection

The increase in the number and type of medical and surgical procedures presents a greater opportunity for fungi to cause infection. Often it is the susceptibility of the host rather than the pathogenicity of the fungus that allows an infection to become established. Factors contributing to the probable occurrence of systemic fungal disease include the degree of exposure to the fungus

involved, the site implicated, the portal of entry into the body and the immunological competence of the patient.

The most common source of infection of systemic candidosis is believed to be the host's own gut flora, for example *Candida* can enter the blood stream by persorption of the gut wall (Stone *et al.*, 1974). Administration of oral antibiotics results in an increase in gut carriage of *Candida* and the incidence of perianal candidosis. Oesophageal candidosis occurs in 5% of cancer patients. It may interfere with their nutrition and lead to fatal dissemination. The oesophagus is a common site of candidosis in patients suffering from the aquired immuno deficency syndrome (A.I.D.S.) (Chandler, 1985). Other common paths of entry are contaminated infusion fluids and cannulae which are contaminated at the time of insertion. The latter can cause local inflammation and lead to the spread of infection. Prolonged parenteral nutrition using deep veins provides another potential seat of infection. The lines may remain in place for a long time and the concurrent use of broad spectrum antibiotics supresses competing flora. The yeast may have been present at the catheter entry site or may originate in the gut and adhere to the catheter during a bout of candidaemia. Removal of the catheter and therefore the focus of infection, may help to clear the condition but this is not always possible and is often done only after the fungus has become established.

Aspergillus spores form a large part of the normal air spora (Gregory, 1973). The respiratory tract is the part of the body most regularly exposed to the greatest volume of air and indeed *Aspergillus* infections most often originate in the lungs. The spores are expelled from healthy lungs or are destroyed by the host defences. However, when these mechanisms are not functioning correctly, or if the lungs are coated with mucus, for example in individuals with cystic fibrosis, the spores may settle and cause irritation and or infection. The immune defences are adept at protecting man from diseases caused by aspergilli and this is reflected in the relative rarity of infections caused by these organisms in contrast to their ubiquity in the environment.

The lung is the organ most commonly implicated in *Aspergillus* infections and is an ideal site from which to spread since the fungus is provided with a readily accessible transport system to every part of the body.

In the United Kingdom, mucormycosis is seen less often than either candidosis or aspergillosis but the zygomycetes are potent pathogens of debilitated patients. If a patient becomes infected, mucoraceous fungi can spread with alarming speed. Mucoraceous fungi refer to fungi belonging to the order Mucorales. The genera most commonly involved in human disease are *Mucor*, *Rhizopus* and *Absidia*. At present antifungal therapy is only successful if administered in the early stages of illness and often

extensive surgery is required as the fungus is associated with extensive necrosis. This group of fungi is most often implicated in rhinocerebral mucormycosis in diabetic patients. However, it has also been associated with colonization and infection of burn wounds (Dennis and Peterson, 1971).

Cryptococcus neoformans is a capsulated yeast which may be present in the atmosphere or can be found colonising the crop of pigeons and is found in their droppings (Evans, 1969). In the United Kingdom it causes problems only in immunocompromised patients, which suggests it is a weak pathogen. However in the U.S.A. and Malaysia *Cryptococcus* has been reported as causing infections in apparently healthy subjects. The yeast may cause sub-clinical infection of the lungs then, in a few cases, spread to the central nervous system. As yet there is no explanation for this difference in the yeast's pathogenic role in different parts of the world.

1.3. Immunology of Systemic Fungal Infection

Mechanisms of defence against fungal infection in a non compromised subject rely on both immunological and non immunological factors. Non immunological factors are non specific and include such protectors as the skin, surface secretions and the host's indigenous flora. The intact skin inhibits establishment of infection by allowing

dessication and turnover of the epithelium sloughs off potential invaders. Sebum contains fatty acids with antifungal properties and the complement factor C_3 in serum inhibits the adherance of *Candida* to corneocytes (Ray, Digre and Payne, 1984).

The flushing action of secretions on mucosal surfaces discourages invasion and the indigenous host flora competes with potential pathogens (Richardson and Warnock, 1982)

In the host at risk, the defects are primarily in the immunological defence system especially neutrophil T and B cell defects. These can be due to an underlying disease or to treatment of the underlying condition.

The decrease in neutrophil numbers is an important factor in the successful outcome of treatment of some solid tumors. When medication inhibits cell proliferation, neutrophil production is also inhibited and this renders the patient more prone to infection (Warnock, 1982). Treatment of malignancies alters the immunological host response. The T cell mediated response can be eliminated, many agents administered to prevent graft or organ rejection abrogate the immunological response of the recipient. Intermittent therapeutic treatment of such people has a less damaging effect on their defence system.

1.4. Candidosis

Candida is a heterogeneous genus and a member of the family *Cryptococcacae* of the fungi imperfecti. Species of the genus *Candida* are predominantly dimorphic fungi often present in the yeast form but most possess the ability to form true mycelium and chains of elongated yeast cells termed pseudomycelium.

Candidosis is the most prevalent of the opportunistic fungal infections of man and it can also be the most difficult to diagnose. *Candida* species can cause superficial, subcutaneous and systemic disease. Unlike the other infections discussed, yeasts of the genus *Candida* form part of the normal microbial flora of man and usually live as commensals, in equilibrium with the host and other micro-organisms. The yeast is never present as a saprophyte in the blood or internal organs but it is a resident of the gastrointestinal tract of a substantial proportion of the population, especially people who are receiving medical attention. It is a facultative pathogen and can only produce disease if the host's defences are impaired otherwise it exists as a commensal with man. There are no geographical limits to the disease as man is the most important reservoir of infection.

Although eight species of *Candida* have been implicated in disease of man, by far the most frequently encountered species is *C. albicans*. It may be involved in superficial infection of the skin, nail folds and oral and vaginal thrush. The largest reservoir of *Candida* in man is the gastrointestinal tract and the principal source of systemic infection is the host's endogenous flora. *Candida* may enter the host's blood by persorption from the digestive tract but the site of invasion for systemic disease is difficult to confirm. Invasive forms of candidosis are rarely primary diseases. They are diseases of ill individuals with some defect of their immune system. The *Candida* adheres to the host tissue, may produce damaging toxins and if present in sufficient numbers, can overwhelm the patient's defence mechanisms.

There are several predisposing factors which contribute to an individual developing systemic or disseminated disease. These include neoplasia, leukaemia, immunosuppressive therapy, surgery especially gastrointestinal (Griffin *et al.*, 1973), diabetes, endocrine disorders, general serious illness and narcotic drug addiction. Griffin *et al.*, in 1973 published a study of 21 patients with candidaemia and ophthalmic lesions, of which 17 had gut and abdominal surgery. Often the

bacterial flora of the gut is suppressed before such procedures but the fungal commensals are not eradicated.

The presence of an indwelling catheter is an important factor in a patient's predisposition to systemic candidal disease and becomes a more important consideration the longer it remains in place, particularly if it is used for parenteral nutrition. There has been speculation that the catheter entry site is the initial site of infection but this is difficult to prove. An alternative hypothesis suggests the plastic surface of the catheter provides a site for adhesion of *Candida* circulating in the blood after persorbtion from the gut and offers a situation from which dissemination can occur. Nutritive solutions entering the blood through these catheters provide the organism with a rich growth medium as hyperalimentation fluids are high in glucose, amino acids and lipids. Their high osmolarity inhibits bacterial growth.

Antibacterial agents are often cited as an important consideration in the dissemination of candidal disease (Warnock, 1982). They may irritate the gut epithelium, disturb the ecosystem of the host's micro-organisms or suppress the host defences directly or indirectly.

The clinical manifestations of candidosis are numerous but several are well recognised.

The superficial forms of *Candida* infection occur when there is damage to the skin, especially if the skin is very moist, or if the balance of micro-organisms on the mucous membranes is disturbed.

1.4.1. Oral

Oral candidosis occurs most frequently in infants who are thought to acquire the infection from their mother's birth canal. It is also a prevalent infection in people receiving radiotherapy for neck and head cancer. In adults it is linked to hormonal or immune problems. Stomatitis in denture wearers and angular cheilitis can be caused by *Candida* although in the latter situation it is usually a secondary invader.

1.4.2. Vaginal

Many otherwise apparently healthy women suffer from vaginal candidosis at some time or other. The incidence is increased during pregnancy when the pH of the vagina is low (Odds, 1979), when the woman is on antibacterial therapy and with the use of oral contraceptives. The symptoms are acute pruritis, discharge, irritation, itching and there may be white lesions on the vulva and vagina. The infection may spread to the perianal and pubic

area and inside thighs. Moreover, *Candida* may coexist with *Chlamydia* and *Trichomonas*.

1.4.3. Cutaneous

This infection occurs, most often, in intertriginous areas where there is local moisture, maceration or occlusion, for example submammary folds, axillae and nail folds. Cutaneous candidosis can be difficult to diagnose by clinical appearance. The skin is often erythematous, scaly and moist, there may be pustules and vesicles and development of pruritis may occur (Roberts, Hay and M^{ac}kenzie, 1984). The disease may resemble eczema, pemphigus, drug eruptions, psoriasis, seborrhoeic dermatitis or dermatophytosis. *Candida* paronychia is an occupational disease associated with longterm immersion of hands in hot water and has been well described by Gentles and La Touche (1969). Paronychia is a painful chronic infection of the nail folds and may be associated with bacterial co-infection. Superficial disease may also present as intertrigo in infants and incontinent patients and as onychia in previously damaged nails.

1.4.4. Chronic Mucocutaneous Candidosis

This malady first manifests itself in childhood, with candidosis often being detected before the immune

disorders which predispose the individual to this chronic candidal infection. *C. albicans* is the only species implicated. The underlying factor in this disease is a cellular immune defect associated with a genetic complaint involving the thymus. It is believed to be a T-cell defect, as antibodies to *C. albicans* are found in these patients. B-cell immunity is intact and the activity of the host's macrophages is unimpaired. The first indication of the disease is oral thrush in the white pseudomembraneous form, then angular cheilitis and lip fissures develop. There may be spread to the larynx and occasionally the oesophagus. Vaginal thrush is also seen in female patients. *Candida* onychia and paronchyia arise and there may be dry, mild erythematous skin lesions (Dwyer, 1981).

C. albicans is the species most frequently involved in systemic disease but several other species have also been implicated including *C. guillermondii*, *C. krusei*, *C. parapsilosis*, *C. tropicalis* and *C. glabrata (Torulopsis glabrata)*. Recently *C. tropicalis* and *C. krusei* have become noticeably more involved in infections of leukaemic patients.

There may be involvement of a number of deep sites or there may be a focal site of infection. Candidaemia may be

a danger sign as it might be a prelude to dissemination in immunocompromised hosts.

1.4.5. Gastrointestinal Tract

The most frequently infected site associated with this form of candidosis is the oesophagus (Blackwell, 1964). Oesophageal candidosis is often a precursor to disseminated candidal disease and this has become an important factor to look for in patients suffering from AIDS (Chandler, 1985).

1.4.6. Respiratory Tract

The lungs are an unusual site for primary infection although they may become involved in disseminated disease. It is a rare site for focal infection and it is difficult to diagnose this presentation unless a biopsy is obtained and the presence of fungal elements demonstrated in tissue sections.

1.4.7. Urinary Tract

The symptoms of urinary tract infection are frequency and dysuria. The infection usually follows catheterisation with obstruction of the bladder. The kidneys are the organs for which *Candida* has a predilection, although the reasons for this have not as yet been elucidated. This

clinical picture is reflected in experimental animals as kidney metastases are frequently found in animals infected with *C. albicans*. Symptoms of renal infection are typical of microbial kidney infection with candiduria, fever and not infrequently positive blood culture.

1.4.8. Endocarditis

Endocarditis is a relatively rare form of the disease but its grave prognosis and the difficulties experienced in its diagnosis have led to a considerable amount of investigation. It is generally encountered in the middle aged and twice as often in men as in women. The most common predisposing factors are prexisting heart disease, especially involving open heart surgery, or drug addiction. The most likely reason for this nidus of infection is the adherence of yeast, from the circulation, to the traumatised tissue of the heart or to a prosthetic implant. Joachim and Polayes (1940) were the first to describe the disease in a drug addict and identified the aetiologic agent as *C. parapsilosis*.

There is a confused clinical picture but the features are embolic phenomena, chills, fever vomiting, nausea, anorexia and heart failure. Endocarditis involves the aortic valve more often than the mitral valve but the tricuspid valves are infrequently implicated. This

correlates with experimental findings that show the left side of the heart is more often involved than the right (Sande, Bowman and Calderone, 1977)

1.4.9. Central Nervous System

The central nervous system (CNS) as the principal focus of the infection is very rare although the brain and meninges can be involved in disseminated infection. The main clinical symptoms are head and neck pain.

1.4.10. Bone and Joint

Infections of bones and joints due to *Candida* species are rarities. In adults they are usually associated with myelomas and are infrequently cured. In neonates the common site is the knee and it occurs predominantly in infants with prolonged intravenous catheterisation. It is a manifestation of disseminated candidosis and often CNS involvement is a concomitant feature. This manifestation of candidosis has also been reported in heroin addicts (Collignon and Sorrell, 1983; Dupont and Drouhet, 1985)

1.4.11. Septicaemia

Candidal infections are often spread via the blood stream and although candidaemia is unusual it is by no means a rare complication in seriously ill patients. In

adults the infection is often fatal and probably causes more deaths than are reported. In infants it is not often fatal if treated. In these patients there may be evidence of joint and CNS involvement. Odds (1979) reviewed the literature. The frequently encountered debilitating features of patients with *Candida* septicaemia are trauma of the thorax and abdomen, multiple antibiotic therapy or catheters. Any of these criteria indicate the severity of the illness of the host. Odds (1979) summaries the most susceptible case as "a seriously debilitated patient treated with a multiplicity of iatrogenic, medical and surgical procedures."

Clinically the first signs may be general detioration and intermittant fever. There is pronounced leucocytosis in the early stages but leukopenia develops in terminal stages of the infection.

1.4.12. Endophthalmitis

Candida endophthalmitis presents in two forms. The rarer exogenous form, results from inoculation of the eye with an environmental source of the fungus or follows infection after an operative procedure (Rosen and Friedman, 1973; Gilbert and Novak, 1984; Edwards, 1985). An outbreak of *Candida* endophthalmitis caused by *C. parapsilosis* occurred in California, the source was

attributed to contaminated irrigating solution which had been used during intraocular surgery (O'Day, 1985; McCray *et al.*, 1986).

Endogenous endophthalmitis infection arises from *Candida* being delivered to the eye via the blood stream. The endogenous form is becoming an increasingly important presentation of endophthalmitis. This is probably a reflection of the increase in patients with disorders rendering them susceptible to attack by opportunistic pathogens. Jones, Richards and Morgan, (1970) reviewed the literature of all cases of fungal endophthalmitis in the United Kingdom from 1880 - 1970 and could only find 30 cases. *C. albicans* was the most commonly implicated organism and accounted for 12 of the cases. *C. albicans* is the species with the greatest propensity to cause infection although other species such as *C. rugosa* have been implicated (Reinhardt *et al.*, 1985). Septicaemia caused by yeasts other than *C. albicans* is less likely to lead to eye infection (Edwards, 1985).

Endogenous *Candida* endophthalmitis was first described by Miale (1943) in a histopathological study. The disease is not common in healthy subjects but has been reported in isolated cases in women with vaginal candidosis in association with child birth (Michelson, Rupp and Efthimiadis, 1975; Cantrill *et al.*, 1980).

The majority of cases of *Candida* endophthalmitis reported in the literature have been described as complications of systemic candidosis (Fishman *et al.*, 1972; Weinstein, Johnson and Moerllering, 1973; Griffin *et al.*, 1973; Meyers, Lieberman and Ferry, 1973). Endophthalmitis occurs mainly in post operative patients who, have undergone gastrointestinal surgery (Freeman, Davis and M^{a}^{c}Lean, 1974) and are being treated by multiple antibiotics and intravenous hyperalimentation subsequent to their surgery (Dellon, Stark and Chretein, 1975; Abel and Baxter, 1976; Montgomerie and Edwards, 1978; Nahata *et al.*, 1981; Henderson *et al.*, 1981). These patients develop infection after febrile episodes and often yield *Candida* on blood culture (Abel and Baxter, 1978; Montgomerie and Edwards, 1978). Henderson *et al.* (1981) described the factors favouring development of *Candida* endophthalmitis in the group of patients they studied as: the alteration of pulmonary clearing mechanisms by use of certain catheters, a high incidence of gastrointestinal haemorrhage, the propensity of *C. albicans* to cause endophthalmitis and the lack of immunosuppression. In non-immunosuppressed patients the eye lesions are easily seen compared to those found in neutropenic patients who may lack the ability to produce a sufficient immune response

of polymorphonuclear leukocytes to develop visible lesions (Henderson *et al.*, 1981).

However, endophthalmitis has been reported in immunosuppressed people. Greene and Wiernik (1972) described their success in treating *Candida* endophthalmitis in a patient with acute leukaemia and Yoshitomi, Inomata and Tanaka, (1983) reported on an autopsy of a case of malignant lymphoma which showed retinal lesions typical of candidosis.

Heroin addicts are emerging as a distinct group which is at risk from *Candida* endophthalmitis. (Section 1. 12.) In such cases this manifestation of *Candida* infection may be present without systemic candidosis (Dally, Thomas and Mellenger, 1982; Collignon and Sorrell, 1983).

Although *Candida* endophthalmitis is a well recognised complication of candidosis, it occurs less frequently in children and babies despite newborn children having a deficient host defence system which probably makes them more at risk from infection by *Candida* species. The factors which predispose the infants to *Candida* endophthalmitis appear to be the same as for older patients. Palmer (1980) described a case of endophthalmitis after abdominal surgery and Speer, Hitter and Rudolph, (1980) reported a case associated with venous feeding and antibiotic therapy. Baley, Annable and

Kliegman, (1981) looked at the incidence of the disease in premature infants. Four of eight premature infants with systemic candidosis had eye lesions. *Candida* sepsis was prevalent in low birth weight premature babies treated by antibiotics and the authors thought the course of the eye lesions reflected the prognosis for the patient. Baley, Kliegman and Fanaroff, (1984) stated that the clinical features of candidosis in these babies were poorly defined but that the underlying causes included total parenteral nutrition, the use of catheters and courses of broad spectrum antibiotics.

1.5. Aspergillosis

The aspergilli are filamentous fungi responsible for a variety of diseases in man and animals. These range from toxicity caused by ingestion of contaminated foodstuffs, allergy and colonisation through to invasive disseminated disease.

Although there are 132 species in this genus, as described in the monograph by Raper and Fennel (1965), the species most frequently implicated as a cause of disease in man is *A. fumigatus* followed by *A. flavus, A. niger and A. terreus*. All of these aspergilli are thermotolerant. The disease is seen in immunocompromised patients and the severity and clinical manifestations of the infection apparently depends on the debility of the host rather than the pathogenicity of the fungus.

The aspergilli are soil saprophytes which utilise decaying plant and animal debris for their nutrition. They produce massive numbers of airborne spores and so form part of the normal air spora in all areas of the world. They rarely invade tissue but can cause systemic infection in profoundly compromised individuals. *Aspergillus* infections are not communicable from one host to another. As these fungi are common laboratory contaminants a single isolation is not sufficient to establish it as the cause

of disease. Contamination is a risk at every stage when handling specimens and appropriate steps must be taken to prevent contamination whenever possible; for example, consecutive early morning samples of sputum are less liable to be contaminated than those taken over a 24 hour period. The major clinical manifestations of aspergillosis are as follows.

1.5.1. Pulmonary

Aspergillus infection of the lungs is the most common manifestation of the disease in man and there are several different types; primary invasive, secondary invasive, secondary noninvasive and primary allergic bronchopulmonary (Al-Doory and Wagner, 1985).

Primary invasion of the lungs in a normal individual with an intact immune system is very rare and as a rule only occurs after repeated, prolonged exposure to massive numbers of *Aspergillus* spores which overwhelm the immune system.

Individuals with a low resistance to disease who have a severely debilitated immune system, for example patients with leukaemia or lymphomas, individuals with neutropenia and children with chronic granulomatous disease, are susceptible to secondary invasive aspergillosis. *A. fumigatus* is the species most often implicated. The

severity of symptoms depends on the part of the lung infected and is sometimes overshadowed by the underlying condition. Patients may be acutely or chronically ill, they develop a non-productive cough, dyspnoea and fever. Dissemination to the brain, kidney and heart may occur. Radiological findings reveal a broad spectrum of radiological changes but typically there is a rounded area of consolidation with a radio-dense periphery around the flare that extends into the lung parenchyma.

The secondary noninvasive form of the disease entails colonisation of pre-existing cavities of the lungs caused by previous disease usually tuberculosis, sarcoidosis or ectatic bronchus. The British Tuberculosis Association in 1970 reported that 12% of TB cases had radiological evidence of aspergillomas. The fungus grows as a compact ball either freely or it may be attached to the wall by fibrous exudate. The fungal ball is called an aspergilloma. The *Aspergillus* rarely invades but the vegetation may enlarge resulting in an inflammatory reaction surrounding the cavity with fibrosis. Clinically it may be asymptomatic or there may be slight haemoptysis with a chronic productive cough with some weight loss or, in extreme examples, severe haemoptysis causing exsanguination and death. The principal distinctive feature is recurrent haemoptysis. In cases with increasing

severity surgical resection is indicated. Within the aspergilloma there may be zonation of mycelial elements with pytnotic layers of host cells. This is indicative of dynamic evolution of the lesion. Furthermore, it has been postulated that endotoxins may play a role in the pathogenesis (Henrici, 1939; Bardana 1978).

The asthmatic and allergic bronchopulmonary forms of the disease occur in people previously sensitised by contact with the spores. The bronchopulmonary form of the disease is an exaggeration of the allergic condition seen in late onset asthma and atopic disease. Clinically, the signs are similar but in the bronchopulmonary state they are chronic and more severe. The symptoms are recurrent febrile episodes, severe cough, wheezing, production of purulent sputum with eosinophils and bronchial plugs harbouring fungal elements. Associated with this is pulmonary consolidation and peripheral blood eosinophilia. The mucous membranes of the bronchioles and bronchi are often red and congested but not invaded.

1.5.2. Paranasal

There are two forms of this disease. The first is noninvasive and mimics nonspecific sinusitis. It is unusual in the United Kingdom and is predominantly found in the tropics. The features that distinguish it from true

invasion are that the patients are apparently healthy or may have a long history of sinusitus. It is slow to progress and develops over months or years. Histologically it is distinct as it forms a fibrosing granuloma and only small fragments of hyphae are found. The morphology of the organism in this manifestation of the disease may lead it to be mistaken for yeast. The most common species of *Aspergillus* causing this infection is *A. flavus*. The patient presents with unilateral facial pain and in the early stages surgical removal may be curative. Alternatively, lavage with or intravenous administration of amphotericin B may prove useful. It is fatal if it spreads to invade the CNS.

The second presentation of paranasal disease is the invasive form when the sinuses and the adjacent tissue are involved, this can simulate neoplastic disease. This form develops and spreads more rapidly.

Paranasal aspergillosis may be related to the inhalation of contaminated marijuana (Schwartz, 1985). It has been cultured from samples of the drug and is considered a source of infection and allergy (Kagen, 1981).

1.5.3. Otomycosis

A. niger is most frequently responsible for otomycosis. The fungus grows on epithelial scales, debris and cerumen in the external ear canal where it causes irritation, pruritis and can impair hearing. Although it is a benign condition it can be chronic and recurrent.

1.5.4. Onychomycosis

Invasion of nail tissues by *Aspergillus* species occurs in previously damaged finger and toe nails. Culture of aspergilli from these sites is not significant unless fungus has been seen on direct microscopical examination of the nail and no yeast or dermatophyte has been cultured (English, 1976).

1.5.5. Central Nervous System

Aspergillus infections of the CNS can follow iatrogenic procedures or can be found in drug addicts possibly from contamination of the injected drug. It may also be a secondary problem to paranasal aspergillosis. Acute *Aspergillus* meningitis is rapidly fatal. The meningeal form of the disease may present with symptoms which may mimic a cerebral tumor with a slow onset of symptoms showing neurological dysfunction such as epilepsy.

Granulomatous lesions may develop in the CNS of less debilitated patients such as diabetics who are able to mount some defence.

1.6. MUCORMYCOSIS

Mucormycosis is the most rapidly fulminating of all the fungal diseases. The classic description of the disease was written in 1943 by Gregory, Golden and Haymaker, when they delineated the syndrome. Pulmonary mucormycosis had been described previously by Furbringer in 1876 who attributed the infections to mucoraceous fungi. In 1885 Paltauf described the first disseminated case and the rhinocerebral form.

When a person becomes infected with a fungus from the family mucoraceae an acute disease develops, which if left untreated will spread with alarming speed causing death in seven to ten days or less. The establishment of the disease may be due to a delay in phagocytic cell migration or a defect in phagocytic function. Amphotericin B is apparently the only antifungal drug able to halt the spread of the disease. Unfortunately drastic surgery is also required as the fungus causes extensive necrosis. (Meyer and Armstrong, 1973; Pillsbury and Fischer, 1977; Meyer *et al.*, 1979).

The mucoraceous fungi are thermotolerant and are very common in the environment. They are poor pathogens since, although they are ubiquitous and are not fastidious with respect to their nutrient requirements or growing conditions, relatively few cases of mucormycosis have been

reported. In the United Kingdom there are on average four per annum (Roberts *et al.*, 1984).

The fungus has a world wide distribution but environmental sources have not been linked to outbreaks although potted plants in hospital wards have been suspected sources on occasions.

It tends to be the hosts underlying susceptibility, not so much the organisms pathogenic potential, which leads to an infection being established. The fungi most frequently implicated in mucormycosis are *Rhizopus oryzae* and *R. arrhizus*. Mucormycosis occurs most frequently in patients with acidosis due to uncontrolled diabetes. Other people at risk are malnourished children, patients suffering from burns, leukaemia or lymphomas (Meyer, Rosen and Armstrong, 1972).

The fungus has a predilection for blood vessels, causing emboli which result in massive haemorrhagic infarction, with subsequent necrosis of the surrounding tissue. The fungus elicits a non-specific tissue response with a suppurative pyogenic reaction, granulomatous reactions are not generally found. Penetration through arterial walls and thrombus formation are characteristic features of this form of mycosis.

There are five major clinical forms of mucormycosis associated with different abnormalities in host defence mechanisms (Wong and Armstrong, 1982).

1.6.1. Rhinocerebral

This is the most frequently encountered form of mucormycosis and is most often seen in patients with diabetes mellitus and acidosis. It is characterised by unilateral facial pain and swelling, progressive invasion produces blindness and perforation of the palate. *R. oryzae* and *R. arrhizus* are the only agents to be implicated in this form of the infection (Rippon, 1974).

Spores settle on the mucosa of the nasopharynx, palate or paranasal sinuses where they germinate and invade. The likelihood of infection is related to the ability of the host's defence system to provide adequate protection. The diabetic, for example, may respond to treatment but the fungus may remain quiescent and then be reactivated during a subsequent episode of acidosis.

Clinical signs are a thick nasal discharge and necrotic areas in the septum and fistulas in the palate. This is accompanied by headache, tender sinuses, facial swelling, decreased vision and fever. Tissues show extensive ischaemic infarction and dry gangrene which facilitates the spread of the fungus across anatomical boundaries eg. into bone. With haemorrhagic necrosis and venous obstruction the extension of the disease often follows the routes of blood vessels and occasionally nerves or the lymphatic system. By these means the fungus progresses to the orbit and the frontal lobes of the brain. Orbital cellulitis is associated with a poor prognosis as it

indicates that the organism has invaded the orbit and the central nervous system leading to loss of function of the fifth and seventh cranial nerves. Necrotic sequelae of the brain are irreversible and lead to coma and death.

The characteristics of this form of the disease are uncontrolled diabetes, orbital cellulitis and meningoencephalitis.

The mechanisms that reduce host resistance in diabetics are poorly understood but abnormal phagocyte function may play a role. Human serum contains a factor which inhibits the growth of *Rhizopus* but this factor does not operate in serum from diabetic patients (Gale and Welch, 1961). In rabbits, microscopic examination of the infection reveals neutrophil exudates in normal animals and very few neutrophils in diabetic animals (Diamond *et al.*, 1978). *Rhizopus* hyphae and spores have been shown to be damaged by neutrophils and ingested and killed by rabbit alveolar macrophages (Levitz *et al.*, 1986).

1.6.2. Pulmonary

Sporangiospores of causal fungi are inhaled and as they are small in size (2.5 - 8μm) they can reach the bronchioles and alveoli of the lower respiratory tract. After the fungus germinates, invading the mucosa and the blood vessels, it forms a tangled mass in the lumen which causes infarctions where the hyphae invade the surrounding tissue. The tissue necrosis leads to cavitation and death

from pulmonary insufficiency, massive haemoptysis or occasionally from dissemination of the disease.

This form of mucormycosis is seen most usually as a complication of patients with leukaemia or lymphomas. Treatment with corticosteroids increases the likelihood of infection. The disease presents as a progressive nonspecific pneumonia with thrombosis and infarction.

1.6.3. Gastrointestinal

This is a rare manifestation of mucormycosis and is linked to malnutrition or gastrointestinal disease, the result of which is damage to the gut mucosa. Children appear to be most at risk. The infection usually progresses rapidly, ending in death.

1.6.4. Disseminated

Dissemination of the disease can occur in all forms unless treated but secondary lesions are most likely if the lungs are the metastatic site. On three occasions a predilection for the brain has been reported in heroin addicts who were apparently non immunocompromised (Adelman and Aronson, 1969; Hameroff, Eckholdt and Lindenberg, 1970; Chmel and Grieco, 1973).

1.6.5. Cutaneous

The largest group at risk from cutaneous mucormycosis is burn patients. Transient colonisation of the injured

areas is not infrequent, spores invade through breaks in the skin. Swelling of the wound occurs and vascular penetration allows spread to the underlying tissues and the development of gangrene (Dennis and Peterson, 1971).

1.7. CRYPTOCOCCOSIS

Fungi of the genus *Cryptococcus* have a worldwide distribution and infections attributable to these fungi have been reported in both immunocompetent and immunocompromised individuals. *C. neoformans* is the species most often involved in infection. *C. neoformans* is the imperfect state of *Filobasidiella neoformans*. There are two varieties of *F. neoformans*, the mating strains of which are compatible with the four serotypes of *C. neoformans*. *F. neoformans* var *neoformans* is compatible with serotypes A and D while *F. neoformans* var *gatti* is compatible with serotypes B and C.

The disease is seen more frequently in the U.S.A. and Australia than in the United Kingdom and northern Europe. In Britain there are eight to 13 new cases every year, 85% of which are patients with underlying disorders (Hay *et al.*, 1980). The infection generally occurs in young adults and the middle aged, the most common debilitating conditions are sarcoidosis, Hodgkins lymphomas, malignancies and systemic lupus erythematosus and in the USA, also AIDS.

The disease is not contagious and is generally acquired by inhalation of the infecting yeasts. The number of infections is low in relation to the yeast's wide

distribution, this suggests that the fungus is not a virulent pathogen.

C. neoformans is a yeast that produces capsules in culture and in tissue. It is believed that the polysaccharide capsule confers an advantage since it impedes phagocytosis.

The saprophytic habitat of *C. neoformans* was established by Emmons in 1955 when he isolated the yeast from pigeon droppings, nests and lofts and the soil in their vicinity. Further work has shown that it can survive dessication and has a wide distribution (Staib, 1962). The fungus utilises low molecular weight nitrogen compounds such as uric acid, urea and creatine but the moisture in the environment, along with the pH and exposure to ultra violet light may influence its survival (Evans, 1969). *C. neoformans* is not good at surviving in competition with other micro-organisms which could be one reason for it not being isolated from the soil with greater frequency.

Cryptococcosis is primarily a disease of the lungs which is usually mild, transient and unrecognised. However, patients with the most commonly diagnosed form of the disease present with a slow progressive meningitis. The primary infection is always pulmonary but there may be dissemination with the CNS the prime target.

1.7.1. Pulmonary

There are two forms of the pulmonary disease, a self-limiting, mild infection and a chronic form with lesions which appear as lesions of several cm in diameter on X-ray. The most common is a subclinical form in normal immunocompetent people which usually resolves rapidly, although if the host is exposed to a high concentration of *C. neoformans*, a chronic infection may develop. Sensitisation without clinical illness has been studied (Fink, Barboriak and Kaufman, 1968; Muchmore *et al.*, 1968) using intradermal skin tests with cryptococcal extracts.

In debilitated patients *C. neoformans* may disseminate from the lungs and the lungs may either become a focus of infection or the pulmonary lesions may heal after the spread of infection. There are no diagnostic symptoms but in most cases the signs are an asymptomatic cough with scanty sputum production, low grade fever, weight loss and pleural pain. The radiological picture is variable but infection tends to be in the lower lung fields, calcification is rare and most lesions heal as granulomatous foci without treatment. Massive infiltrates raise the possibility of dissemination especially in immunocompromised hosts.

1.7.2. Central Nervous System

Infection of the CNS by *C. neoformans* is the most frequently diagnosed form of the disease although as yet there is no explanation for the predilection of the fungus for this site. There is an incidence of underlying disease in 85% of cases in the United Kingdom but underlying disease is not as common in the U.S.A.. The drug amphotericin B has reduced the number of fatalities and flucytosine has been used successfully on occasions but there is recurrence in 25% of surviving cases. The patients present with pyrexia, frontal and temporal headaches, which become increasingly severe and frequent. There is often tenderness of the skull and neck. The patients experience nausea and vomiting with mental changes, then paralysis and coma develop. Ocular manifestations include papillodema, blurring of vision, vertigo and photophobia. The papillodema may necessitate a spinal tap to prevent optic damage. The cerebral spinal fluid (CSF) may show raised pressure, excess protein, low sugar concentration and a proliferation of lymphocytes with the occasional neutrophil. Encapsulated yeasts may be seen but if the lesion is restricted to the brain they will not be present.

1.7.3. Cutaneous

Cutaneous lesions may accompany dissemination in 10-15% of cases (Roberts *et al.*, 1984). The lesions are acneform pustules or papules which form ulcerated subcutaneous abcesses with time and often resemble those caused by other micro-organisms.

1.7.4. Osseous

Bone involvement occurs in 5-10% of cases and is accompanied by pain and swelling for several months. There is no characteristic radiological picture and the lesions are usually multiple, discrete and widely disseminated. This is a destructive, chronic form of the disease.

1.7.5. Visceral

In disseminated disease, any organ of the body may become infected and in such cases the clinical picture resembles tuberculosis. After the central nervous system the heart, testes, prostate and the eye are the most common sites invaded (Rippon, 1974). Infections of the kidney, adrenal glands, liver and spleen are less common. Eyes become involved by direct extension of the organism from the subarachnoid space or by haematogenous spread from other foci.

1.8. Diagnosis of Opportunistic Fungal Infections

The diagnosis of opportunistic systemic fungal infections often presents difficulties because the fungi causing the disease may form part of the normal endogenous flora of man or may be common laboratory contaminants. The mycologist must examine all the available evidence before deciding if the presence of an organism is of relevance. The patient's clinical history may help to decide the significance of a fungus seen on direct microscopical examination, on isolation in culture or from indication of its presence by serological techniques for detection of antigen or immunoglobulins associated with the fungus

1.8.1. Candidosis

Of all the opportunistic fungal infections this is perhaps the most difficult to diagnose, since yeasts of the genus *Candida* comprise part of the endogenous human flora. The presence of *Candida* in sputum, faeces or from superficial sites is therefore of little diagnostic significance unless isolated repeatedly in quantity and supported by other evidence. Serology must also be interpreted with care as many healthy people in the normal population have antibodies to *C. albicans* but do not have candidal infections.

The diagnosis of superficial candidal infections is relatively simple if fungus is seen on direct microscopical examination of the skin or nail sample and is grown in culture. The same procedures are required for vaginal smears and swabs.

The picture is less clear cut for patients with systemic infection. Clinically patients with invasive disease have pyrexia of unknown origin (PUO) which does not respond to antibiotics and is associated with night sweats and weight loss. Blood cultures may give a useful indication that the potential for dissemination exists but these are often negative in cases of systemic infection and transient candidaemia may occur without an established infection. Urine cultures may indicate the extent of spread of the fungus and are an important sample to examine as kidneys are a major target organ for *Candida*.

In immunosuppressed patients at risk from opportunistic infections, the isolation of *Candida* from skin, sputum or oral mucosa, although not diagnostic for candidosis, should alert the physician to the possibility of future infection. If culture results are indicative of heavy colonisation, there is obviously a large reservoir of potentially pathogenic organisms. Biopsy specimens are useful for the diagnosis of disseminating disease, although since this procedure is invasive it is not

advisable for seriously ill patients. Examination of bronchial secretions or washings, collected by a bronchoscope, to avoid invasive procedures and to eliminate contamination by mouth flora, is helpful.

Candida species grow readily over a wide range of pH values and on most solid media. The yeast can be isolated selectively from clinical specimens contaminated with bacteria by the addition of antibiotics such as chloramphenicol to the medium. Some yeasts are susceptible to this compound at levels of 6.25 µg/ml or greater (McGinnis, 1980). After purifying the culture the yeast is identified using biochemical tests for assimilation and fermentation (Lodder, 1970).

As *C. albicans* is the yeast most frequently isolated from man, procedures have been developed for its rapid identification. These include the formation of germ tubes when incubated in serum at 37°C and the production of chlamydospores on a dalmau plate or Czapek Dox agar (Dawson, 1962).

Serology may be useful in many instances, especially when the results are examined in conjunction with other information such as cultural investigations and the patient's clinical condition. Antibodies and antigens associated with *Candida* may be detected in a number of ways: double diffusion, counterimmunoelectrophoresis,

indirect immunofluoresence and latex particle agglutination.

The detection of candidal antigens is a method which is being investigated with interest but these tests are in the developmental stage. The latex agglutination test appears to be the most promising (Richardson and Warnock, 1982; Hopwood, Evans and Carney, 1985; Fung, Donata and Tilton, 1986). However, experience is required to interpret the results as there may be false positive results, problems with the sensitivity of the antibody coated latex with storage and cross-reaction of this reagent with rheumatoid factor.

A single positive antibody test result is not indicative of infection since, for example, patients who have undergone cardiac surgery have high titres in the absence of overt systemic infection (Evans and Forster 1976). The number of yeasts present in these patients is high and increases post-operatively (Evans, 1975). Seroconversion and rising titres may be helpful in diagnosis (de Repentigny and Reiss, 1984).

The presence of *Candida* endophthalmitis may indicate that the patient has systemic candidosis. Patients present with ocular pain, blurred vision, photophobia and may be conscious of floaters in their visual field. The eyes often appear blood-shot with circumlimbal injection and

episcleral vessel dilation. On ophthalmic examination off-white cotton wool-like masses may be seen. These lesions may resemble "a string of pearls" (Aguilar *et al.*, 1979) extending into the occasionally hazy vitreous. If there has been circumfrential haemorrage then the lesions may resemble Roth spots. Clinical appearance is often used to make the diagnosis but three procedures employed to obtain a definitive diagnosis are: anterior chamber aspiration, although this is rarely helpful in cases of haematogenously spread *Candida* endophthalmitis; a vitreous tap and a diagnostic vitrectomy through the pars plana. The last method is the most effective (Edwards 1985). Microscopical examination of smears or culture from such samples provide good evidence of infection. A negative result may be due to the relatively small number of yeasts present, the difficulty in aspirating a lesion through a fine needle or the vitrectomy not being lesion directed.

1.8.2. Aspergillosis

A single isolation of an *Aspergillus* species from a clinical specimen is not sufficient evidence that it is causing disease as the aspergilli are commom laboratory contaminants. The fungus must be demonstrated in body tissues or fluids by direct microscopical examination of a potassium hydroxide mount of the sample or histological

examination of biopsy material. Moreover, it must be isolated from repeated specimens and identified by morphological characteristics.

Examination of the sample may also help to elucidate the form of aspergillosis, for example the peripheral blood and sputum of allergic cases may exhibit eosinophilia.

Contamination is possible at every stage of specimen collection, transportation and processing as the aspergilli are ubiquitous in the environment. The traditional 24 hour sputum collection gives rise to contamination while the sample is being collected. The time interval allows any yeast contamination from the mouth flora to multiply thus obscuring filamentous fungi which are usually slower growing and present in smaller numbers.

Isolation of aspergilli is relatively simple on most mycological laboratory agars as they possess few special nutritional requirements. Identification is most frequently performed on 2% malt or Czapek Dox agar at 28° C, there are several texbooks that detail the identification of the aspergilli but the definitive text is Raper and Fennel's "The genus Aspergillus" (1965). With non sporing varieties of the fungus, exoantigen tests may prove helpful in identification (Kaufman, 1981).

The fungus is not susceptible to chloramphenicol, which, if included in the media reduces bacterial contamination. However, there may be problems with overgrowth of the sample with yeasts. The pathogenic *Aspergillus* species are thermotolerant and will generally grow at 28, 37 or 45 °C. *A. fumigatus*, the most frequently encountered species causing disease in man, will grow at 45° C while yeasts will not.

Biopsy specimens are invaluable in the diagnosis of aspergillosis particularly if invasive disease is suspected. Both transthoracic and open lung biopsy have been used.

Serological tests can prove useful in diagnosis. Longbottom and Pepys (1964) correlated clinical types of infection with skin tests and double diffusion for the detection of precipitins. Double diffusion and counter-immunoelectrophoresis are the most widely used but enzyme linked immunosorbent assay (ELISA) for antibody levels may also be used. However, in patients with invasive aspergillosis only a small proportion of cases produce detectable antibodies as the patients tend to be immunosuppressed and if antibodies are present they usually have a low titre. In allergic aspergillosis elevated levels of specific IgE are frequently found. ELISA methods are being developed for antigen detection

(Sabetta, Minter and Andriole, 1985). In cases of aspergilloma, skin tests are of little use, although the immediate and late skin tests might be positive in some cases. However, these tests are often positive in cases of allergic bronchopulmonary disease, showing a type 3 Arthus reaction.

Radiological techniques can be helpful in the diagnosis of aspergillosis particularly the pulmonary presentations. On radiography the picture of an aspergilloma is that of a round opaque mass clearly delineated within a cavity. When the patient is tilted the mass is seen to move. A crescent shaped radiolucent area called Monod's sign may be present over the upper part of the mass (Rippon, 1974). In the allergic form of the disease there may be only transient lung shadows, segmental shadowing or extensive fibrosis and some cavitation. Whereas with invasive pulmonary aspergillosis the picture is one of focal consolidation with a broad spectrum of radiological changes. Round the flare at the area of consolidation there is a radiodense periphery extending into the lung parenchyma (Roberts *et al.*, 1984).

1.8.3. Mucormycosis

The rapid diagnosis of this disease is imperative since of all the fungal infections occurring in the United

Kingdom it is the most rapidly fatal and to prevent spread immediate excision of the infected tissue and the earliest possible treatment with the antifungal amphotericin B is required.

Clinical signs depend on the underlying condition of the host. Its most common manifestation is as rhinocerebral mucormycosis in diabetic patients. Diagnosis at an advanced stage is relatively easy but for a good prognosis early diagnosis is vital. The most useful clue may be a dark necrotic discharge which resembles dried blood eminating from the patient's nasal pharynx or palate. Swabs of the area may be negative and may be of little value. Multiple biopsies are the ideal as they provide enough material to allow for both culture and histological examination. Care should be taken when obtaining biopsies since the causal fungi have a predilection for invading blood vessels and the subsequent necrosis which occurs means the organism is unlikely to be found in the centre of a lesion. It is often more fruitful, therefore, to look for the fungus at the edge "upstream" from the centre. In patients with the cutaneous form of the disease biopsies should be taken from the growing edge of the lesion.

Often direct examination, after clearing in potassium hydroxide, provides enough evidence to initiate treatment

without having to wait for culture results, which unfortunately are positive in a relatively low proportion of cases. Although failure to see fungi in a potassium hydroxide mount does not mean it is not present (Greer and Rodgers, 1985). Cryostat sections can accelerate the diagnosis of mucormycosis and the time they save could be critical.

Biopsies should be processed immediately, whenever possible but if storage is necessary, this should be at room temperature as mucoraceous fungi do not survive for longer than a few hours on refrigeration.

The mucorales will grow on most media wich does not contain cycloheximide, however other antibiotics are required in the media to prevent bacterial overgrowth, especially when purulent exudates are cultured. The numbers of viable organisms present in tissue is small, so a heavy inoculum is required and minicing the tissue is preferable to homogenisation as it kills fewer hyphae. When cultures are obtained, *R. oryzae* is most often the fungus isolated.

Other aids that have been used in the diagnosis of mucormycosis include radiology, which delineates the extent of infection but does not identify the etiologic agent and angiography, which has been used to define the extent of vascular destruction. Serological studies for

antibodies have shown that precipitins are not always present in patients with proven mucormycosis. However, this could be related to the small numbers of reports, as the work is only in the preliminary stages (Jones and Kaufman, 1978). Skin tests have not proved to be specific enough for diagnostic purposes.

Examination of faeces and gastric aspirates have sometimes proved useful in the diagnosis of the gastrointestinal form of the disease.

As fungal elements are not always present or are small in number in discharges and in tissue, diagnosis is often based on clinical evidence. As these fungi are ubiquitous in the environment a positive culture result, in addition to being difficult to obtain, cannot be taken as irrefutable evidence of infection but, in any patient at risk, an isolation of a mucoraceous fungus cannot be dismissed as transient flora or contamination.

1.8.4. Cryptococcosis

Cryptococcal meningitis is the usual presentation of this disease and it is the form with the poorest prognosis especially in a debilitated individual. The outcome will be fatal unless the condition is diagnosed and treated. Unfortunately there are no symptoms that will give an accurate differential diagnosis. Chronic disease of any

organ is marked by recurrence and exacerbation of the infection which renders the assessment of treatment difficult.

In the pulmonary disease the x-ray picture is variable, although the organism is generally seen in the lower lung fields. The picture may be that of a solitary dense infiltrate or of a broader more diffuse infiltrate that may show fibrosis following healing. The disease may appear as extensive peribronchial infiltrates or, as often occurs in patients with leukaemia and lymphomas, there may be lesions in all areas of the lungs. Walled off lesions may require surgery to exclude carcinoma. Active lesions are granulomatous masses containing yeasts which can displace the host tissue mechanically. If the lesion penetrates the wall of the bronchus, masses of yeast cells may be present in the sputum but in general the scanty sputum which is produced does not contain yeast cells.

As there are no clinical features which are pathognomic for cryptococcosis the identification of the aetiologic agent starts with the microscopical examination of a specimen. Direct microscopical examination might not permit identification to species but will provide useful early clues, for example the presence of a capsule, the size and shape of the yeast, whether pseudomycelium is found and the presence or absence of buds and how they are

attached. A minimum of five ml of cerebral spinal fluid is required for adequate microscopy and culture. The sample should be centrifuged or membrane filtered to concentrate the solids. A wet mount made in India ink can show up the capsule but experience is needed to differentiate yeasts from blood cells and lipid granules. Alcian blue and mucicarmine stains are useful in the detection of cryptococci. Fluorescent antibody techniques have also proved useful as have calcofluor white stains (Hollander *et al.*, 1984).

C. neoformans is not a fastidious organism but it is sensitive to cycloheximide. The yeast will grow on many routine laboratory media and can develop in culture after 24-48 hours although it is best to retain the culture plates for six weeks. Birdseed agar is a differential medium for *Cryptococcus* on primary isolation. It is made with an extract of the Indian thistle plant and *C. neoformans* develops as brown colonies within a week. The colour is caused by a phenol oxidase mediated reaction which leads to the formation of a melanin pigment in the yeast cell walls.

In a urine sample catheterised from the lower urogenitary tract more than 10^4 colony forming units of yeast per ml are required for convincing diagnosis of

urinary tract infection. However a single colony of *C. neoformans* from CSF is suggestive of infection.

Samples of sputum rarely yield *C. neoformans* on culture, Tynes *et al.* (1968) have reported asymptomatic carriage, indicating that a positive culture may not be indicative of an infection but must be regarded with suspicion in patients who are at risk of infection.

Examination and culture of biopsy specimens can be helpful in the diagnosis.

In osseous infections the diagnosis is indicated by the relatively stationary appearance of the lesions and lack of periosteal proliferation.

Skin tests are of little value in the diagnosis of cryptococcosis, as the capsular antigen does not elicit a response in a significant number of patients. A positive skin test is not indicative of an active infection.

Serology is very useful in detecting cryptococcosis. The most valuable test has proved to be the latex agglutination test to detect the polysaccharide capsule antigen. The titre of this test can be employed to monitor the progression or cure of the disease in response to treatment.

For noncompromised patients the guidelines recommended to ensure that there is an active infection and not merely colonisation, include: pyrexia, the absence of any other

chronic pulmonary disease and the detection of cryptococcus antigen in serum or CSF. Other investigations which maybe helpful are full physical examination, including examination of the skin for rashes, a blood screen including biochemistry for liver function tests and electrolytes, culture of blood and CSF, protein and sugar levels in the CSF.

Another test for *C. neoformans* is pathogenicity for mice, in which infection of the brain leads to death in four to five days if injected intracranially and in one week if injected in the tail vein with a suspension of 10^4 *C. neoformans*.

As *C. neoformans* elicits minimal inflammatory reactions, the gross pathology of an infected organ especially the CNS is often normal. Tissue reaction is sparse but in an active infection a focus may evolve into a tuberculoid granuloma. Early lesions are gelatinous and contain aggregates of encapsulated yeasts while older lesions are granulomatous with the degree of granuloma formation being related to the resistance of the host to infection. Necrosis and cavitation are rarely seen in a cryptococcal infection. An enlarging brain lesion has the appearance of a growing neoplasm and often patients may have to undergo neurosurgical procedures before the correct diagnosis is made.

1.9. Histology of Fungal Infections

The fungi capable of causing opportunistic infections in man can usually be isolated from tissues, exudates and body fluids but often diagnosis is based on a combination of clinical history, serological investigations, cultural studies and histopathological evidence. Isolation of a fungal pathogen may take several days and on occasions may take a few weeks, which may be too long to wait before initiating treatment, if the patient is to survive the fungal challenge. The rapid diagnosis of a fungal infection is particularly important if the patient is seriously ill with an underlying disease or is immunocompromised, as is often the case with individuals susceptible to opportunistic infections.

When a fungal infection has not been suspected, the entire available biopsy or autopsy material may be fixed for histological examination and therefore culture cannot be attempted. In such instances, the recognition and accurate identification of fungal elements in frozen or paraffin embedded sections is important. Histological recognition of opportunistic infections also provides proof of invasion by the fungus which may have been mistaken for a laboratory contaminant or part of the commensal flora.

Histopathology will reveal tissue reactions, detect the presence of fungi and disclose morphological characteristics which enable the fungus to be identified. The size, chemical composition and morphology of fungi can be studied by light microscopy and, when the classical form of the fungus is seen, the mycosis can be named. The appearance of fungal lesions in tissues depends on many variables such as the organ involved, the underlying disease, the resistance of the host and the duration of the infection. In the prevailing conditions, inflammatory reactions to fungal invasions are similar regardless of which agent is involved but an inflammatory reaction does alert the pathologist to the fact that there is an infection present. However, in immunosuppressed patients the host response may be minimal.

1.9.1. Stains

(i) Haematoxylin and Eosin (H & E)

This stain is used to show the tissue response to the aetiologic agent and will also allow distinction between hyaline and dematiaceous fungi. As the special fungal stains do not visualise tissue reactions a H & E counterstain with the Gomori method is recomended by some experts (Chandler *et al.*, 1980; Roberts *et al.*, 1984). H and E is the stain of choice for demonstrating Splendore-

Hoeppli material. However, although aspergilli and zygomycetes do stain with this method other fungi stain poorly. Small numbers of fungi may be overlooked and some difficulty is encountered in distinguishing fungal elements from tissue components.

(ii) Grocott's/Gomori's (GMS)

This stain shows the fungi as brown or black. In the presence of chromic acid, the hydroxyl groups of the polysaccharides in the hyphal walls are oxidised to aldehydes which reduce the methenamine silver nitrate complex, resulting in the deposition of reduced silver at these foci. Light green is a frequently utilised counterstain as it gives a uniform background, however, light green does not demonstrate the presence of any tissue reactions. If sections are overstained then erythrocytes and naked nuclei may resemble yeast cells and blood vessels, especially if branched, are easily confused with zycomycetes

(iii) Periodic Acid Schiff's (PAS)

With this stain the polysaccharides of the fungal cell walls are oxidised to aldehydes in the presence of periodic acid. The aldehydes react with Schiff's reagent to give a reddish purple colour. To eliminate some of the

nonspecific staining seen with PAS the sections can be pretreated by diastase digestion to remove glycogen, this results in a better contrast with the background. The PAS technique stains calcific bodies which can resemble yeasts.

(iv) Gridley

This stain utilises chromic acid to oxidise the fungal polysaccharides which are then reacted with Schiff's reagent. The mycelium stains a deep blue and any conidia, on the rare occasions when they are present, stain rose to purple.

(v) Fluorescent Techniques

Fluorescent agents have been used to enhance the visibility of fungal elements in specimens. Fluorescent brighteners such as calcofluor white and Blankophor, possess a diaminostilbene backbone structure and have high affinities for cellulose and chitin, as they bind to β1-3 and β1-4 glycosidic linkages of polysaccharides. Moheit, Cowan and Moore, (1984) demonstrated *C. albicans*, *A. fumigatus*, *R. oryzae* and *C. neoformans* in paraffin embedded and frozen sections of tissue from humans and animals using the nonspecific fluorescent stain calcofluor white. Monheit *et al.* (1985; 1986) used the brightener in

conjunction with papanicolaou stain to detect fungi in cytology specimens.

Hollander *et al.* (1984) employed Blankophor BA to examine samples of skin and nail. Frozen and paraffin tissue sections were also examined. *C. albicans*, *C. neoformans*, *Mucor sp.* and several dermatophytes were readily detected with this agent.

Although calcoflour white is the brightener most often used, other whiteners with different backbone structures have been tested. Three compounds with a diaminostilbenedisulfonic acid-cyanuric chloride backbone structure have been reported as having superior fluorescence on fungal wet mounts (Green and Moore, 1985).

Fluorescent agents are technically simpler to employ than other histological methods, however a fluorescence microscope is required and the preparations are not permanent. There may be false positive reactions due to contamination of the slides with cotton wool, paper fibres, pollen and spores.

1.9.2. Candidosis

The diagnosis of candidosis may rely on demonstrating invasion of the tissues by the fungus, since culture results do not differentiate between invasion and colonisation. Therefore, candidosis is sometimes not

diagnosed until autopsy when the predominance of *Candida* species in a lesion assumes a pathogenic significance and the large number of yeasts may result in inflammatory reactions due to cellular components. Once invasion of tissue occurs, although there are both yeasts and mycelial forms present, the proportion of hyphal elements increases with the age of the lesion (Okudaira, 1985).

Terminal infection is frequently acute with lesions characterised by suppuration and necrosis. The multifocal microabscesses have an abundance of degenerating neutrophils associated with pseudomycelium, hyphae and blastospores. The abscesses are associated with acute pyogenic reaction and radiating colonies of *Candida* may also be present (Chandler *et al.*, 1980). Fibrin and erythrocytes are other components of these lesions. Small masses of yeast cells surrounded by tissue macrophages are seen throughout the tissue especially in the brain. The characteristic abscesses may not have time to develop in early stages of infection or they may not be formed in immunocompromised patients, due to suppression of the host's cellular response. In such cases haemorrhagic necrosis associated with hyphae may be seen. Aspergilli and Zygomycetes have a great predilection for invading blood vessels but this also is seen on occasions with *Candida*.

In the rare cases of chronic candidal infection multiple abscesses are ringed by epithelioid cells, giant cells and fibroblasts. *Candida* may also be seen in solid granulomas which simulate tuberculosis but the candidal lesions are not so well organised.

The kidneys are the organs most frequently involved in systemic candidal infection and may become so invaded that they cease to function. Infection may be restricted to the cortex, sometimes the medulla may also be invaded. Gut lesions lead to ulcer formation and necrosis. In lung sections *C. glabrata* can resemble *Histoplasma* and specific immunohistological stains may be required to differentiate the two fungi.

In superficial infections *Candida* is restricted to the stratum corneum but hyperkeratosis and acanthosis of the epidermis with chronic inflammatory reaction in the dermis is often seen.

The picture in chronic mucocutaneous candidosis is different from other forms of candidosis. There are masses of hyphae and budding yeast cells with blastospores more numerous in the epithelium and hyphal forms more prevalent in the deeper epidermis. Inflammatory cells are present in the underlying tissues. The epidermis may exhibit varying degrees of hyperkeratosis, acanthosis and pseudoepitheliomatous hyperplasia. Dense collections of

lymphocytes, plasma cells, macrophages and foreign body giant cells are present in the dermis.

Candida stains poorly with H & E and may be easily overlooked. The fungal stains PAS, GMS and Gridley's demonstrate the organism well, although, since they are non specific stains, they do not differentiate *Candida* from other fungi.

1.9.3. Aspergillosis

The demonstration of *Aspergillus* in biopsy or autopsy tissue is irrefutable evidence of infection by the organism. If there is an airspace adjacent to the fungus then conidial heads may be seen but these are not formed if the fungus is invading tissue.

The aspergilli stain with H & E if the tissue is properly fixed and not necrotic but they can be difficult to see (Chandler, *et al.*, 1980). PAS may be disappointing (Rippon, 1974).

The aspergilli in tissue are characterised by repeated dichotomous branching at a 45° angle. The hyphae are septate but the septae may not be readily evident and the mycelium may resemble a mucoraceous fungus. The hyphal walls are parallel and smooth and the tip is refractile. Conidia are generally not seen but transverse sections of hyphae often resemble yeasts. The centre of a radial

lesion may contain swollen cells and yeast-like hyphae which can measure up to 8μmin diameter.

The histological picture in aspergilloma is a tangled mass of hyphae which may show zonation of mycelial elements with pyknotic layers of host cells, this presentation indicates the dynamic evolution of the lesion. There may be little tissue destruction as these fungal balls tend to form in pre-existing lung cavities. The ball of fungus is surrounded by a thin fibrotic capsule with a few inflammatory cells.

Invasive aspergillosis is an acute disease displaying necrotising, pyrogenic lesions and infarcts. The appearance of the disease is similar to mucormycosis as the fungus readily invades blood vessels causing thrombosis. Hyphae appear to penetrate the vessel walls allowing direct haematogenous spread from a primary pulmonary lesion. The classic appearance of invasive aspergillosis is of hyphae radiating from a central focus. This starburst appearance is the actinomycetoid form of the fungus. Necrosis of the surrounding tissue is due to endotoxin production in addition to vascular obstruction (Rippon, 1974; Chandler *et al.*, 1980). The host response in cases of invasive aspergillosis may be absent due to the depression of the immune system but there is usually a dense infiltrate of neutrophils.

Early lesions develop as aggregates of mycelium with radiating hyphae. The peripheral fungus is surrounded by neutrophils. A zonation pattern may be formed by slow and rapid phases of growth and variation in the density of the fungus.

Chronic lesions contain scattered giant cells, neutrophils and eosinophils. The fungal elements may be short, globose and distorted. Fibrosis is progressive and may be severe. Isolated foci are walled off by granulomatous reactions and contain giant cells similar to those seen in tuberculosis.

Sinus granulomas do not show the regular branching pattern of acute invasion but these hard fibrosised bodies are composed of aggregates of foreign body giant cells and fibrous tissue associated with the fungus. Splendore-Hoeppli material may coat the hyphal fragments. The histology is distinctive, only small fragments of mycelium are found and as a result of this morphology the infection may easily be attributed to a yeast. Occasionally, extensive local deposits of calcium oxalate crystals may be seen. These are birefringent when viewed under polarised light. The crystals are formed from oxalic acid produced by the fungus and they are seen only when the organism is associated with necrotic tissue.

1.9.4. Mucormycosis

The mucoraceous fungi are readily seen using the H & E stain and GMS but their detection rate when stained with the conventional specialised fungal stains of Gridleys and PAS is uneven and barely adequate.

The pathology does not vary with the site of infection. Gangrene involvement is common, surfaces show massive haemorrhage with recent emboli and in older lesions the appearance is putty-like. The tissue reaction is not consistent. In some cases no cellular response is seen but usually there are varying degrees of oedema and necrosis with neutrophil accumulations, plasma cells and some giant cells. The acute reaction shows the diffuse infiltration of polymorphonuclear leukocytes. Eosinophils are not often seen. Tissues often exhibit suppurative reactions but may show granulomatous changes. The characteristic feature is the involvement of the blood vessels with mucoraceous fungi invading the walls of arteries, causing thrombosis and necrosis of the adjacent tissue.

The hyphae are irregularly and haphazardly branched and lateral mycelium often grow at nearly 90°; lateral vesicles may be seen. The hyphae are sparsely septate and although the mycelium is broad, its width may vary greatly and the hyphal walls also vary in thickness. The mucoraceous fungi can be mistaken for sclerosed

capillaries, nematodes and they may also be confused with empty spherules of *C. immitis* in endemic regions. The small amount of fungus present in tissue sections and the oblique angles at which they are often cut, makes their identification by histological morphology alone difficult.

1.9.5. Cryptococcosis

In tissues cryptococci are present as unicellular, yeasts, producing buds and if surrounded by a capsule can be detected fairly readily. The size of the capsule varies from 4-15μmand may be five times the size of the cell.

There are two forms of lesions, granulomatous with a few organisms or thick walled cysts with large numbers of yeasts. In active lesions there are multiple budding yeasts.

Tissue reactions vary a good deal from little inflammatory response to granulomatous reaction. Although reactions vary from case to case and with different organs, the tissue reactions tend to be mild, with few macrophages appearing in active infection; suppuration and necrosis are infrequent.

If there is no tissue response then the yeast growth develops rapidly. In the lungs they are attacked by alveolar macrophages and giant cells. Poorly encapsulated forms elicit granulomatous inflammatory reactions,

neutrophils are not numerous and the tissue response is usually non-suppurative.

In the gelatinous histological pattern there are masses of organisms and mucoid degeneration of the tissue. There is no walling-off, the infected tissue and the yeast lie free. In these active lesions there is multiple budding.

In the granulomatous type of reaction there are smaller numbers of yeasts, almost all of which are surrounded by macrophages and giant cells. The fungi are intracellular with fragmented cell walls and poorly stained cryptococci can be seen in phagocytes. The yeasts in these lesions are "dry" variants, very small and poorly encapsulated.

When tissues are stained by H & E, cells of *C. neoformans* stain pale blue to pink and are 2-20 μmin diameter. H & E does not stain the capsular material so the yeast cells are seen surrounded by a clear zone. They have thin walls, are of varying shape and the internal contents cannot be seen in detail. A presumptive diagnosis of *C. neoformans* infection can be made on histopathological examination if the sections are stained with mucicarmine or alcian blue since other pathogenic yeasts do not absorb these stains. When stained by Mayers mucicarmine, the capsule stains bright red and the background is blue or yellow depending on the counterstain used.

1.10. Immunohistochemical Staining

Immunohistochemical techniques help to alleviate some of the problems encountered when attempting to identify fungi in tissue sections by their morphology and tissue reactions they elicit.

Van Noorden and Polak (1979) defined immunocytochemistry as "the identification of a tissue constituent *in situ* by means of a specific antigen-antibody reaction tagged by a visible label." If it can be proved the antigen-antibody reaction is specific, it is possible to identify the tissue constituent.

1.10.1. Immunofluorescence

The original method described was a direct immunofluorescent technique developed by Coons and Kaplan (1950). A fluorescent dye, fluorescein isothiocyanate, attached to an antibody, was used to identify the corresponding antigen in tissue sections. The sections were incubated with the dye in phosphate buffered saline and then examined using a microscope fitted with an ultraviolet light source. Coons and his co-workers refined the technique with the result it became widely employed by the 1960's (Coons and Kaplan, 1950; Coons, Leduc and Conolly, 1955).

The direct method requires the application of the fluorescent antibody to deparaffinised sections. With the

indirect method, the unconjugated initial antibody is washed off, then a conjugated antibody, raised against the primary animal species, is applied enabling the reaction to be seen. The indirect method produces a brighter fluorescence although the direct method is preferred by some workers (Chandler *et al.*, 1980).

Hotchi (1967) was the first to use immunofluorescence to demonstrate *Aspergillus* in lung sections. The aspergilli can be differentiated from the zygomycetes and *Candida* but as yet the identification of *Aspergillus* species is not possible (Warnock, 1979; Kaplan, 1985). *Candida* has been studied using immunofluorescence but the fluorescent conjugated antisera crossreact with the other members of the genus and other fungi eg. *H. capsulatum* (Warnock, 1979: Kaplan, 1985). Some *Candida* species may be identified by a compilation of fluorescent antibody regimes (Gordon, Elliott and Hawkins, 1967).

In contrast to the mycelial fungi, reagents have been developed for *Cryptococcus, Blastomyces, Coccidioides* and *Sporothrix* (Chandler *et al.*, 1980). Fluorescent antibody techniques are used routinely for the detection of *C. neoformans* in tissue and in CSF. Marshall *et al.* (1961) were the first to stain histological tissue sections from cases of cryptococcosis using direct immunofluorescence.

The indirect method has been used to demonstrate *Trichophyton rubrum* in frozen sections of biopsy material from chronic dermatophyte infections (Hay and Saeed,

1981). However cross-reaction with *T. mentagrophytes* var *interdigitale* and *T. mentagrophytes* var *mentagrophytes* occurs.

The antigenic properties of the fungi in tissue sections are not altered by prolonged storage (Kaplan, 1985). As a result retrospective immunohistochemical staining can be performed to test new antibodies and examine old case histories. It is possible to restain H & E stained sections by an immunohistochemical method, if no other material is available. PAS and GMS stained sections cannot be restained as the oxidation of the wall polysaccarides alters their antigenicity, therefore there is no reaction with the antibody.

Ideally, the sections to be examined are most satisfactory if they are thin, 4-6μm; the tissue is not dense and the fungal elements are numerous. If the material is not satisfactory, the sections may need to be treated with trypsin to expose more antigenic sites. If there is calcification of the tissue the sections will have to be decalcified before staining (Kaplan, 1985; Chandler *et al.*, 1980). Other disadvantages of this technique are: a fluorescent microscope is essential, the background details may be difficult to discern and the slides are not permanent as the stains cannot withstand dehydration.

1.10.2. Peroxidase Staining Methods

Nakae and Pierce (1966) introduced the use of enzyme labels to immunohistochemistry employing the enzyme peroxidase conjugated to an antibody. Histochemical development of the antigen-antibody reaction results in a coloured end product which can be seen using a light microscope. The reaction is made visible with diaminobenzidine (DAB) using the reaction described by Graham and Karnovsky (1966). Development of enzyme reactions can be monitored and stopped when the "signal to noise" ratio is at its optimum. That is when there is sufficient strength of specific staining with as little background staining as possible. There is no requirement for specialised equipment and the preparations can be stored indefinitely. Disadvantages of the technique are, the danger that sections may become detached during the procedure, the endogenous peroxidase activity must be blocked and DAB may be carcinogenic on prolonged exposure at high concentrations.

(i) Direct Method

The direct method employs a labelled primary antibody which is applied directly to the section, it then attaches to antigenic sites.

(ii) Indirect Method

The indirect method is more sensitive, the primary antibody is not conjugated and it is the secondary antibody which is tagged with the enzyme. The first antibody is applied to the section, washed off, then the second antibody raised in another species against the IgG of the primary species is applied to the section. A substrate is used to visualise the reaction, and the primary antigen site is thus revealed. The indirect method, apart from being more sensitive, may be more economical as the primary antibody is used at lower strength and, provided the antisera are all raised in the same species, one labelled antibody can be utilised with many antigens. The disadvantages of this method are the additional washing steps and incubation stages required. They make the test more time consuming and there is a greater danger that the sections may float off during the staining proceedure.

(iii) Peroxidase Anti-peroxidase Method

One of the more sensitive of the immunoperoxidase methods is the peroxidase anti-peroxidase (PAP) technique (Sternberger, 1979). This involves the use of a bridging antibody between the primary antiserum and the final conjugated antibody. The final layer, a peroxidase anti-peroxidase combination, is a cyclic complex with three peroxidase molecules to two antibody molecules. As this

enables more peroxidase to attach at the reaction site than in the indirect technique, the method is extremely sensitive. The technique allows for higher dilution of the primary antibody as a result of the magnification by the third antibody and this reduces the background staining. The second layer antibody must be in excess of the primary antibody to achieve competition for the binding sites. This means that only one site of the second antibody is occupied by the primary antibody, leaving one site free to combine with the PAP complex.

As the peroxidase is attached to the complex by immunological and not chemical bonds, it loses none of its enzymatic activity. This reduces nonspecific staining as the higher dilutions employed eliminate unwanted antibodies present at low titres.

(iv) Immunoperoxidase Stains in Diagnosis of Fungal Infections

Immunoperoxidase staining methods have not been employed routinely in diagnostic medical mycology. However, the indirect method of immunoperoxidase staining has been used to recognise *Sporothrix schenckii*, *C. neoformans* (Russell, Beckett and Jacobs, 1979) and *A. fumigatus* (Saeed and Hay 1981) in tissue sections.

The reports so far described for fungi have used dried fungal smears as the positive control material. The negative control material for comparison was provided by

air dried smears from fungal suspensions (Russell *et al.*, 1979) or sections from cases of mucormycosis or candidosis (Saeed and Hay, 1981).

Controls have an important role in histochemical staining to ensure that the staining components are working and that the technique has been carried out correctly. Control material is also valuable in the determination of the specificity of the method to stain the organisms involved. The lack of availability of optimal control material, especially for fungal infections, can be a problem. Jung (1985) developed a fairly complicated system to produce control material by culturing micro-organisms in clotted plasma. Moreover he noted staining differences when other types of positive *in vitro* controls such as lyophilized fungi were used.

With all immunohistochemical techniques the specificity of the antibody determines the specificity of the test. The antigen injected into the rabbits, to raise the antiserum, ideally should be as pure as possible to raise as specific an antigen as possible. Despite this, the antiserum will not be active solely against the challenge antigen. The serum will contain natural antibodies which may cross-react with tissue components. Therefore an antiserum cannot be deemed specific unless stringent controls are applied.

Tests for the presence of antibodies in the serum include counterimmunoelectrophoresis (CIE) (de Repentigny

and Reiss, 1984), ELISA and, most importantly, the staining of known positive tissue. The serum must be evaluated against staining of the background and tissue constituents other than the ones intended to be stained.

Characteristics of a good antibody include good affinity to the antigen, it must have strong avidity in order not to be washed away during the washing procedure. The antiserum should have a high titre to allow dilution of unwanted antibodies to below a significant level and to allow for less "background to noise" ratio.

The antigen in the tissue must be insoluble yet available to the antibody. The architecture of the tissue should remain recognisable after processing to help with orientation of the sections. Fixation may cause problems as cross-linkages of tissue proteins are formed with aldehyde fixatives. Pretreatment with a protease such as trypsin prior to staining breaks cross-linkages to reveal more antigenic sites. However it is possible that some large protein molecules are cleaved to liberate sites not available before the pretreatment and so resulting in false positive results.

1.11. Cross-reactivity Among Fungal Antigens And Antibodies

Fungi have a wide range of antigenic determinants, which may allow their differentiation if antigenic factors peculiar to a genus or species can be isolated. However the complexity and variability of fungal antigens still leads to controversy about the relative value of the majority of the antigens.

Fungal antigens may be proteins associated with the cytoplasmic contents, or if derived from the cell wall, they may be polysaccharides or glycoproteins. It is not yet possible to distinguish those antigens implicated in the disease process from pathologically irrelevant antigens. Studies on the nature and substrate specificity of fungal antigens may lead to a greater understanding of the pathogenicity of fungi and help to identify those antigens produced when a particular fungus is involved in a disease process.

1.11.1. Mucoraceous fungi

Only a few investigations into the antigenic relationships of the mucoraceous fungi have been made. Jones and Kaufman (1978) analysed filtrate and homogenate antigens from *Absidia*, *Mucor* and *Rhizopus spp.*. They demonstrated common antigens and also antigens which would permit differentiation of the fungi. Immunodiffusion tests with serum from proven systemic cases reacted with

antigens from each species. Immune serum showed considerable cross-reactivity if a filtrate antigen was used, whereas more genera-specific antigens were seen with homogenate antigens.

Unique and common antigens were found when 10 species of mucoraceous fungi were compared by crossed immunoelectrophoresis (Hessian and Smith, 1982). The investigators found little cross-reactivity of mucoraceous sera with *A. fumigatus* and no cross-reactivity with *C. albicans* . *R. oryzae* and *R. arrhizus* exhibited a great deal of antigenic similarity, but the *Rhizopus spp.* had only slight cross-reactivity with *Mucor, Mortierella* and *Absidia.* Moreover *Absidia* and *Mucor* exhibited relatively high antigenic sharing.

1.11.2. *Candida*

Candida expresses protein, carbohydrate and mannan antigens. The mannan antigens are responsible for the majority of the false positive reactions but they may be the only antigen to react with sera from some invasive cases (Kaufman, 1981). Mannan-free antigen is more specific but its sensitivity is poor in immunodiffusion tests.

Different serotypes of *C. albicans* appear to possess differing antigens. Serotype A and *C. tropicalis* and serotype B and *C. stellatoidea* have close antigenic similarities (Hansenclever and Mitchell, 1961).

In later studies, when tested by ELISA, patients with *C. tropicalis* infections gave positive results for *C.albicans*. *C. albicans* also cross-reacted with *Cryptococcus*. Spinal fluid and serum from patients with cryptococcal infections gave a wide range of titres when tested for antibody to *Candida* but there was no evidence of mannan antigenemia (Meckstroth *et al.*, 1981).

Reaction of human serum against antigens from *H. capsulatum*, *C. albicans* and *S. cerevisiae* occurred most frequently against the antigens shared by the three fungi, although the intensity of the reactions varied. Reactivity to antigens specific for individual fungi was uncommon. Antibodies in immunized rabbits also cross-reacted with antigens of the other two fungi (Kumar *et al.*, 1985). Magaldi and M^{a}c^{c}kenzie (1984) noted a strong cross-reaction between a rabbit antiserum raised against *C. albicans* and *Aspergillus* antigens.

Monoclonal antibodies have been employed to clarify the specificity of antigenic determinants. Polonelli and Morace (1986) demonstrated a 12 Kda antigen common to all the *C. albicans* strains tested that was not produced by the other yeasts tested. Miyakawa *et al.* (1986) produced monoclonal antibodies against a *C. albicans* mannan antigen but cross-reactivity patterns were shown with *C. tropicalis* and *C. glabrata*.

Recent work has suggested that there may be some cross-reactivity of *C. albicans* and mammalian antigens (Manning-

Zweerink *et al.*, 1986). Numerous *C. albicans* antigens were detected, by immunoblot analysis, in serum samples from patients with systemic candidosis but the same antigens were also detected in normal subjects. Hyperimmune rabbit serum was used to detect several antigens of *C. albicans* in human sera from patients with systemic candidosis, the same antigens were detected from normal sera. Pre-immune rabbit serum did not react with any antigens suggesting that human serum may contain shared antigenic components with *C. albicans*.

1.11.3. *Aspergillus*

There is a remarkably wide range of antibodies against antigens of aspergilli. *Aspergillus* species have been shown to posses 16-26 antigenic components for each species analysed electrophoretically. The immunological similarities within groups of these fungi reflect the botanical classification of Raper and Fennel (1965), (Vernes and Biguet, 1980). *A. fumigatus* is the species most commonly associated with infection in man and its antigenic determinants have been studied in some detail. Differences in individual patients and the variety of different antigens from different strains of the fungus make it desirable to use a battery of antigens for serological testing of clinical specimens.

Longbottom and Pepys (1964) studied the significance of antigens of *A. fumigatus*. Sera giving a positive

precipitin response to *A. fumigatus* also demonstrated some cross-reactions with antigens from *A. terreus*, *A. flavus*, *A. nidulans* and *A. niger* in immunodiffusion tests. Patients with evidence of an *A. nidulans* infection not only had a precipitin response to *A. nidulans* but also had a positive response with *A. fumigatus*.

Reed (1978) found that sera positive against *A. fumigatus* antigens were often also positive with *A. clavatus* antigens. Different strains and varieties of *A. fumigatus* have high antigenc similarities. *A. flavus* immune serum reacts with *A. fumigatus* . It would appear that *A. fumigatus* is more closely related to *A. flavus* than *A. niger* based on fused rocket electrophoresis and skin tests (Kim and Chaparas, 1979).

Cross-reaction of patient sera is common between *A. fumigatus* and *A. flavus* also between *A. fumigatus* and *A. nidulans* (Vernes and Biguet, 1980).

Fractionation of antigens from *Aspergillus* species into, water soluble antigen, triton detergent extracted fractions and a triton/water soluble component has been attempted. The water soluble/triton extracted fractions were the most specific when tested by double diffusion against antiserum raised in animals. However, when the sera from patients were tested against the same antigens there was cross-reactivity amongst the different species indicating shared antigens in these extracts.

Magaldi and M^{c}kenzie (1984) noted marked cross-reactivity between species of *Aspergillus* when examined by ELISA and immunofluorescence. Antiserum raised against *A. terreus* was the most specific as it only reacted with its homologous antigen. However, the *A. terreus* antigen did cross-react with the sera raised against other species of *Aspergillus*. Antigens of *A. flavus* cross-reacted only with antiserum raised to *A. fumigatus* showing lines of identity. *A. fumigatus* antigens cross-reacted with the other three sera producing several lines of identity when examined by line immunoelectrophoresis (Magaldi and M^{c}kenzie, 1984). In general, there were only weak reactions of heterologous fungal sera against antigens of *Aspergillus* species with the exception of *C. albicans* antiserum which gave a strong response to *A. fumigatus, A. flavus, A. terreus and A. niger* antigens.

The complexity of immunological relationships of fungi necessitates the use of carefully monitored and controlled experiments when employing immune serum or fungal antigens as markers or bridges in systems for identification of fungal disease. The use of immune serum to detect fungi in tissue sections by means of immunoperoxidase staining techniques is one such system.

1.12. Fungal Infections in Drug Addicts

Drug addicts have been reported as being susceptible to mycosis caused by various fungi at different body sites. *Candida* is the most commonly reported fungus associated with infections of these individuals. Pulmonary candidosis has been reported in a heroin addict, on one occasion (Lazzarin *et al.*, 1985). Other fungal infections include cryptococcal meningitis in an intravenous cocaine abuser (Schuster, Valentine and Holzman, 1985). *Penicillium* sp. was the cause of endophthalmitis following parenteral drug abuse on one occasion but no attempt was made to establish the source of infection (Swan *et al.*, 1985).

Candidal endocarditis is a recognised complication of heroin abuse and was first described in a heroin addict by Joachim and Polayes (1940). However, of the 11 cases of endocarditis described by Luttgens (1949) only one involved *Candida* and that was in a mixed infection. Of the 28 cases of heroin associated endocarditis reported by Dreyer and Fields (1973) only one was attributed to *Candida*. These authors suspected that earlier reports overemphasised the fungal infections.

Heroin associated candidal endocarditis causes predominantly left sided valvular infection with the aortic and mitral valves most frequently involved (Sande *et al.*, 1977).

1. 12. 1. Endophthalmitis in Drug Addicts

Fungal endophthalmitis is not a common disease. Jones *et al.* (1970) reviewed all the cases of fungal endophthalmitis reported in the United Kingdom between 1880 and 1970 and could find only 30. *C. albicans* was the most commonly implicated fungus, accounting for 12 of the cases. Recently *Candida* endophthalmitis has been seen more frequently and many of the patients have been heroin addicts (Salmon, Partridge and Spalton, 1983; Servant *et al.*, 1985). Fungal endophthalmitis in heroin addicts, particularly if *Candida* is the aetiologic agent, appears to present with a different picture to that found in other patients. In addicts it is frequently unilateral with little other evidence of candidal infection and no evidence of life threatening deep seated disease.

Candida endophthalmitis was first described in 1943 by Miale and was a histological study. The first diagnosis based on clinical evidence was made by Van Buren (1958). However, the disease was not described in association with drug abuse until much later. Fungal endophthalmitis associated with intravenous drug abuse was first attributed to an *Aspergillus* sp. and no yeast forms were evident (Sugar, Mandell and Shalev, 1971). The diagnosis of the first case of endophthalmitis attributed to *C. albicans* in a drug abuser was based on an agglutination test for *Candida* in the serum and recognition of yeast elements in a sample of vitreous. Although no yeast was cultured and fluorescent

antibody tests could not confirm the identity of the yeast (Getnick and Rodrigues, 1974). Vastine *et al.* (1976) reported a cluster of four addicts with presumed candidal endophthalmitis.

The histopathology of candidal endophthalmitis was examined in a drug abuser who died as a result of a car accident seven weeks after commencement of antifungal treatment. A presumptive clinical diagnosis had been made and was confirmed by culture of a pars plana vitrectomy sample. No fungus was visible in the histological material but there were lymphocytes, neutrophils and macrophages, infiltrating the vitreous. Granulation material was seen at the optic cup extending into the vitreous, forming a fibrovascular nodule of chronic inflammatory cells and multinuclear giant cells (Stern, Fetkenhour and O'Grady, 1977).

It was Aguilar and co-workers (1979) who first suggested that the presentation of endophthalmitis in drug addicts may be different from that seen in other patients. The anterior chamber reaction seen in the three cases they described was not typically seen in other forms of the disease. These authors also emphasised the benefits of concentrating the vitreous sample before attempting culture. They suggested that *Candida* endophthalmitis might often be missed in drug addicts as there may be a long latent period between inoculation and manifestation of the clinical disease. The patients may not admit to being drug addicts and the

diagnosis may not be considered as the patients may respond initially to steroids administered for uveitis (Aguilar *et al.*, 1979).

The first report of related cases of mycotic endophthalmitis in drug abusers described a man and wife (Elliott *et al.*, 1979). The vitreous from the man grew *C. albicans*. His wife also presented with endophthalmitis associated with glaucoma, she had a history of candidal infection of the scalp, nails and mucous membranes. The wife had a positive skin test reaction to *C albicans* extract. However vitrectomy samples yielded *Aspergillus flavus, Torulopsis glabrata* and a *Helminthosporium sp.*.

Salmon *et al.*(1983) described a British addict with candidal endophthalmitis. No speculation was made as to the source of the yeast but it was thought to follow intravenous infection. Tarr (1980) also reported a case and he described the addict as being healthy apart from slight backache, The cause of this back pain was not investigated but it may have indicated another site of candidal infection. Localised candidal infection, not associated with the generalised systemic disease in heroin addicts was first alluded to by Dally *et al.*, (1982) when he reported not only endophthalmitis but hair loss and skin rashes. This theme was expanded by Collignon and Sorrell (1983) who thought that this may be a distinctive syndrome in heroin abusers. They saw seven addicts who developed eye, skin and costal cartilage lesions. The eye was involved in every instance.

Cultures from skin and costal cartilage grew *C. albicans*. The authors regarded lesions on the scalp associated with regional lymphadenopathy as being a feature of candidosis in heroin adicts. Dally, Thomas and Danan, (1983) agreed with Collignon and Sorrell's observations.

1.12.2. Clusters of *Candida* Endophthalmitis in Drug Addicts

The first evidence that candida endophthalmitis and other manifestations of candidosis could occur as epidemics in communities of heroin abusers was provided by Mellenger *et al.* (1982) who described 35 cases in the Paris region. There were cutaneous lesions in 88% of cases, eye involvement was seen in 65% of the patients and there were instances of osteoarticular and pleuropulmonary infection. The skin lesions manifest themselves as papulo-nodular sores often located in the hairy regions of the body. If present in the scalp they are likely to cause pain. Eight patients were involved in one outbreak in Australia (Hoy and Speed, 1983) and four in a small cluster in New Zealand (M^{mc}Kay, 1983). It was speculated in both these reports that the source of the yeast may have been the fresh lemon juice used to dissolve the heroin. Other outbreaks were recorded in 15 American heroin addicts (Cuandra *et al.*, 1984), in 17 in Australia (Sorrell *et al.*, 1984 : Gallo *et al.*, 1985) and 11 cases were reported in Spain (Podzamczer *et al.*, 1985). Two heroin addicts with extensive folliculitis were described from Switzerland (Calandra *et al.*, 1985) and 38 Parisian

addicts were described as having candidosis by Dupont and Drouhet, (1985). Clusters of cases have been reported in several areas of the world; in ten addicts from two groups in the Netherlands (Hogeweg and De Jong, 1983; Malecaze *et al.*, 1985) and in Glasgow in 8 addicts (Servant *et al.*, 1985). These reports predominantly involved skin papules and nodules although the eyes were infected in some instances.

1.13. Epidemiology of fungal Infections in Drug Addicts

The transmission of infection amongst heroin addicts has been ascribed to several sources but the most commonly cited hypothesis is the sharing of needles and syringes, although the prevalence of this practice is difficult to ascertain.

Bacterial infection of a married couple who shared needles was reported by Hussey *et al.* in 1944, and a malaria outbreak in heroin addicts was attributed to the communal use of injection equipment by Most (1940). The high proportion (70%) of addicts with evidence of current or past hepatitis B virus infection (Follett *et al.*, 1986) is related not only to the addicts' disregard of hygienic injection technique but the repeated and communal use of the syringes and needles. In one recent study, it was found that 68% of intravenous drug abusers engaged in needle sharing (Black *et al.*, 1986). The recent advent of AIDS and its spread through the heroin addict community, in all parts of the world, has highlighted the problems associated with sharing blood contaminated injection paraphernalia (Paine, Tonuma and Monheit, 1985; Friedland *et al.*, 1985; Follett *et al.*, 1986). Currently, 17% of all AIDS patients in the United States are intravenous drug users (Mulleady and Green, 1985).

The sharing of syringes and needles has also been implicated in the epidemiology of *Candida* endophthalmitis

in heroin addicts. This manifestation of candidosis has been reported in isolated incidents and in clusters and outbreaks, with as yet no proven common link, in the heroin communities throughout the world.

Although syringes, needles and other paraphernalia have been proposed as being the source of *Candida* endophthalmitis, on circumstantial evidence, (Vastine *et al.*, 1976; Hogeweg and De Jong, 1983; Sugar *et al.*, 1971) little effort has been made to verify this hypothesis. Sorrell *et al.* (1984) described *Candida* endophthalmitis in seven young men and reported that they had shared two syringes. Unfortunately they did not culture the needles or syringes.

In a further study, 100 random sets of injection paraphernalia, not necessarily taken from addicts with infections, confiscated from the street were cultured to identify any microbial contamination. Only 11% yielded no organism, 9% grew fungi, the rest yielded bacteria. The most frequently isolated fungus was *Saccharomyces spp.* which was isolated five times. *Aspergillus spp.* was isolated three times and *Rhodotorula* sp. once. *C. albicans* was not recovered (Tuazon, Hill and Sheagren, 1974).

The attempts that have been made at culturing syringes and needles from patients with evidence of candidosis have had varying degrees of success. Calandra *et .al.* (1985) found the culture of a syringe shared by two heroin

addicts with candidosis was positive for *C. albicans*. Podzamczer and Gudiol (1986) also isolated *C. albicans* from syringes, whereas Elliot *et al.*, (1979) cultured some injection paraphernalia and recovered *Cryptococcus laurentii* and a *Rhodotorula* species. Dupont and Drouhet (1985) believe these findings are exceptional and do not account for the epidemic nature of *Candida* endophthalmitis in heroin abusers.

Another likely source of *Candida* is contaminated street heroin and this might account for the sporadic outbreaks of the disease. In all published reports of candidosis in addicts, in which the type of heroin has been described, it has been brown heroin, regardless of where in the world the cases have presented. (Vastine *et al.*, 1976 (Chicago); Mellinger *et al.*, 1982 (Paris); Dally *et al.*, 1982; 1983 (Paris); Lopez *et al.*, 1983 (Rome); Calandra *et al.*, 1985 (Switzerland); Dupont and Drouhet, 1985 (Paris); Malecaze *et al.*, 1985 (Toulouse); Podzamczer and Gudiol, 1986 (Barcelona)). This form of the drug has appeared on the market only in the last few years during which time a syndrome of candidosis has become more frequently recognised in drug abusers.

Three patients, from seven cases described in the Netherlands, obtained their heroin, independently of each other but via the same dealer and developed *Candida* endophthalmitis about the same time, suggesting that the heroin might be the source of the infection (Hogeweg and

De Jong, 1983). Heroin obtained from a single dealer was implicated in ocular candidosis in two Australian addicts. In neither of these two instances was an attempt made to culture the drug (Sorrell *et al.*, 1984).

Culture of heroin has been attempted on several occasions without success. Malecaze *et al.* (1985) reported that all the samples they procured were sterile. Dally *et al.* (1982) cultured four samples with a negative result. Podzamczer *et al.* (1985;1986) were also unsuccessful when they cultured the drug. There is little detail as to how these investigations were performed and the size and number of samples and their relationship to the infected addicts are seldom indicated. Mellinger *et al.* (1982) reasoned that the negative findings in their study were due to the small sample size and inadequate methodology. However, Lopez *et al.* (1983) cultured 50 samples and although they found bacterial contamination in 37 no fungi were recovered. In Barcelona 210 samples of street heroin were cultured without isolation of *C. albicans* (Miro *et al.*, 1986).

To try to find a correlation between infection and street heroin, all heroin confiscated by the Chicago police department during a two week period was cultured and although no *Candida* was recovered, four unidentified fungi were isolated (Vastine *et al.*, 1976). One hundred heroin samples seized by the Bureau of Narcotics and Dangerous Drugs in the USA were cultured at random, no

Candida species, nor any other yeasts, were isolated but *Aspergillus fumigatus* was the second most common organism recovered (Tuazon, Miller and Shamsuddin, 1974).

In another study, 31 samples of street drug that were examined for chemical adulterants and microbiological contamination. 61% were positive for microbial growth. No yeasts were recovered but *Aspergillus* spp. were recovered on three occasions and *Penicillium* once. On three occasions unidentified saprophytic fungi were cultured (Moustoukas *et al.*, 1983).

Heroin has been shown to have some antimicrobial activity, although it is limited and variable (Tuazon *et al.*, 1980). The survival of *C. albicans* in heroin was studied by mixing a suspension of 120-150 colony forming units (CFU) of the yeast in 100 mg of the drug and culturing the inoculated paste one week later. *C. albicans* was not recovered (Dupont and Drouhet, 1985).

Another possible source of the *Candida* causing outbreaks of endophthalmitis, is the diluent addicts use to dissolve their drug. The brown heroin now commonly used by the addicts is not as soluble in water as the white heroin previously available. As a result many addicts use a slightly acidic solvent, such as vinegar or lemon juice (Mellenger *et al.*, 1982; Malecaze *et al.*, 1985). Sorrell *et al.*, (1984) reported two addicts who bought their drug from the same dealer also used the juice from the same lemon and similar situations have been noted elsewhere.

Three Australian patients who developed endophthalmitis had shared a lemon (Hoy and Speed, 1983) and four drug addicts from New Zealand who had used the same lemons, presented with *Candida* endophthalmitis (M^{mc}Kay, 1983). Dally *et al.*, (1982) find this phenomenon is unlikely to explain the contamination and are supported by Dupont and Drouhet (1985) who suggest that gentle heating of the mixture could have a sterilising effect . Other investigators have found fresh lemons to be an excellent culture medium for *C. albicans* (Hoy and Speed, 1983; Podzamczer and Gudiol, 1986). Fresh lemons experimentally inoculated with *C. albicans* promoted the rapid growth of the organism with even a very small inoculum resulting in a heavily contaminated lemon after one week (Newton-John, Wise and Looke, 1984). However, Miro *et al.*(1986) cultured 25 samples of lemon juice used by heroin addicts and failed to isolate *C. albicans*.

1.14. Biotyping of *Candida albicans* Isolates

Biotyping divides species of fungi into groups which may not be phylogenetically accurate. It is a procedure whereby isolates of a species may be grouped or divided according to morphological, biochemical or physiological criteria which cannot be directly attributed to genetic varieties. A strain is a physiological race but a biotype has no formal taxomonic status.

Biotyping medically important fungi has practical and epidemiological applications and a number of attempts have been made to differentiate strains of *C. albicans* (Odds, 1985). These range from serotyping into groups A and B by their antigenic composition (Hasenclever and Mitchell, 1961) to elaborate techniques distinguishing hundreds of groups. Ideally these systems will determine enough groups of *C. albicans* to enable the isolate to be "fingerprinted". The system must be sufficiently sensitive to recognise as many characteristics as required to select several groups which highlight the differences of the isolates. Unfortunately the larger the number of characteristics tested the more unwieldy the test becomes and the fewer the number of isolates which can be handled.

Colony morphology was the basis of Brown-Thomsen's (1968) biotyping system but his 18 morphological forms reverted to the form from which the majority of the isolates originated.

Warnock *et al.*, (1979) devised a method of differentiating *C. albicans* based on differences of resistance to six chemicals at concentrations which gave a differential effect. If a chemical inhibited growth then a letter code assigned to that chemical was included in the isolate's resistotype description. This biotyping method allowed for the ten control and 15 unknown strains to be tested on one plate. The system had the potential to distinguish 1092 strains but in practice it revealed a restricted number. A further disadvantage of the method was the requirement for biological titration, as there was batch to batch variation in the critical concentrations of the chemicals. In addition it proved impossible to obtain a precise pattern of inhibition for the reference strains.

This resistogram method was employed to differentiate strains of *C. albicans* in patients with vulvovaginitis. It was found that 16 of the 30 patients harboured a single type while 14 carried more than one strain (Warnock *et al.*, 1979). M^{c}Creight and Warnock (1982) modified the resistogram method and replaced four of the original chemicals to enhance the differentiation of isolates. The improved method was used to investigate cases of denture induced stomatitis and discovered one resistotype to be most prevalent (M^{c}Creight, Warnock and Watkinson, 1984).

An alternative system for the presumptive identification and strain differentiation of *C. albicans* was devised by Odds and Abbott (1980). The test was

designed for routine screening, as it can be applied to large numbers of clinical isolates. Ten biotyping agars were used, one allowed the identification of *C. albicans* and the other nine differentiated the strain types. The agar media constituents and the inoculum size were very precisely controlled in this method. Depending on the growth or non-growth or pigment production of the yeasts on the media the fungi were given a number which reads out as a digital sequence which is unique for that combination of test results. The group of numbers constitutes the biotype. This system has been modified slightly to enhance the test and to permit the identification of other medically important *Candida spp.*. Four other differentiation test plates containing inhibitory chemicals have been introduced. The glycerine assimilation test has been replaced by a test for resistance to boric acid (Odds and Abbott, 1983).

It has been shown that most people carrying *C. albicans* have the same biotype at different body sites and often for long periods of time (Warnock *et al.*, 1979; Odds, 1982; McCreight *et al.*, 1984). Biotypes have not yet been linked to pathogenicity of *C. albicans* strains but they have been shown to be informative in studying the epidemiology of infections. The results indicated that some biotypes which are isolated more frequently than others may have a greater pathogenic potential. (Burnie *et al.*, 1985).

In this study the biotyping system of Odds and Abbott (1980; 1983) was used to assist the discovery of the source of an outbreak of *Candida* endophthalmitis in Glasgow.

1.15. Animal Models For *Candida* Endophthalmitis

Investigations of the eye are performed with animal models to correlate normal and abnormal structure and function. Although there are numerous similarities amongst the visual systems of vertebrate animals, there are significant differences which must be taken into consideration when selecting a research model and when extrapolating results from one species to another. A subhuman primate provides the ideal laboratory model for eye research (Peiffer, Armstrong and Johnson, 1981) but in practice this is not possible for financial and logistic reasons.

Duke-Elder (1958) wrote a description of the comparative anatomy and physiology of animals in which he included the classification of placentals according to their retinal vascularization.

The retina of both man and the mouse is holangiotic and the entire retina receives a direct blood supply. In rabbits and hares the blood vessels are limited to the horizontal expanses and such retinae are termed merangiotic. In animals with paurangiotic retinae only small vessels are present and these extend just a short distance from the optic disc. This group includes the guinea pig. Anangiotic retinas have no retinal vessels and this is seen in some primitive mammals.

There are numerous animal models for the study of candidosis (Guentzel, Cole and Pope, 1985) and for various ophthalmic conditions (Peiffer *et al.*, 1981) but models for fungal eye diseases have been infrequently described. Studies of keratitis have been made in rabbits with both *Fusarium solani* (Forster and Rebell, 1975) and *C. albicans* and in the latter fungus corticosteroids were used to induce keratitis. (Stern, Okumamoto and Smolin, 1979).

Anterior uveitis due to *F. solani* (O'Day *et al.*, 1979) was initiated by direct transcorneal chamber inoculation. The intravitreal route has also been used for posterior segment infection by introducing the organism via a needle through the pars plana. This technique requires the animal to be heavily sedated or anaesthetised.

Histoplasmosis has been studied in several animals (Smith *et al.*, 1978) and ocular cryptococcosis has been induced in rabbits, primates and cats, Blouin and Cello (1980) successfuly reproduced lesions similar to those occuring in man. Metastatic *Candida* endophthalmitis has been produced in both rabbits and mice (Hoffman and Waubke, 1961; Hoffman, 1966). In experimental animal models designed to compare the ocular pathogenicity of *Cryptococcus neoformans, Candida glabrata* and *Aspergillus fumigatus* in relation to *C. albicans*, only *C. albicans* and *C. neoformans* could be recovered from eye cultures, and no lesions were seen on ophthalmic examination of any of the

animals infected with fungi other than *C. albicans* (Fujita *et al.*, 1982).

Candida endophthalmitis has become of increasing interest in view of its association with candidaemia, heroin addiction and its use as a diagnostic aid in disseminated candidosis. The rabbit, despite its retinal vasculature, has proved to be the most popular animal model for candidal endophthalmitis. Endophthalmitis has been induced by direct inoculation of *C. albicans* into the eye and this method has been used to assess treatment of the infection with amphotericin B and flucytosine (Peyman, Vatine and Meisels, 1975). However, this method of establishing the disease is not analogous to the route of inoculation in drug addicts or patients receiving hyperalimentation, when the organism is spread *via* the bloodstream to the eye.

Intravenous inoculation of *Candida* into the rabbit has proved useful in establishing a model for experimental haematogenous candidal endophthalmitis. In one study 88% of rabbits had ocular lesions two weeks post infection (Edwards *et al.*, 1975) and it was consistently found by culture that the numbers of organisms isolated from both the eyes and kidneys in each animal were approximately symmetrical. There was some discrepancy between the numbers of organisms seen on histological examination of tissue sections from the lesions and the number of organisms isolated on culture. This was attributed to the

sampling error caused by examining a single central section of the lesions. Demant and Easterbrook (1977) employed a rabbit model similar to that of Edwards *et al.* (1975) in an attempt to correlate quantitative culture results with the number, size and severity of the animals' clinical lesions. They did not obtain consistent results although the eyes with the worst clinical appearance did yield the highest colony count on culture.

Animal models have been designed to investigate the reliability of diagnostic vitrectomy in experimental *Candida* endophthalmitis. Henderson *et al.*, (1979) found vitreous aspiration to be a most useful procedure, since culture results correlated well with the clinical picture in experimental disease.

Although ophthalmic assessment is of diagnostic value in many patients it may not prove to be so reliable in severely immunocompromised or neutropenic patients. Henderson *et al.* (1980) reported that immunosuppression alters the development of ocular lesions in experimental haematogenous endophthalmitis and any lesions seen are small and atypical.

Species of *Candida* other than *C. albicans* have been implicated in endophthalmitis (O'Day, 1985; McCray *et al.*, 1986) but the relative ocular pathogenicity of other species of *Candida* was shown to be poor when examined using a model for haematogenous endophthalmitis. *C. parapsilosis*, *C. krusei* and *C. guillermondii* failed to

produce any ocular lesions in this model. *C. tropicalis* and *C. stellatoidea* caused extensive renal infection but not significant eye infection, they did have a low level of ocular pathogenicity but the vitreous was not infected (Edwards *et al.*, 1977).

A model of endogenous *Candida* endophthalmitis in rabbits has been used to look at the possibility of treating the disease by laser photocoagulation. Regression and healing were seen when the laser was applied to early lesions and although larger lesions did not heal they were less inflammed than those in the control animals. No damaging effects were reported (Santos, Buen and Juarez, 1967).

Many animal models have been established for the purpose of examining the penetration of drugs to the eye. Ocular structures are easily accessible and as a result a number of routes of administration are available that are not possible with other organs. However, the hydrophobic corneal epithelial layer and the hydrophilic corneal stroma over external surfaces act as barriers to topical drugs. Systemically administered drugs are hindered by the blood-ocular barrier similar to that of the blood-brain barrier (Rapoport, 1976). Contributing to the blood ocular barrier are the endothelial cells of the iris and the retinal vessels and the pigment epithelium. Inflammation may also affect ocular penetration of drugs (Peiffer *et al.*, 1981).

Toxicity of antifungal drugs has been evaluated by means of animal models of exogenous endophthalmitis. Axelrod *et al.*, (1973) found doses of 5 and 10 μg could be injected into normal rabbit eyes without change but if the dose was increased to 25 μg it was etremely toxic. In a further study Axelrod and Peyman, (1973) reversed the course of an exogenous *Candida* endophthalmitis infection in rabbits by a single intravitreal administration of 5 - 10 μg of amphotericin B. Intravitreal amphotericin B was tested in uninfected and infected vitrectomised eyes and was found to be nontoxic at the highest dose used, 5μg/0.1 ml (Huang, Peyman and M^{c}Getrick, 1979). Intravitreal amphotericin B methyl ester was not toxic at 10 μg and reversed the course of exogenous candidal endophthalmitis.

Amphotericin B was found to be toxic at concentrations greater than 15 μg per eye, it produced vitreous opacities which were dose dependant (M^{c}Getrick, Peyman and Nyberg, 1979). Raichand *et al.* (1980) concluded that amphotericin B methyl ester at 10 μg/ml in vitrectomy infusion fluid is a safe effective agent in the treatment of experimental endophthalmitis. These two sets of results are contradictory, as the volume of the vitreous is 4 ml and at a dose of 10 μg/ml the dose per eye would be 40 μg above the toxicity limit described by M^{c}Getrick *et al.* (1979). One study which compared miconazole, ketoconazole, amphotericin B and flucytosine found oral ketoconazole and

intravenous amphotericin B in combination to be an effective treatment for the disease (Jones *et al.*, 1981).

The eye is immunologically unique as it is devoid of a lymphatic system and has no directly associated lymph nodes. Some work indicates that uveal and limbal tissues play a role similar to that of secondary lymphoid tissue elsewhere in the body. Some studies have shown that blood borne lymphocytes and splenic lymphoid tissue play a role in the recognition of intra-ocular antigens. This is termed the splenocameral axis (Kaplan and Streilein, 1978; Kaplan, 1983).

2. OUTBREAK OF *CANDIDA* ENDOPHTHALMITIS IN GLASWEGIAN HEROIN ADDICTS

2.1. *Candida* Endophthalmitis in Glaswegian Heroin Addicts

Glasgow, like many large cities has an increasing problem with heroin addiction (Ditton and Spiers, 1982). Heroin is readily available in the city and a conservative estimate is that there are a 100 new addicts each year.

In this study we were concerned with one area of the city called Castlemilk. This estate is situated to the south of the city and was built in the 1950's (Figure 1).

2.1.1 Street Heroin

There are two types of street heroin; white from Asia, mainly Pakistan and brown which may originate in Pakistan, Iran or Mexico.

The most common type used in Glasgow is brown heroin. It contains many diluents such as sugar, quinine and talcum, which tend not to be soluble. The heroin is a dirty brown colour when the main dealer sells it to the "pushers" who adulterate it further with impurities such as, scouring powder and cement before selling it to the addicts (Figure 2).

The addicts in Glasgow are therefore at present buying approximately 15% pure heroin. A 5mg bag costs £5 and some addicts eventually spend as much as £150-£200 per day on heroin. They finance their habit by theft and by becoming "pushers".

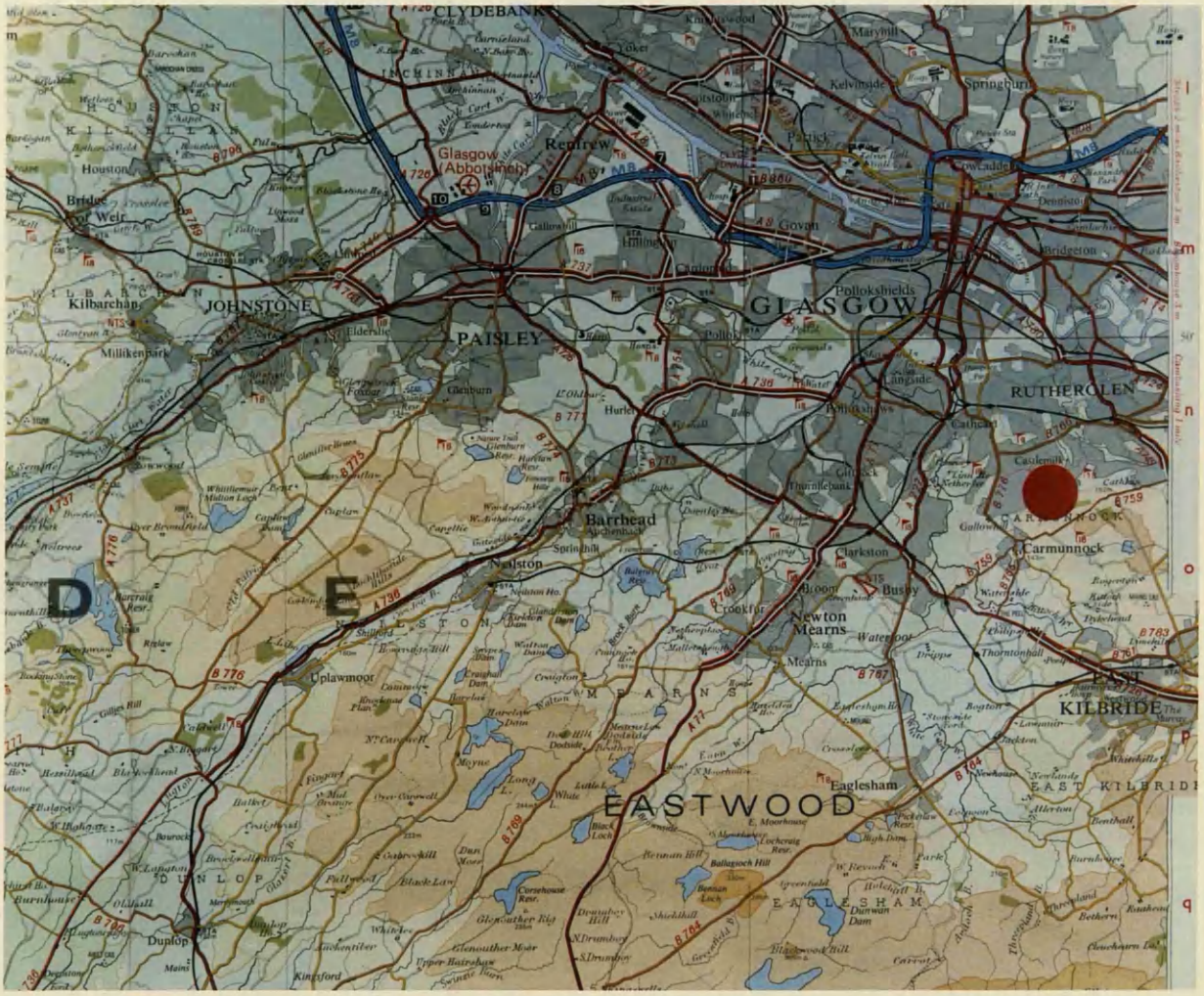


Figure 1 Position of Castlemilk

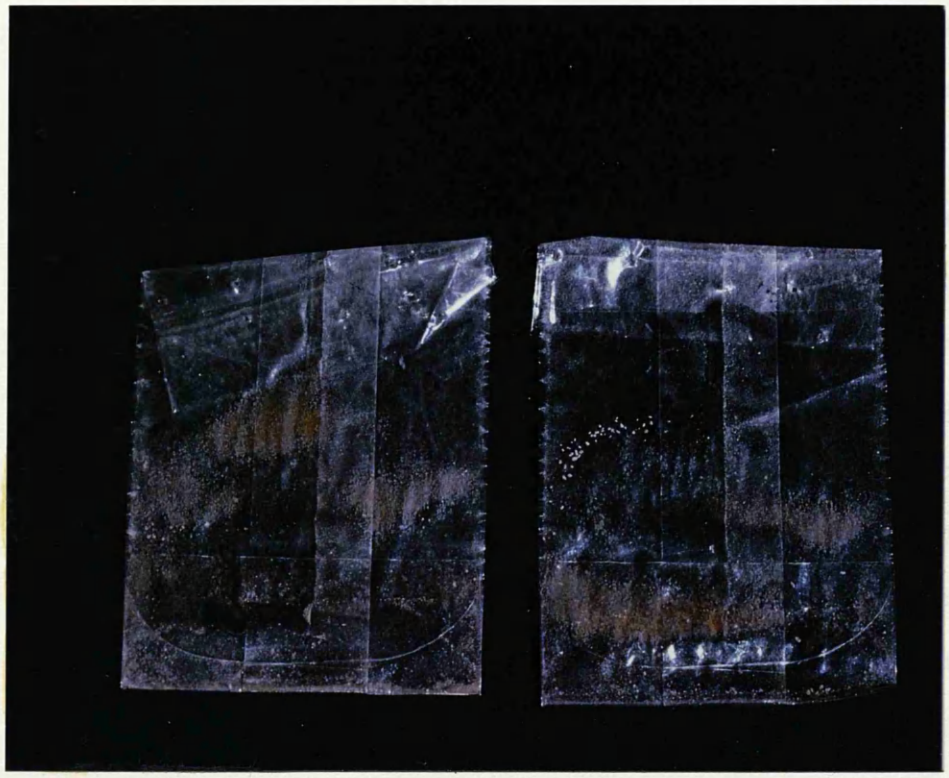


Figure 2 Heroin confiscated from a "pusher"

2.1.2. Injection Technique

The injection technique is to dissolve their drug in lemon juice, vinegar or water on a spoon. Sometimes they heat this mixture, not in order to reduce any microbial contamination but to help to dissolve the powder. They stir the mixture with the plunger from the syringe, break the filter from the end of a cigarette which they place on the spoon and draw the fluid through it into the syringe. After injection, the addicts often rinse out the syringe with their own blood, sometimes using saliva to lubricate the needle and sometimes licking their finger and holding it over the needle mark. Addicts use the same syringe repeatedly and it is suspected, share them with their friends.

2.1.3. Clinical Picture

In November 1982 the eye departments of a number of Glasgow hospitals saw several cases of what appeared to be fungal endophthalmitis. The patients presented with quite a wide range in the severity of their symptoms. Some had a red painful eye with deteriorating vision; some could just detect hand movements or count fingers at a distance of two metres (Figure 3). In other patients the eye was painless but they had blurred vision and annoying floaters in the visual field. The floaters were caused by yeasts and white blood cells in the normally clear vitreous.

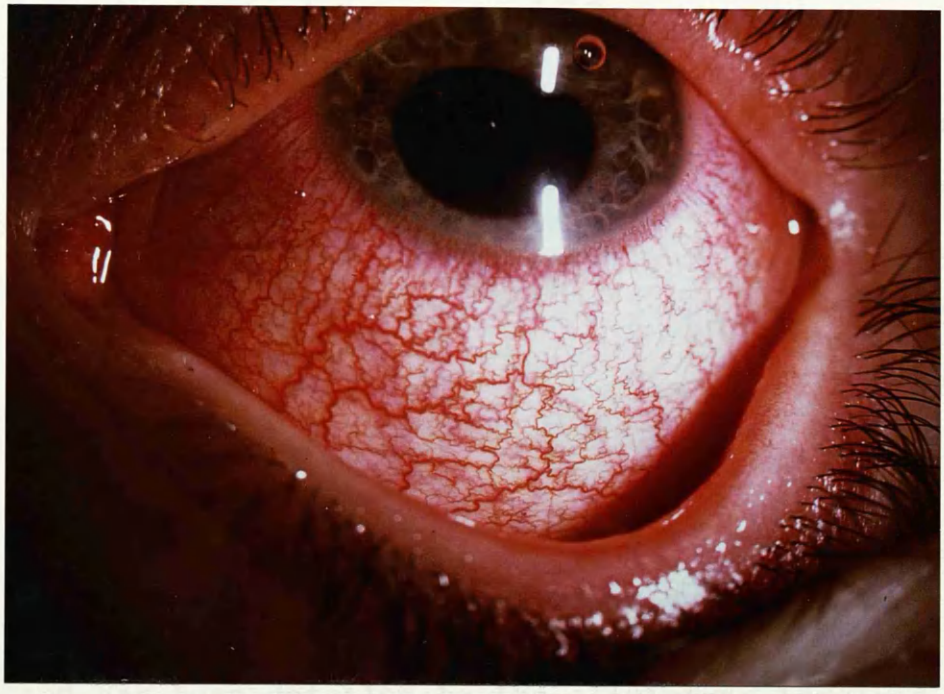


Figure 3 Addict's eye showing circumlimbal injection and episcreral vessel dilation

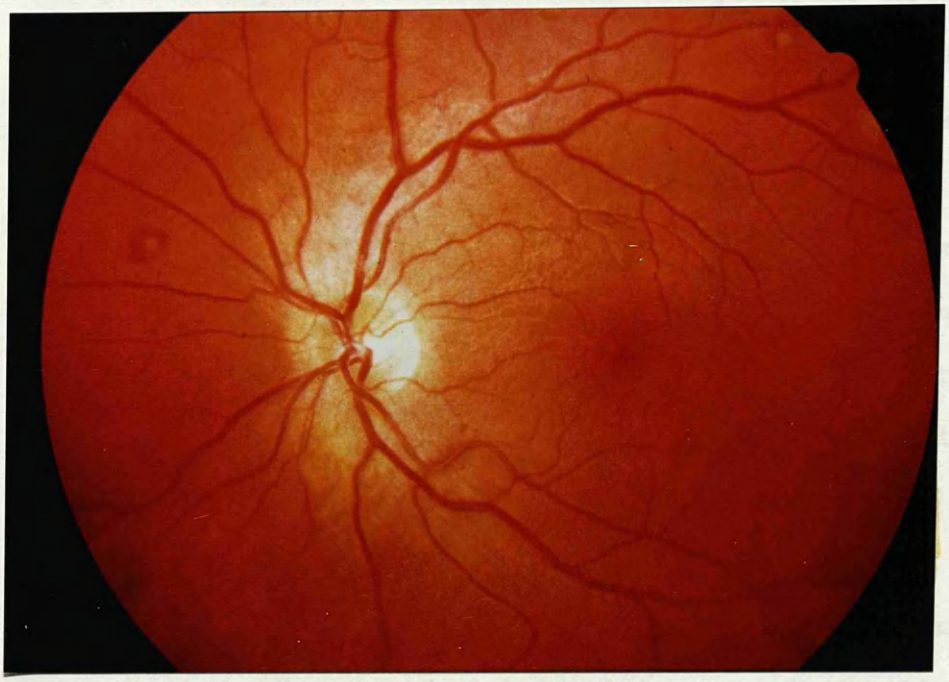


Figure 4 Photograph of a healthy retina taken through a normal vitreous.

A photograph of a retina taken through a normal vitreous shows a clear picture of blood vessels (Figure 4). In a patient with *Candida* endophthalmitis the hazy vitreous results in the retinal vasculature being out of focus. White fluffy snowballs of fungus cause refractive disturbances resulting in opacity (Figures 5 and 6).

2.1.4. Treatment of *Candida* Endophthalmitis

Treatment of the Glasgow patients varied with each case and has been detailed by the clinicians involved (Servant *et al.*, 1985). Some of the cases resolved spontaneously. Ketoconazole was used but problems of hepatotoxicity made the clinicians wary about using it with patients who already had a history of jaundice. With flucytosine, although the levels of the drug in the vitreous are therapeutic, there is a possibility of wild type and drug induced resistance.

Intravitreal amphotericin B was the treatment of choice if the condition persisted. The addicts were usually treated as out-patients. If further deterioration occurred then intravenous amphotericin B was used; sometimes in conjunction with a vitrectomy to remove some of the fungal load. There have been inconsistant reports about ocular penetration of amphotericin B after intravenous administration. Using a bioassay method on vitreous samples amphotericin B was not detected in any of the Glasgow patients. However, the samples of neat vitreous were extremely small and if the amount of drug present was low it

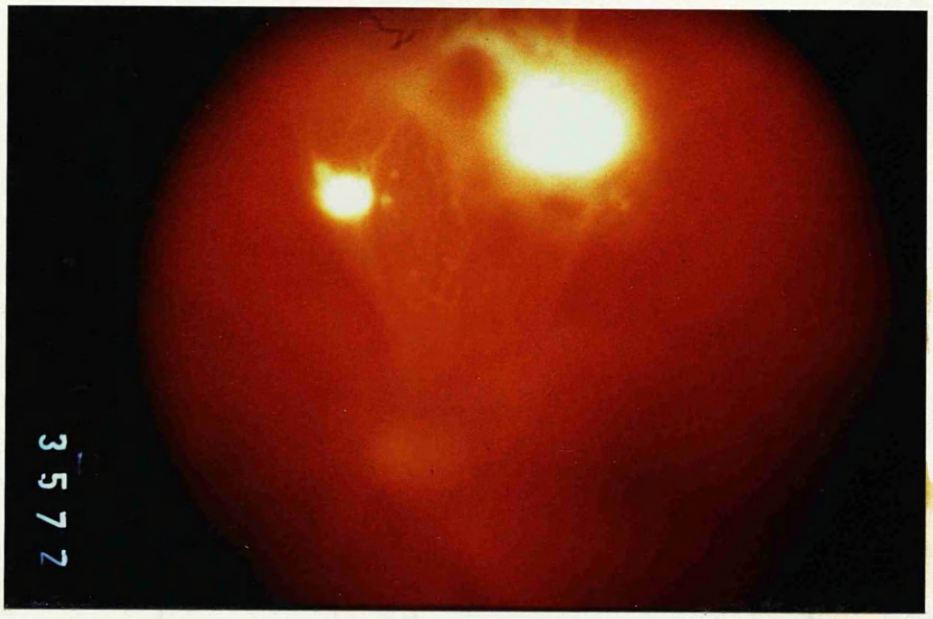


Figure 5 Characteristic "snowball" appearance of Candida endophthalmitis lesion.

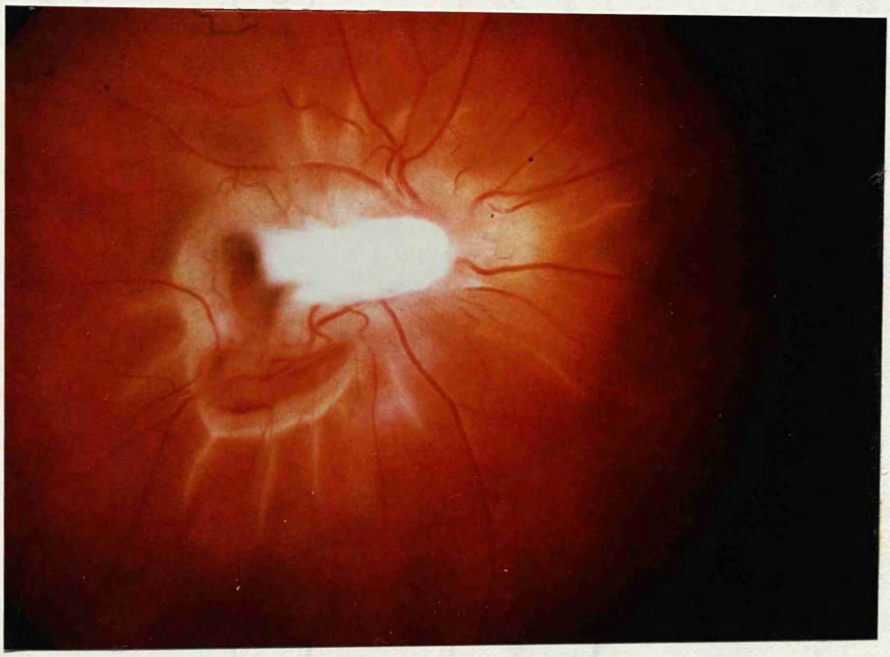


Figure 6 Lesion caused by Candida endophthalmitis.

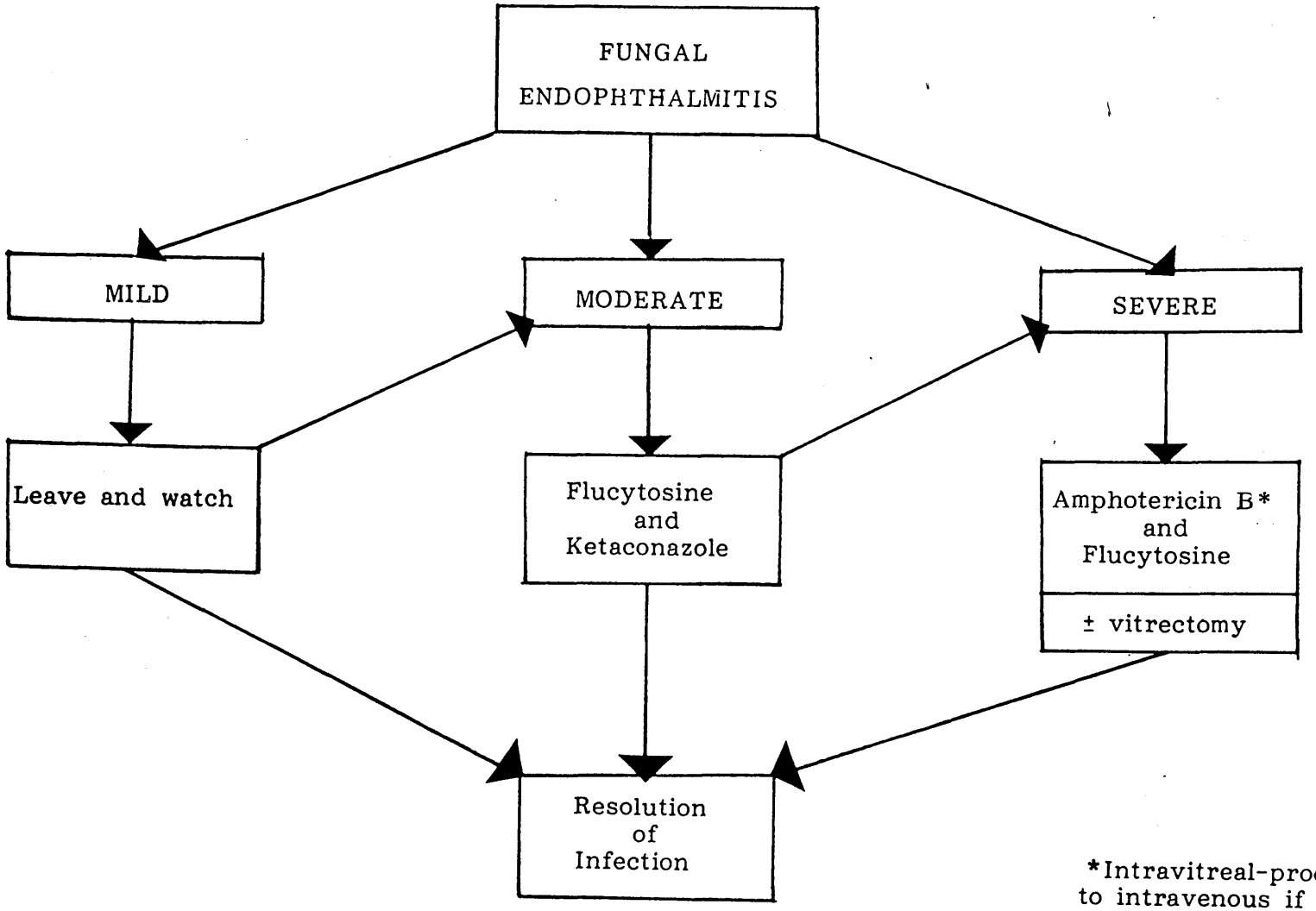


Figure 7 Treatment regimen for Candida endophthalmitis in heroin addicts in Glasgow.

was not too surprising that it was not detected as the bioassay system used was not sufficiently sensitive (Figure 7).

Even after successful treatment the patient may be left with damaged sight. In some treated eyes there is macular scarring and fibrosis which leads to retinal folding and distorted vision (Figure 8). The scarring damages the most important region of the eye which leaves the patient with only peripheral vision.

2.1.5. Patients

Twenty four Glasgow heroin addicts have now been diagnosed as having had *Candida* endophthalmitis.

Table 1: Castlemilk heroin addicts.

Male	20
Female	4
Age Range	16 - 33 years
Heroin Abuse	6 weeks - 5 years
Left Eye	15
Right Eye	2

The patients were asked to complete a questionnaire to help to discover the source of the outbreak (see page 111). The majority of the infected addicts had a connection with Castlemilk (Figure 9). Eighteen of the addicts lived in

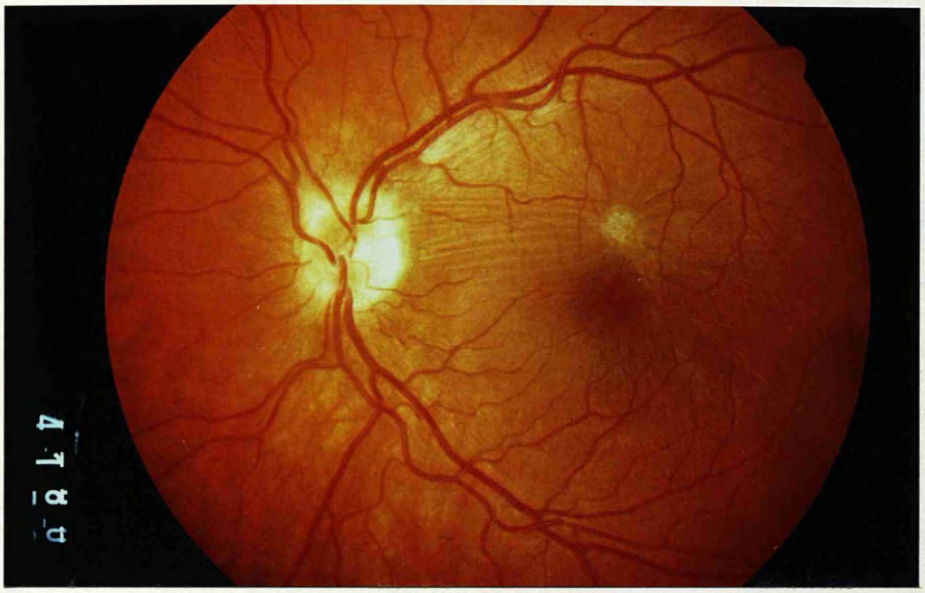


Figure 8 Photograph of a retina from a treated case of endophthalmitis, showing retinal folding and macular scarring.

Castlemilk, two admitted buying their heroin from Castlemilk, three others stayed in the south of the city. The green dot on the map shows the location of a female "pusher" who supplied several of the addicts. With the exception of a married couple and one friend, the heroin addicts claimed not to know each other.

All the addicts with *Candida* endophthalmitis seemed to dissolve their drug in "Jif" squeezy lemons and did not use water or vinegar. A number of the addicts suspected that the "pusher" may have been using shop-bought yeast to cut the drug before selling it.

The possible sources of infection appeared to be :- the drug itself, the lemon juice used as a solvent, the injection paraphernalia or they were infected from their own gut.

Figure 9 Map of Castlemilk showing the homes of heroin addicts and the suspected location of a "pusher".

CANDIDA ENDOPHTHALMITIS IN HEROIN ADDICTS

PATIENT DATA SHEET

Name:

Address:

Age: Sex

Which eye is involved?

Visual acuity:- Left eye
Right eye

Are there any lesions on the scalp or costochondral region?

Are they experiencing any hair loss?

How long have they used heroin?

What type of heroin? e.g., brown/white

What diluent do they use? e.g., lemon juice/vinegar/water.

Is the injection paraphernalia available for culture? e.g., syringes, diluent, spoon, drug wrapper.

Do they, or have they ever shared syringes or drug diluent with their friends?

Have any of their addict friends suffered deteriorating vision recently?

Have they any connection with the Castlemilk area of Glasgow?

oooOooo

The following samples would be extremely useful to help with our understanding of the aetiology of this disease.

Mouthwash

Mouth swab

Faecal sample

Vitreous

Hair with roots/skin scrapings for lesions on scalp

Venous blood for candida serology. (Please indicate Hep B and HIV test results)

Please return this form with any available samples to Gillian S. Shankland, Department of Medical Mycology, Anderson College, 56 Dumbarton Road, Glasgow. (Tel. 339-8855 Ext. 4010/4011).

3. MATERIALS AND METHODS

3.1 Preparation of Antigens

The antigens used in this study to raise antisera to *Rhizopus oryzae*, *Candida albicans* and *Aspergillus fumigatus*, or for use in tests for serological specificity, were prepared in accordance with a method described by M^{c}kenzie, Philpot and Proctor (1980). The isolates were stored as silica gel preserved cultures (Gentles and Scott, 1979). Several of the isolates had been stored in this way for many years. The antigens used are detailed in the following table.

Table 2: Antigens of *Aspergillus*, *Rhizopus*, and *Candida* species with their corresponding antigen number, details of the isolates involved with their sources and silica gel number, if stored in the Glasgow culture collection.

Antigen	Name/Number	Silica gel Number	Source
A. fumigatus	Bencard (1)		Commercial
A. fumigatus	Bencard (2)		Commercial
A. fumigatus	Indian		Commercial
A. fumigatus	GAF 5	AF5	CLV Weybridge
A. fumigatus	GAF 83	AF5	CLV Weybridge
A. fumigatus	GAF 84	AF60	Air Derm. Dept.
A. fumigatus	GAF 85	AF59	Air Derm. Dept.
A. fumigatus	GAF 86	AF60	Air Derm. Dept.
A. flavus	GAFl 2	AFL6	Sputum
A. clavatus	GAC 2	ACL1	Barley grain
A. nidulans	GANid 1	ANi1	IMI 75006
A. niger	GAN 2	ANig4	Sputum

Table 2 continued

Antigen	Name/Number	Silica gel Number	Source
A. terreus	GAT 2	ATe8	CLV, Weybridge
R. oryzae	GRh1	Rhsp.1	Soft palate
C. albicans	Mercia (somatic)		Commercial
C. albicans	MRL 3153 (cytoplasmic)		
C. albicans	GCA003	CA9	Throat swab
C. albicans	GCA406	CA6	Skin biopsy
C. parapsilosis	MRL 304 (cytoplasmic)		

3.1.1. Antigens of Filamentous Fungi

Glucose peptone broth (300 ml) was inoculated with the fungus. Magnetic stirrers were used to ensure adequate aeration and the cultures were kept at 37°C for three days by which time the growth was dense.

The cultures were decanted into sterile 5 litre flasks containing 3 litres of glucose peptone broth and these were incubated on magnetic stirrers for a further three days.

Glucose Peptone Broth

D - glucose	20 g
Bacteriological peptone	5 g
Distilled water	1,000 ml

The fungus was harvested by filtering through nylon and the hyphal mat was stored frozen at -20°C. The fungus was pulverised using mortar, pestle and sand that had been autoclaved for 30 minutes at 15 lbs p.s.i. pressure then dried in a sterilizing oven for 1 hour.

The fungus, sterile sand and volatile buffer were mixed, frozen in the mortar, pounded by the pestle until liquid, refrozen and ground again.

Volatile Buffer

Ammonium hydrogen carbonate	8 g
Water	1,000 ml

The pH was adusted to 7.2 by dropwise addition of glacial acetic acid.

The slurry was centrifuged at 2,000g to remove the sand and debris, the supernatant was frozen overnight and recentrifuged.

The second supernatant was dialysed against daily changes of 10% polyethylene glycol 600 in volatile buffer at 4°C for 2 days. It was then frozen overnight at -20°C, centrifuged at 2,000g for 1 hour, the supernatant was decanted and spun for a further 30 minutes. The supernatant from this was freeze-dried to give a powdered antigen preparation.

3.1.2. Antigens of *Candida* Species

The *C. albicans* antigen used to raise antiserum to *C. albicans* was supplied, as lyophilised powder, by Prof. D.W.R. M^{a}^{c}kenzie of the Mycological Reference Laboratory, Central Public Health Laboratory, 61, Colindale Avenue, London (MRL). The *C. parapsilosis* antigen was also received from this source.

Other *C. albicans* antigens were prepared from glucose peptone agar cultures grown in roux bottles. The yeasts were incubated for 24 hours at 37°C, they were then harvested by washing in saline before centrifugation to recover the organisms. The yeasts were frozen, thawed then disrupted in a Braun MSK homogeniser. A suspension of yeast and an equal volume of number 12 (0.124-0.176 mm diameter) ballotini glass beads were placed in a homogeniser bottle. Homogenisation of the cells was conducted under continuous carbon dioxide cooling. At minute intervals the bottle was removed to check for freezing or overheating. After four minutes the homogenate was examined microscopically to ensure that disruption had occurred. The homogenate was centrifuged at 2,000 g. The supernatant was then dialysed against distilled water for 24 hours before being freeze dried.

3.1.3. Commercial Antigens

The following commercial antigens were used in this study: Bencard numbers 1 and 2 *A. fumigatus* Bencard Ltd.,

Brentford, Middlessex; *A. fumigatus* Indian culture filtrate V.P. Chest Institute, University of Delhi.; *C. albicans* somatic Mercia Diagnostics Ltd., Guildford U.K.; *C. albicans* and *C. parapsilosis* cytoplasmic Mycological Reference Laboratory, London.

3.2. Production of Antisera

3.2.1. Laboratory Raised Antisera

The antisera were raised in New Zealand white rabbits housed in Forth-Tech rabbit cages 10x18x24 inches. They were maintained on a diet of Labsure R14 plus a supply of hay and water *ad libertum* from drop bottles.

A 10 ml test bleed was collected from the marginal ear vein of each rabbit to test for fungal antibodies prior to the start of immunisation. *A. fumigatus* and *R. oryzae* freeze dried antigens were reconstituted in phosphate buffered saline (P.B.S.) to a concentration of 30 mg/ml.

Phosphate Buffered Saline (PBS) for Reconstitution of Antigens

Sodium chloride	8 g
Potassium chloride	0.2 g
Disodium hydrogen phosphate (anhydrous)	1.15 g
Potassium dihydrogen phosphate	0.2 g
Magnesium chloride (hydrated)	0.1 g
Calcium chloride (add last)	0.1 g
Distilled water	1,000 ml

The reconstituted antigens were mixed with equal volumes of Freund's incomplete adjuvant (Difco Laboratories) and 0.2 ml was injected subcutaneously into New Zealand white rabbits. This was done five times at intervals of three weeks over a four month period, freshly prepared antigens were used each time.

A 10 ml sample of blood was taken from each rabbit's ear to determine if precipitating antibodies were present for each antigen. The rabbits were exsanguinated 3 weeks after the final injection, provided the serum had a titre of at least 1 in 8 by counterimmunoelectrophoresis. The blood was allowed to clot and the separated serum was stored frozen in 4 ml amounts in bijoux bottles.

In raising antiserum to *C. albicans* a shorter course of injections was used. A 5 mg/ml solution of antigen in P.B.S. was emulsified with an equal volume of Freund's incomplete adjuvant and 0.5 ml was injected subcutaneously into the thigh of New Zealand white rabbits. This was performed at four day intervals for 16 days, the rabbits were rested for three weeks then a booster dose was administered. Blood was taken for precipitin tests after a further two weeks and when the sera had a titre of 1 in 8 the rabbits were exsanguinated.

3.2.2. Commercial Antisera

Antisera raised in rabbits, against *Aspergillus* species, were purchased from the Institut Pasteur, Paris, for use in specificity determinations.

Details of the antisera employed in this study are given in Table 3.

Table 3: Origins of antisera indicating the species and antigen type to which they were raised.

Source	Antiserum	Antigen
Rabbit 1	*A. fumigatus*	GAF 5
Rabbit 2	*R. oryzae*	Rh. sp.1
Rabbit 3	*C. albicans*	MRL
Rabbit 4	*C. albicans*	MRL
Rabbit 6	*A. fumigatus*	GAF 5
Past. Inst.	*A. fumigatus*	Commercial
Past. Inst.	*A. flavus*	Commercial
Past. Inst.	*A. nidulans*	Commercial
Past. Inst.	*A. niger*	Commercial
Past. Inst.	*A. terreus*	Commercial

In the subsequent tables, rabbits 1 - 6 are designated as R1 - R6 and the sera from the Institut Pasteur are designated as PI.

3.3. Strength and Specificity of Antisera

The strength and specificity of laboratory raised or commercial antisera were determined by counter-immunoelectrophoresis, agar gel double diffusion and enzyme linked immunosorbent assay.

3.3.1. Counterimmunoelectrophoresis (CIE).

Endosmosis in a electrophoretic gel ensures that water molecules migrate from anode to cathode, this slows the movement of negatively charged particles and may reverse the direction of flow of the slower moving ones.

In an agarose gel with a high pH of 7.2 - 9, immunoglobulins move to the cathode while most soluble antigens travel towards the anode. Where they converge a precipitin band is formed. This rapid and sensitive method was used to determine the titres of the antisera and to reveal any cross-reactivity with antigens other than those the sera was raised against.

(i) Preparation and Electrophoresis of Gels

Sigma type 111 high electroendosmosis (E.E.O.) agarose was prepared as 1% in barbitone/sodium barbitone buffer, then measured into 4 ml amounts which were melted when required.

Barbitone/Sodium Barbitone Buffer.

Barbitone	3.55 g
Sodium barbitone	7.55 g
Sodium azide	0.5 g
Distilled water	1000 ml

Adjust to pH 8.2

The 1% agarose was poured on to 5 cm square glass slides sitting on coins, this helps to avoid spillage and facilitates handling the slides when the gels have set. Wells were cut in the gels in rows using a 4 mm gel cutter (Figure 10).

Opposing wells were filled with serum and antigen solutions and the slides placed in an electrophoresis tank containing barbitone/sodium barbitone buffer. The serum wells were connected to the anode and the antigen wells to the cathode with lint wicks.

A direct current power supply with a constant voltage of 6 volts per cm width of gel was passed through the gels for 90 minutes.

(ii) Washing of Gels

The slides were removed from the tank and washed in citrate buffer at 37°C for two days before being dried and stained.

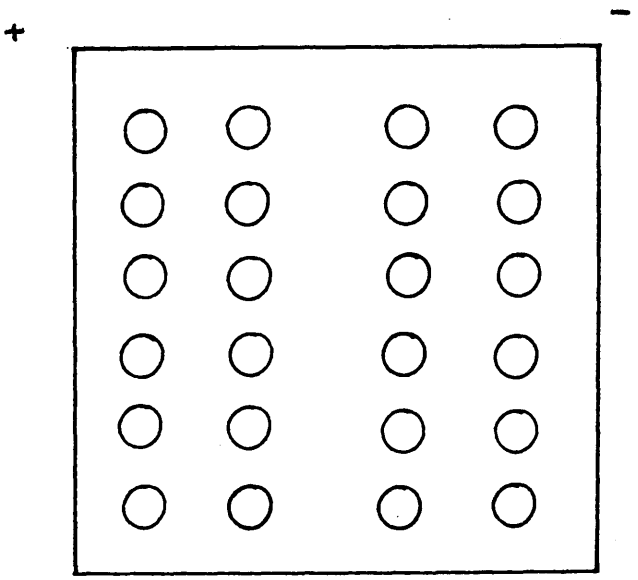


Figure 10 Template used for cutting the wells for counterimmunoelectrophoresis. Antisera were placed in the left hand and antigens in the right hand wells of the two columns.

Citrate Buffer

Sodium chloride	40 g
Trisodium citrate	100 g
Sodium azide	1 g
Distilled water	2,000 ml

Dissolve by stirring

(iii) Drying and Staining Gels

The gels were removed from the washing solution (citrate buffer), rinsed in tapwater and dried beneath filter paper squares using a hair dryer; this binds the gels to their slides. The filter paper was moistened before it was removed and the gels dried further. The gels and slides were stained in Coomassie brilliant blue for five minutes before being removed and destained in methanolic acetic acid. The destaining time varied, the ideal being enough time for the background to clear while allowing any weak precipitin lines to show clearly. Background stain usually cleared within 30 minutes.

Coomassie Brilliant Blue

B.D.H. Page Blue 83	0.5 g
Methanolic acetic acid	100 ml

Methanolic Acetic Acid

Methylated spirits	5 parts
Glacial acetic acid	1 part
Distilled water	5 parts

The antisera and antigens run in CIE to indicate their selectivity and specificity are given in Table 4

Table 4: CIE to demonstrate interactions of different *Aspergillus*, *Candida* and *Rhizopus* antigens with homologous antisera and to indicate possible cross-reactions with heterologous antisera.

Antiserum	Antigen	Antigen Number
A. fumigatus	*A. fumigatus*	Indian
A. fumigatus	*A. fumigatus*	GAF 5
A. fumigatus	*A. fumigatus*	GAF 70
A. fumigatus	*A. fumigatus*	GAF 83
A. fumigatus	*A. fumigatus*	GAF 84
A. fumigatus	*A. fumigatus*	GAF 85
A. fumigatus	*A. fumigatus*	GAF 86
A. fumigatus	*A. fumigatus*	GAF 86B
A. fumigatus	*A. flavus*	GAFl 2
A. fumigatus	*A. clavatus*	GAC 2
A. fumigatus	*A. nidulans*	ANi 1
A. fumigatus	*A. niger*	ANig 4
A. fumigatus	*A. terreus*	ATe 8
A. fumigatus	*C. albicans*	Mercia
A. fumigatus	*C. albicans*	MRL
A. fumigatus	*C. parapsilosis*	MRL
A. fumigatus	*R. oryzae*	Rh 1

Table 4 continued

Antiserum	Antigen	Antigen Number
C. albicans	*C. albicans*	Mercia
C. albicans	*C. albicans*	MRL
C. albicans	*C. albicans*	GCA003
C. albicans	*C. albicans*	GCA406
C. albicans	*C. parapsilosis*	MRL
C. albicans	*A. fumigatus*	Indian
C. albicans	*R. oryzae*	Rh 1
R. oryzae	*R. oryzae*	Rh 1
R. oryzae	*A. fumigatus*	Indian
R. oryzae	*C. albicans*	MRL
A. flavus	*A. fumigatus*	Indian
A. flavus	*A. flavus*	GAFl 1
A. flavus	*A. clavatus*	GAC 2
A. flavus	*A. nidulans*	GANi 1
A. flavus	*A. niger*	GANig 4
A. flavus	*A. terreus*	GATe 8
A. nidulans	*A. fumigatus*	Indian
A. nidulans	*A. flavus*	GAFl 1
A. nidulans	*A. clavatus*	GAC 2
A. nidulans	*A. nidulans*	GANi 1
A. nidulans	*A. niger*	GANig 4
A. nidulans	*A. terreus*	GATe 8
A. niger	*A. fumigatus*	Indian
A. niger	*A. flavus*	GAFl 1
A. niger	*A. clavatus*	GAC 2
A. niger	*A. nidulans*	GANi 1
A. niger	*A. niger*	GANig 4
A. niger	*A. terreus*	GATe 8
A. terreus	*A. fumigatus*	Indian
A. terreus	*A. flavus*	GAF 1
A. terreus	*A. clavatus*	GAC 2
A. terreus	*A. nidulans*	GANi 1
A. terreus	*A. niger*	GANig 4
A. terreus	*A. terreus*	GATe 8

3.3.2. Agar Gel Double Diffusion

Gels for double diffusion tests were poured as described for the CIE method. Wells were cut in the gels using cork borers and gel cutters of the appropriate

sizes. The template used in these tests is illustrated in Figure 11. The gels were incubated in humid chambers for 48 hours to allow diffusion of the antigens and antibodies through the agar. The double diffusion gels were washed and stained as described previously.

The template was used to find any lines of identity common to the antiserum tested against particular antigens the details of which are given in Table 5.

Table 5: Double diffusion experiments to determine cross-reactions between antisera and their homologous and heterologous antigens.

Antiserum	Antigen	Antigen Number
A. fumigatus	*A. fumigatus*	MRL
C. albicans	*A. fumigatus*	MRL
R. oryzae	*A. fumigatus*	MRL
A. fumigatus	*A. fumigatus*	GAF 83
C. albicans	*A. fumigatus*	GAF 83
R. oryzae	*A. fumigatus*	GAF83
A. fumigatus	*A. fumigatus*	GAF 84
C. albicans	*A. fumigatus*	GAF 84
R. oryzae	*A. fumigatus*	GAF 84
A. fumigatus	*C. albicans*	MRL
C. albicans	*C. albicans*	MRL
R. oryzae	*C. albicans*	MRL
A. fumigatus	*C. albicans*	GCA003
C. albicans	*C. albicans*	GCA003
R. oryzae	*C. albicans*	GCA003
A. fumigatus	*C. albicans*	C406
C. albicans	*C. albicans*	C406
R. oryzae	*C. albicans*	C406
A. fumigatus	*R. oryzae*	Rh. 1
C. albicans	*R. oryzae*	Rh. 1
R. oryzae	*R. oryzae*	Rh. 1

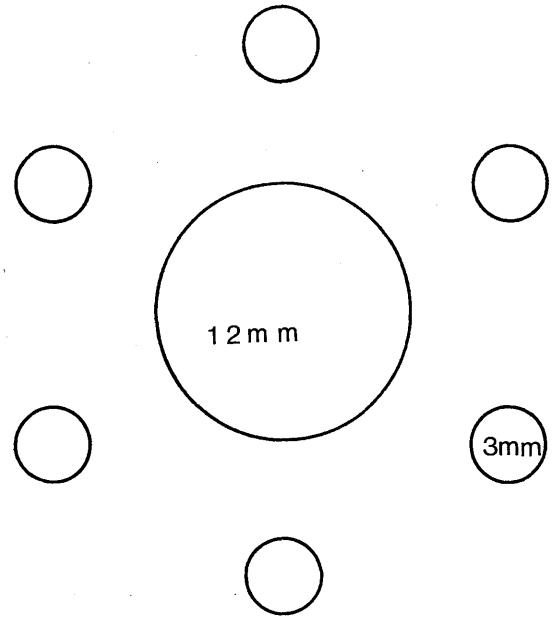


Figure 11 Showing the pattern of wells used in double diffusion experiments. The test serum was placed in the centre well with the antigens in the surrounding smaller wells.

3.3.3. Enzyme-linked Immunosorbent Assay For Antibody Detection (ELISA)

In order to detect the presence of antibodies to antigens of either the same or a different species as those against which the antisera were raised, an ELISA technique was employed. This method had the advantage of being able to quantify the strength of the reaction of the antisera against the antibodies in the assay. A schematic representation of the procedure is shown in Figure 12.

(i) Coating of Solid Phase

The antigen solutions were prepared to provide a range of antigen concentrations from 1.5 mg/ml to 30 mg/ml in sodium carbonate coating buffer, pH 9.6.

Sodium Carbonate Coating Buffer

Sodium carbonate	1.56 g
Sodium hydrogen carbonate	2.93 g
Sodium azide	0.2 g

Make up to 1,000 ml with distilled water and adjust to pH 9.6 with NaOH.

Volumes of 150 µl of antigen solutions were dispensed into wells of 96 well polyvinyl micro titre plates (Sterlin Ltd., Middlesex, England). These were then incubated at 37°C in a shaking incubator cabinet for 1

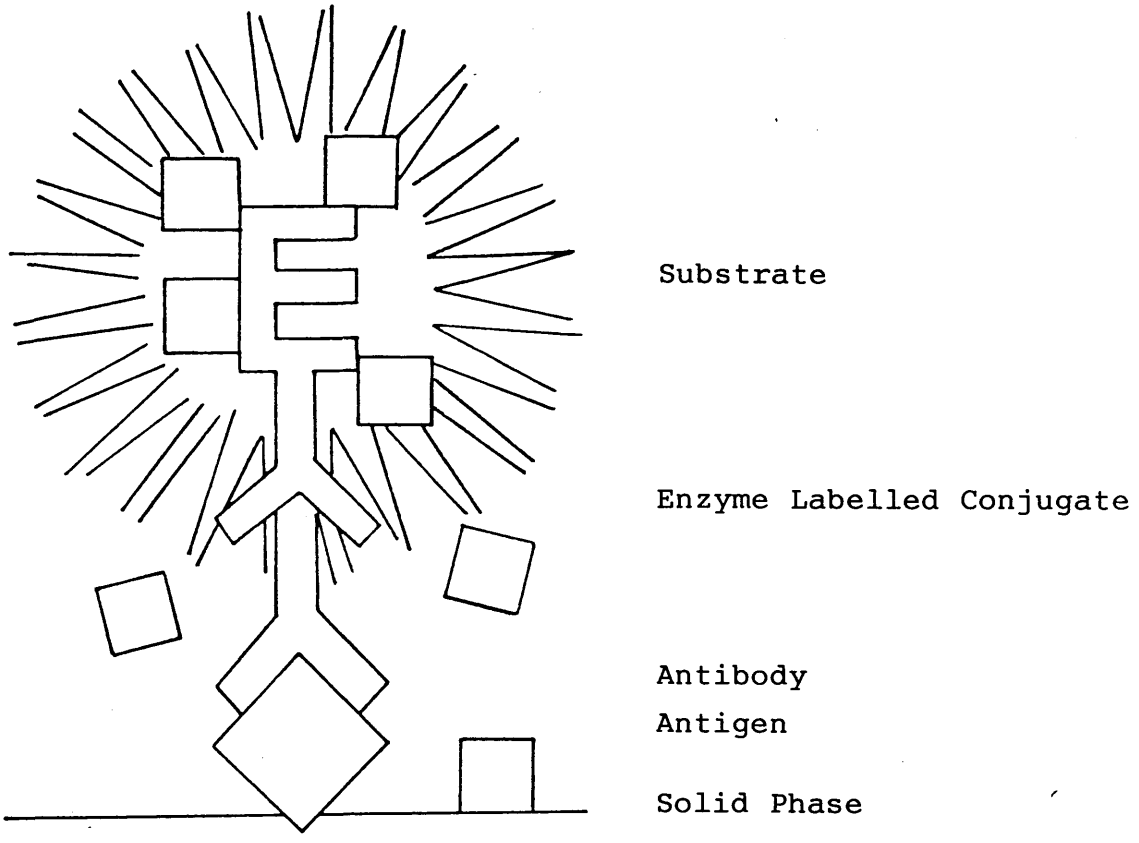


Figure 12 Diagramatic representation of the ELISA technique used to detect antibodies in serum.

hour. This enhances antigen binding to the plastic surface.

(ii) Washing Technique

The contents of the wells were discarded and the plates were washed with phosphate buffered saline Tween 20 (P.B.S.T20) three times for three minutes. This ensures that no unbound antigen remains in the wells.

P.B.S. Tween 20

Sodium chloride	8 g
Potassium dihydrogen phosphate	0.34 g
Dipotassium hydrogen phosphate	1.21 g
Tween 20	0.5 ml

Make up to 1,000 ml with distilled water.

N.B. It is advantageous to heat a little of the water to rinse out the pipette after measuring the Tween 20 as it adheres to the pipette and is difficult to remove completely.

(iii) Addition of Primary Antibody

Dilution series were made of the antisera to be tested and 150 µl of each concentration were dispensed into the wells. The plates were then incubated at 37°C for one hour.

The plates were washed as described previously.

(iv) Application of Conjugate

150 µl of anti-rabbit heavy and light chain IgG-alkaline phosphatase conjugate, (Miles Laboratories, Stoke Pages Slough), diluted 1:2000 in P.B.S.T20, were pipetted into each well and the plates were incubated for a further hour at 37°C.

After incubation the wells were again washed with P.B.S.T20.

(v) Addition of Substrate and Reading of Plates

A substrate solution was prepared using one 5 mg. tablet of p-nitrophenyl phosphate (Sigma 104) dissolved in 5 ml substrate buffer and agitated until it dissolved and 150 µl of this solution was dispensed into the wells. The plates were incubated at 37°C with constant agitation and were checked after 10 minutes, after which time if the positive control had turned visibly yellow the plates were read at a wavelength of 405 nm on a Dynatech MR 950 microELISA plate reader (Dynatech Laboratories, Billingshurst, U.K.). The plates were incubated and the absorbance read after a further 20 minutes.

Substrate Buffer

Sodium azide	0.1 g
Diethanolamine*	48.5 ml
Distilled water	400 ml

Add 1M hydrochloric acid to adjust pH to 9.8

Make up to 500 ml. with distilled water.

Store in a dark bottle as it is light sensitive.

* Store at 37°C as it freezes at 26°C.

The antigens and antibodies which were tested against each other using this method are listed in Table 6. C. *parapsilosis* has been abreviated to *C. parap.*

Table 6: Details of reagents assayed by ELISA to detect reactions of various antigens at different concentrations with homologous and heterologous antisera from laboratory and commercial sources.

Antigen	Antigen Number	Antiserum	Source
C. albicans	MRL 3153 (5 mg/ml)	*C. albicans*	R3
C. albicans	MRL 3153 (5 mg/ml)	*C. albicans*	R4
C. albicans	MRL 3153 (5 mg/ml)	*A. fumigatus*	R1
C. albicans	MRL 3153 (5 mg/ml)	*A. flavus*	IP
C. albicans	MRL 3153 (5 mg/ml)	*A. nidulans*	IP
C. albicans	MRL 3153 (5 mg/ml)	*A. niger*	IP
C. albicans	MRL 3153 (5 mg/ml)	*A. terreus*	IP
C. albicans	MRL 3153 (5 mg/ml)	*R. oryzae*	R2
C. albicans	MRL 3153 (30 mg/ml)	*C. albicans*	R3
C. albicans	MRL 3153 (30 mg/ml)	*C. albicans*	R4
C. albicans	MRL 3153 (30 mg/ml)	*A. fumigatus*	R1
C. albicans	MRL 3153 (30 mg/ml)	*A. flavus*	IP
C. albicans	MRL 3153 (30 mg/ml)	*A. nidulans*	IP
C. albicans	MRL 3153 (30 mg/ml)	*A. niger*	IP
C. albicans	MRL 3153 (30 mg/ml)	*A. terreus*	IP
C. albicans	MRL 3152 (30 mg/ml)	*R. oryzae*	R2
C. albicans	mannan (30 mg/ml)	*C. albicans*	R3
C. albicans	mannan (30 mg/ml)	*C. albicans*	R4
C. albicans	mannan (30 mg/ml)	*A. fumigatus*	R1
C. albicans	mannan (30 mg/ml)	*A. fumigatus*	R6
C. albicans	mannan (30 mg/ml)	*A. flavus*	IP
C. albicans	mannan (30 mg/ml)	*A. nidulans*	IP
C. albicans	mannan (30 mg/ml)	*A. niger*	IP
C. albicans	mannan (30 mg/ml)	*A. terreus*	IP
C. albicans	mannan (30 mg/ml)	*R. oryzae*	R2
C. parap.	MRL (5 mg/ml)	*C. albicans*	R3
C. parap.	MRL (5 mg/ml)	*C. albicans*	R4
C. parap.	MRL (5 mg/ml)	*A. fumigatus*	R1
C. parap.	MRL (5 mg/ml)	*R. oryzae*	R2
A. fumigatus	Indian	*C. albicans*	R3
A. fumigatus	Indian	*C. albicans*	R4
A. fumigatus	Indian	*A. fumigatus*	R1
A. fumigatus	Indian	*R. oryzae*	R2
A. fumigatus	AF 5 (5 mg/ml)	*C. albicans*	R3
A. fumigatus	AF 5 (5 mg/ml)	*C. albicans*	R4
A. fumigatus	AF 5 (5 mg/ml)	*A. fumigatus*	R1
A. fumigatus	AF 5 (5 mg/ml)	*A. fumigatus*	R6

Table 6 continued

Antigen	Antigen Number	Antiserum	Source
A. fumigatus	AF 5 (5 mg/ml)	*A. flavus*	PI
A. fumigatus	AF 5 (5 mg/ml)	*A. nidulans*	PI
A. fumigatus	AF 5 (5 mg/ml)	*A. niger*	PI
A. fumigatus	AF 5 (5 mg/ml)	*A. niger*	PI
A. fumigatus	AF 5 (5 mg/ml)	*A. terreus*	PI
A. fumigatus	AF 5 (5 mg/ml)	*R. oryzae*	R2
A. fumigatus	AF 5 (30 mg/ml)	*C. albicans*	R3
A. fumigatus	AF 5 (30 mg/ml)	*C. albicans*	R4
A. fumigatus	AF 5 (30 mg/ml)	*A. fumigatus*	R1
A. fumigatus	AF 5 (30 mg/ml)	*A. flavus*	PI
A. fumigatus	AF 5 (30 mg/ml)	*A. nidulans*	PI
A. fumigatus	AF 5 (30 mg/ml)	*A. niger*	PI
A. fumigatus	AF 5 (30 mg/ml)	*A. terreus*	PI
A. fumigatus	AF 5 (30 mg/ml)	*R. oryzae*	R2
A. fumigatus	MRL (1.5 mg/ml)	*C. albicans*	R3
A. fumigatus	MRL (1.5 mg/ml)	*C. albicans*	R4
A. fumigatus	MRL (1.5 mg/ml)	*A. fumigatus*	R1
A. fumigatus	MRL (1.5 mg/ml)	*A. flavus*	PI
A. fumigatus	MRL (1.5 mg/ml)	*A. nidulans*	PI
A. fumigatus	MRL (1.5 mg/ml)	*A. niger*	PI
A. fumigatus	MRL (1.5 mg/ml)	*A. terreus*	PI
A. fumigatus	MRL (1.5 mg/ml)	*R. oryzae*	R2
A. fumigatus	MRL (30 mg/ml)	*A. fumigatus*	R1
A. fumigatus	MRL (30 mg/ml)	*A. fumigatus*	R6
A. fumigatus	MRL (30 mg/ml)	*A. flavus*	PI
A. fumigatus	MRL (30 mg/ml)	*A. nidulans*	PI
A. fumigatus	MRL (30 mg/ml)	*A. niger*	PI
A. fumigatus	MRL (30 mg/ml)	*A. terreus*	PI
A. flavus	GA FL 1 (3 mg/ml)	*C. albicans*	R3
A. flavus	GA FL 1 (3 mg/ml)	*C. albicans*	R4
A. flavus	GA FL 1 (3 mg/ml)	*A. fumigatus*	R1
A. flavus	GA FL 1 (3 mg/ml)	*A. fumigatus*	R6
A. flavus	GA FL 1 (3 mg/ml)	*A. flavus*	PI
A. flavus	GA FL 1 (3 mg/ml)	*A. nidulans*	PI
A. flavus	GA FL 1 (3 mg/ml)	*A. niger*	PI
A. flavus	GA FL 1 (3 mg/ml)	*R. oryzae*	R2
A. flavus	GA FL 1 (30 mg/ml)	*C. albicans*	R3
A. flavus	GA FL 1 (30 mg/ml)	*C. albicans*	R4
A. flavus	GA FL 1 (30 mg/ml)	*A. fumigatus*	R1
A. flavus	GA FL 1 (30 mg/ml)	*A. fumigatus*	R6
A. flavus	GA FL 1 (30 mg/ml)	*A. flavus*	PI
A. flavus	GA FL 1 (30 mg/ml)	*A. nidulans*	PI
A. flavus	GA FL 1 (30 mg/ml)	*A. niger*	PI
A. flavus	GA FL 1 (30 mg/ml)	*R. oryzae*	R2

Table 6 continued

Antigen	Antigen Number	Antiserum	Source
A. nidulans	GA Nid 1 (30 mg/ml)	*C. albicans*	R3
A. nidulans	GA Nid 1 (30 mg/ml)	*C. albicans*	R4
A. nidulans	GA Nid 1 (30 mg/ml)	*A. fumigatus*	R1
A. nidulans	GA Nid 1 (30 mg/ml)	*A. flavus*	PI
A. nidulans	GA Nid 1 (30 mg/ml)	*A. nidulans*	PI
A. nidulans	GA Nid 1 (30 mg/ml)	*A. niger*	PI
A. nidulans	GA Nid 1 (30 mg/ml)	*A. terreus*	PI
A. nidulans	GA Nid 1 (30 mg/ml)	*R. oryzae*	R2
A. niger	GA N 2 (3 mg/ml)	*C. albicans*	R3
A. niger	GA N 2 (3 mg/ml)	*C. albicans*	R4
A. niger	GA N 2 (3 mg/ml)	*A. fumigatus*	R1
A. niger	GA N 2 (3 mg/ml)	*A. flavus*	PI
A. niger	GA N 2 (3 mg/ml)	*A. nidulans*	PI
A. niger	GA N 2 (3 mg/ml)	*A. niger*	PI
A. niger	GA N 2 (3 mg/ml)	*A. terreus*	PI
A. niger	GA N 2 (3 mg/ml)	*R. oryzae*	R2
A. niger	GA N 2 (30 mg/ml)	*C. albicans*	R3
A. niger	GA N 2 (30 mg/ml)	*C. albicans*	R4
A. niger	GA N 2 (30 mg/ml)	*A. fumigatus*	R1
A. niger	GA N 2 (30 mg/ml)	*A. flavus*	PI
A. niger	GA N 2 (30 mg/ml)	*A. nidulans*	PI
A. niger	GA N 2 (30 mg/ml)	*A. niger*	PI
A. niger	GA N 2 (30 mg/ml)	*A. terreus*	PI
A. niger	GA N 2 (30 mg/ml)	*R. oryzae*	R2
A. terreus	GA T 2 (30 mg/ml)	*C. albicans*	R3
A. terreus	GA T 2 (30 mg/ml)	*C. albicans*	R4
A. terreus	GA T 2 (30 mg/ml)	*A. fumigatus*	R1
A. terreus	GA T 2 (30 mg/ml)	*A. fumigatus*	R6
A. terreus	GA T 2 (30 mg/ml)	*A. flavus*	PI
A. terreus	GA T 2 (30 mg/ml)	*A. nidulans*	PI
A. terreus	GA T 2 (30 mg/ml)	*A. niger*	PI
A. terreus	GA T 2 (30 mg/ml)	*A. terreus*	PI
A. terreus	GA T 2 (30 mg/ml)	*R. oryzae*	R2
R. oryzae	G Rh1 (30 mg/ml)	*C. albicans*	R3
R. oryzae	G Rh1 (30 mg/ml)	*C. albicans*	R4
R. oryzae	G Rh1 (30 mg/ml)	*A. fumigatus*	R1
R. oryzae	G Rh1 (30 mg/ml)	*A. flavus*	PI
R. oryzae	G Rh1 (30 mg/ml)	*A. nidulans*	PI
R. oryzae	G Rh1 (30 mg/ml)	*A. niger*	PI
R. oryzae	G Rh1 (30 mg/ml)	*A. terreus*	PI
R. oryzae	G Rh1 (30 mg/ml)	*R. oryzae*	R2

3.3.4. Streptavidin-Biotin Enzyme Linked Immunosorbent Antigen Assay for the Testing of Antiserum Specificity

(i) Principal of Assay

A new generation of immunoassays with high sensitivity has evolved from the attraction between proteins and water soluble proteins. Streptavidin from *Streptomyces avidinii* is a tetrameric protein with four affinity binding sites for biotin. Exploitation of this high affinity results in the sensitivity of immunoassays being enhanced.

(ii) Assay Method

To test for cross-reactions among the *Aspergillus* antigens a streptavidin-biotin capture antibody system was used. *Aspergillus* antigens from *A. fumigatus*, *A. clavatus*, *A. flavus*, *A. niger*, *A. nidulans* and *A. terreus* were diluted in normal human serum at levels of 100 μg to 100 ng/ml before being tested in the system.

The capture antibody was human anti-*A. fumigatus* from an aspergilloma patient (indirect ELISA titre 1:10,000) used at 1/500 dilution in sodium carbonate coating buffer. The detection antiserum was a rabbit anti-*A. fumigatus* antisera (Institut Pasteur) diluted to 1/500 in P.B.S. incorporating bovine serum albumen (B.S.A.)

Other reagents were biotinylated donkey anti-rabbit Ig G (R.P.N. 1004; Lot 19; Amersham Int. p.l.c.; Amersham)

used at 1/1000 dilution in P.B.S.-B.S.A. and streptavidin-biotin-horseradish peroxidase (S-B-HPRO) (1/1000 dilution: R.P.N. 1051, Lot 23: Amersham Int.p.l.c.; Amersham). The reaction was made visible by the addition of 1,2-phenylene-diamine dihydrochloride (Dakopatts; Dako Ltd., High Wycombe)

Assay Schedule

1. Solid phase coated with 200 µl human anti-*A. fumigatus* capture antibody for 1 hour
   Wash, the same technique was used as described previously.
2. Antigen dilutions added 200 µl for 1 hour.
   Wash
3. Detection antibody, rabbit anti-*A. fumigatus* 200 µl for 1 hour
   Wash
4. Biotinylated donkey anti-rabbit Ig G 200 µl added for 1 hour
   Wash
5. Biotin-streptavidin horseradish peroxidase preformed complex 200 µl for 1 hour
   Wash
6. Substrate (1,2 phenylene-diamine dihydrochloride) added for 30 minutes.

The intensity of the reactions was measured on a Dynatech MR950 microELISA mini reader at light wavelength 490 nm.

3.4. Preparation of Material for Histopathology

3.4.1. Tissue Fixation

Unfixed material from biopsy, post mortem or animal sources was immersed in neutral buffered formalin until adequately fixed (at least 24 hours) before being processed.

Fixative

Formaldehyde(40%)	10 ml
Calcium chloride	1 g
Water	90 ml

3.4.2. Tissue Processing and Paraffin Embedding

The fixed tissue was impregnated in paraffin wax using an automatic tissue processor.

Paraffin Processing Schedule

Tissues were processed with an automatic tissue processor (Histokinette) using the following schedule:-

1. 50% methylated spirit	1 hour
2. 80% methylated spirit	1 hour
3. 8% phenol in methylated spirit	2 hours
4. 8% phenol in methylated spirit	2 hours
5. 8% phenol in methylated spirit	3 hours
6. Absolute alcohol	2 hours
7. Absolute alcohol	2 hours

8.	Absolute alcohol:xylene (50:50)	1 hour
9.	Xylene	1 hour
10.	Xylene	1 hour
11.	Paraffin wax Difco polywax M.P.,57°C	3 hours
12.	Paraffin wax Difco polywax M.P.,57°C	3 hours

The tissues were aligned and embedded in fresh paraffin wax in embedding pans. These blocks were trimmed and mounted on wooden chucks using melted wax as an adhesive. "Molifex" was used to soften the tissue face before cutting. Serial 5μ sections of the embedded organs were sliced using a flat cutting Leitz rotary microtome. The wax of the block face was cooled with ice between ribbons, this facilitated cutting.

3.4.3 Special Processing For Mouse Eyes

When mouse eyes were processed the same schedule as above was followed but the xylene in steps 8,9 and 10 was substituted by chloroform as this is a softer reagent and prevents hardening of the sclera which, if friable, makes the eye difficult to section.

3.4.4. Techniques for Preparing Mounted Tissue Sections

(i) Mounting Tissue Sections

Ribbons of sections were floated on warm water in a thermostatically controlled mounting bowl set at 56°C and the individual sections were picked-up upon cleaned,

grease-free glass slides that had been coated with gelatin or in the case of the eye sections coated with poly-L-lysine. To encourage the sections to adhere to the slides they were baked in a 60°C oven for 30 minutes.

Cleaning Solution for Glass Slides

Potassium dichromate	120 g
Water	200 ml
Nitric acid	120 ml

(ii) Gelatinised Slides

Sheet gelatine (5 g) was dissolved in 1 litre warmed distilled water then 0.5 g Chromate potassium sulphate was added. Cleaned slides were immersed in the solution for one minute before being dried in racks in a 37°C incubator.

(iii) Poly-L-lysine Slides

A 1 mg/ml solution of poly-L-lysine (MW>15,0000;Sigma) was prepared and vials stored frozen until required. A 10 µl drop of the solution was placed on the cleaned slides and spread over them with the end of another slide. The solution dried almost at once.

3.4.5. Examination of Tissues for Fungi by the Peroxidase Anti-Peroxidase Immunohistochemical Staining Method

The peroxidase anti-peroxidase (PAP) method of staining is an immunohistochemical technique used to locate and identify fungi present in tissues. Many tissue types were examined for the presence of fungi using the PAP staining method. Several fungal diseases were represented in the tissues examined and these were stained with homologous and heterologous antisera. Table 7 details the types of tissue examined, the diagnosis of the infection and the basis for that diagnosis. Table 8 lists the sections and the antisera used.

In the following tables abbreviations have been used :- A/loma denotes aspergilloma, P/nasal denotes paranasal and L/geal denotes laryngeal.

** and *** in Table 7 are patients whose identities are not known.

Table 7: Details of tissues examined by PAP immunoperoxidase staining, the disease suspected, which fungus, if any, was isolated, the tissue involved and basis on which the diagnosis was made.

Patient	Fungus	Basis for Diagnosis	Tissue
Mucormycosis			
M F		Tissue morphology	Kidney
M F		Tissue morphology	Spleen
M S	*R. oryzae*	Culture and morphology	Soft palate
A G		Tissue morphology	Brain
A G		Tissue morphology	Palate
A H		Tissue morphology	Brain
A H		Tissue morphology	Kidney
Candidosis			
I C	*C. parap.*	Culture and morphology	Lung
I C	*C. parap.*	Culture and morphology	Adrenal
I C	*C. parap.*	Culture and morphology	Kidney
**		Tissue morphology	Larynx
***		Tissue morphology	Liver
I McK		Tissue morphology	Brain
J H	*C. albicans*	Culture and morphology	Liver
Aspergillosis			
V C		Tissue morphology	Brain
H F		Tissue morphology	Brain
A P	*A. fumigatus*	Culture and morphology	Brain
A P	*A. fumigatus*	Culture and morphology	Lung
J D	*A. fumigatus*	Culture and morphology	Lung
W B	*A. fumigatus*	Culture and morphology	A/loma
F DeS	*A. fumigatus*	Culture and morphology	A/loma
B L		Tissue morphology	Lung
Brum		Tissue morphology	Lung
A Hed		Tissue morphology	P/nasal sinuses
A Hug		Tissue morphology	Kidney
A Hug		Tissue morphology	Heart
A Hug		Tissue morphology	Lung
A S	*A. flavus*	Culture and morphology	Lung
W P		Tissue morphology	Lung
J k	*A. fumigatus*	Culture and morphology	Trachia
W R		Tissue morphology	Lung
P B	*A. fumigatus*	Culture and morphology	Lung

Table 8: Histology sections for the validation of the PAP staining method. Details of disease and tissue type and the antiserum employed are listed.

Antiserum	Disease	Organ
A. fumigatus	Aspergillosis (*A. fumigatus*)	Lung
A. fumigatus	Aspergillosis (*A. fumigatus*)	A/loma
A. fumigatus	Aspergillosis (*A. fumigatus*)	Brain
A. fumigatus	Aspergillosis (*A. fumigatus*)	Trachia
A. fumigatus	Aspergillosis	P/nasal sinuses
A. fumigatus	Aspergillosis	Kidney
A. fumigatus	Aspergillosis	Heart
A. fumigatus	Aspergillosis (*A. flavus*)	Lung
A. fumigatus	Candidosis (*C. albicans*)	Liver
A. fumigatus	Candidosis (*C. albicans*)	Kidney (mouse)
A. fumigatus	Candidosis	Liver
A. fumigatus	Candidosis	Brain
A. fumigatus	Candidosis	L/geal biopsy
A. fumigatus	Candidosis (*C. parap.*)	Lung
A. fumigatus	Candidosis (*C. parap.*)	Kidney
A. fumigatus	Candidosis (*C. parap.*)	Adrenal
A. fumigatus	Mucormycosis (*R. oryzae*)	Soft palate
A. fumigatus	Mucormycosis	Brain
A. fumigatus	Mucormycosis	Kidney
A. fumigatus	Mucormycosis	Spleen
A. flavus	Aspergillosis (*A. fumigatus*)	Lung
A. flavus	Aspergillosis (*A. flavus*)	Lung
C. albicans	Candidosis (*C. albicans*)	Liver
C. albicans	Candidosis (*C. albicans*)	Kidney (mouse)
C. albicans	Candidosis	Liver
C. albicans	Candidosis	Brain
C. albicans	Candidosis	L/geal biopsy
C. albicans	Candidosis (*C. parap.*)	Lung
C. albicans	Candidosis (*C. parap.*)	Kidney
C. albicans	Candidosis (*C. parap.*)	Adrenal
C. albicans	Aspergillosis (*A. fumigatus*)	Lung
C. albicans	Aspergillosis (*A. fumigatus*)	A/loma
C. albicans	Aspergillosis	Lung
C. albicans	Aspergillosis	Trachia

Table 8 continued

Antiserum	Disease	Organ
C. albicans	Aspergillosis	P/nasal sinuses
C. albicans	Aspergillosis	Kidney
C. albicans	Aspergillosis	Heart
C. albicans	Aspergillosis (*A. flavus*)	Lung
C. albicans	Mucormycosis (*R. oryzae*)	Soft palate
C. albicans	Mucormycosis	Brain
C. albicans	Mucormycosis	Kidney
C. albicans	Mucormycosis	Spleen
R. oryzae	Mucormycosis (*R. oryzae*)	Soft palate
R. oryzae	Mucormycosis	Brain
R. oryzae	Mucormycosis	Kidney
R. oryzae	Mucormycosis	Spleen
R. oryzae	Aspergillosis (*A. fumigatus*)	Lung
R. oryzae	Aspergillosis (*A. fumigatus*)	A/loma
R. oryzae	Aspergillosis	Lung
R. oryzae	Aspergillosis	Trachia
R. oryzae	Aspergillosis	P/nasal sinuses
R. oryzae	Aspergillosis	Kidney
R. oryzae	Aspergillosis	Heart
R. oryzae	Aspergillosis (*A. flavus*)	Lung
R. oryzae	Candidosis (*C. albicans*)	Liver
R. oryzae	Candidosis (*C. albicans*)	Kidney (mouse)
R. oryzae	Candidosis	Liver
R. oryzae	Candidosis	Brain
R. oryzae	Candidosis	L/geal biopsy
R. oryzae	Candidosis (*C. parap.*)	Lung
R. oryzae	Candidosis (*C. parap.*)	Kidney
R. oryzae	Candidosis (*C. parap.*)	Adrenal

3.4.6. Development of Agar Block Technique for Histopathological Staining and Evaluation of PAP Immunoperoxidase Method.

(i) Inoculation of the Agar

As control sections of fungal infections were not always available and to demonstrate any possible cross-reactivity amongst the antisera used for staining, an agar block technique was developed. This method simulates three dimensional fungal hyphae growth through tissue. The technique is easy to perform and unlike animal models the amount of fungus present in the blocks could be controlled.

Suspensions of filamentous fungi comprising of spores and hyphal fragments were prepared from freshly grown cultures of the organisms in sterile distilled water with 1% Tween 80. The yeast suspensions were prepared in sterile distilled water from overnight cultures.

Molten glucose peptone agar or Czapek Dox Tween 80 agar in 30 ml amounts were inoculated with 3 X 10^7 suspensions of the fungi and poured into 9 cm diameter petri dishes. At least a 30 ml volume of agar was required, as the depth of agar constituted the depth of the block to be cut and thin blocks are more difficult to handle. The plates were allowed to set and were incubated for 24 hours at 28°C. Czapek Dox agar was chosen for the yeasts to encourage the formation of mycelium and pseudomycelium.

Glucose Peptone Agar

D-glucose	20 g
Bacteriological peptone	5 g
Agar	10 g

Sterilise by autoclaving at 15 p.s.i. for 20 minutes.

Czapek Dox Agar

Czapek Dox agar (Oxoid)	22.7 g
Distilled water	500 ml

Dissolve by boiling add 5 ml Tween 80. Sterilise for 20 minutes at 15 p.s.i.

(ii) Preparation and Sectioning of the Blocks

Squares of one cm dimensions were cut from the agar plates and attached to metal cryostat chucks by freezing. The chucks were put in a container with acetone and dry ice, a drop of water was spotted onto the cold chuck and a square of the agar culture was placed on top. A few more drops of water around the agar block ensured it remained frozen in position. The chucks and attached blocks were stored at -20°C until they were cut.

The blocks were sectioned on a cryostat and the blanks were picked-up on gelatinised slides This prevented the sections floating away during the long washing stages of the immunoperoxidase staining procedure. The slides with the agar sections were stored in sealed Coplin jars at

-20°C until they were stained. The staining methods employed for the sections were identical to those for the paraffin sections but the agar sections did not require to be hydrated prior to staining.

Table 9: Details of sections from inoculated agar blocks stained by PAP immunoperoxidase method with information on the source of the primary antibody.

Agar Block Inoculated With	Primary Antibody	Source
C. albicans	*C. albicans*	R3 & R4
C. albicans	*A. fumigatus*	R1
C. albicans	*A. flavus*	P I
C. albicans	*A. nidulans*	P I
C. albicans	*A. niger*	P I
C. albicans	*A. terreus*	P I
C. albicans	*R. oryzae*	R2
C. albicans	Negative Serum	Rabbit
C. parap.	*C. albicans*	R3 & R4
A. fumigatus	*C. albicans*	R3 & R4
A. fumigatus	*A. fumigatus*	R1
A. fumigatus	*A. flavus*	P I
A. fumigatus	*A. nidulans*	P I
A. fumigatus	*A. niger*	P I
A. fumigatus	*A. terreus*	P I
A. fumigatus	*R. oryzae*	R2
A. fumigatus	Negative Serum	Rabbit
A. flavus	*C. albicans*	R3 & R4
A. flavus	*A. fumigatus*	R 1
A. flavus	*A. flavus*	P I
A. flavus	*A. nidulans*	P I
A. flavus	*A. niger*	P I
A. flavus	*A. terreus*	P I
A. flavus	*R. oryzae*	R2
A. flavus	Negative Serum	Rabbit
A. nidulans	*C. albicans*	R3 & R4
A. nidulans	*A. fumigatus*	R1
A. nidulans	*A. flavus*	P I
A. nidulans	*A. nidulans*	P I
A. nidulans	*A. niger*	P I

Table 9 continued

Agar Block Inoculated With	Primary Antibody	Source
A. nidulans	*A. terreus*	P I
A. nidulans	*R. oryzae*	R2
A. nidulans	Negative Serum	Rabbit
A. niger	*C. albicans*	R3 & R4
A. niger	*A. fumigatus*	R1
A. niger	*A. flavus*	P I
A. niger	*A. nidulans*	P I
A. niger	*A. niger*	P I
A. niger	*A. terreus*	P I
A. niger	*R. oryzae*	R2
A. niger	Negative Serum	Rabbit
A. terreus	*C. albicans*	R3 & R4
A. terreus	*A. fumigatus*	R1
A. terreus	*A. flavus*	P I
A. terreus	*A. nidulans*	P I
A. terreus	*A. niger*	P I
A. terreus	*A. terreus*	P I
A. terreus	*R. oryzae*	R2
A. terreus	Negative Serum	Rabbit
R. oryzae	*C. albicans*	R3 & R4
R. oryzae	*A. fumigatus*	R1
R. oryzae	*A. flavus*	P I
R. oryzae	*A. nidulans*	P I
R. oryzae	*A. niger*	P I
R. oryzae	*A. terreus*	P I
R. oryzae	*R. oryzae*	R2
R. oryzae	Negative Serum	Rabbit
Mucor hiemalis	*C. albicans*	R3 & R4
Mucor hiemalis	*A. fumigatus*	R1
Mucor hiemalis	*A. flavus*	P I
Mucor hiemalis	*A. nidulans*	P I
Mucor hiemalis	*A. niger*	P I
Mucor hiemalis	*A. terreus*	P I
Mucor hiemalis	*R. oryzae*	R2
Mucor hiemalis	Negative Serum	Rabbit
A. glauca	*C. albicans*	R3 & R4
A. glauca	*A. fumigatus*	R1
A. glauca	*A. flavus*	P I
A. glauca	*A. nidulans*	P I
A. glauca	*A. niger*	P I
A. glauca	*A. terreus*	P I
A. glauca	*R. oryzae*	R2
A. glauca	Negative Serum	Rabbit

3.5. Staining Methods for the Examination of Sections from Tissues and Agar Blocks

3.5.1. Periodic Acid-Schiffs

1.	Paraffin sections to water	5 minutes
2.	70% Methylated spirits	10 minutes
3.	Alcoholic periodic acid	5 minutes
4.	70% Methylated spirits	5 minutes
5.	Acid reducing rinse	5 minutes
6.	70% Methylated spirits	5 minutes
7.	Rinse briefly in tap water	
8.	Schiff's reagent (in dark staining jar)	25 minutes
9.	Rinse in tap water	
10.	Haematoxylin (Mayers)	1 minute
11.	Rinse in water	
12.	Blue quickly in Scott's tapwater substitute	
13.	Rinse in tap water	
14.	Dehydrate, clear and mount	

Using this method fungi stain red/purple.

3.5.2. Gomori's Methenamine Silver Nitrate (Armed Forces Institute of Pathology U.S.A.)

1.	Paraffin sections to water	
2.	5% chromic acid	1 hour
3.	Wash for a few seconds	

4. Rinse in 1% sodium bisulphate 1 minute
5. Wash 5 minutes
6. Wash with 4 changes of distilled water
7. Methenamine silver nitrate in oven at 58°C for 30 minutes until section turns yellowish brown. Use paraffin coated forceps to remove slide from this solution. Dip slide in distilled water and check for adequate silver impregnation with microscope. Fungi should be dark brown at this stage.
8. Rinse in 6 changes of distilled water
9. Tone in 1% gold chloride solution for 2-5 minutes
10. Rinse in distilled water
11. Remove unreduced silver with 2% sodium thiosulphate (hypo) solution for 2-5 minutes.
12. Wash thoroughly
13. Counterstain with working light green 30-45 seconds.
14. Dehydrate, clear and mount.

This technique sharply delineates fungi in black.

Solutions

5% Chromic Acid

Chromic acid	5 g
Distilled water	100 ml

3% Methenamine

Hexamethylenetetramine	3 g
Distilled water	100 ml

Stock Methenamine-Silver Nitrate

Silver Nitrate 5% solution	5 ml
Methenamine 3% solution	100 ml

A white precipitate forms but disperses immediately on shaking. Clear solutions remain usable for months if kept in a refrigerator.

1% Sodium Bisulphate

Sodium bisulphate	1 g
Distilled water	100 ml

2% Sodium Thiosulphate (Hypo)

Sodium Thiosulphate	2 g
Distilled Water	100 ml

Working Light Green

Light green stock solution	5 g
Distilled water	100 ml

5% Silver Nitrate

Silver Nitrate	5 g
Distilled water	100 ml

5% Borax

Borax (photographic or USP)	5 g
Distilled water	100 ml

Working Methenamine - Silver Nitrate

Borax 5% solution	2 ml
Distilled water	25 ml
Mix and add:	
Methenamine-Silver Nitrate stock solution	25 ml

0.1% Gold Chloride

Gold chloride, 1% solution	10 ml
Distilled Water	90 ml

(May be used repeatedly)

Stock Light Green

Light Green S.F. (Yellow)	0.2 g
Distilled water	100 ml
Glacial Acetic Acid	0.2 ml

The stained sections were mounted in harleco synthetic resin (H.S.R.) and covered with coverslips of the appropriate size.

The remaining unstained sections were stored as blanks until they were stained using the PAP immunocytochemical staining method.

3.5.3. Peroxidase Anti-Peroxidase Staining Technique for the Examination of Tissue and Agar Block Sections.

(i) Principal of the Method

Although the PAP method is more complex and time consuming than the direct or indirect methods of immunoperoxidase staining it is far more sensitive.

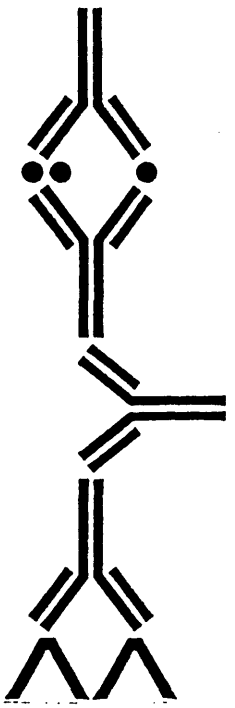
The primary antibody is followed by a second layer of serum raised in another animal species. The third and final antiserum comprises of IgG from the same species as the first layer but which has been raised against and conjugated to, horseradish peroxidase. Peroxidase is therefore bound to the Fab portion of the IgG and the Fc portion should bind with the Fab sites on the second layer. The reaction is made visible by the substrate diaminobenzidine (D.A.B.). (Figure 13)

(ii) Titration of Antisera

The dilution of each antiserum which gave optimal staining was used. This was determined by checkerboard titrations and was different for each antibody.

(iii) Peroxidase Anti-Peroxidase Staining Procedure

AS DAB staining was conducted in the aqueous phase the sections were first brought to water. The labelled slides were dewaxed in xylol then alcohols. The endogenous



PAP Method.

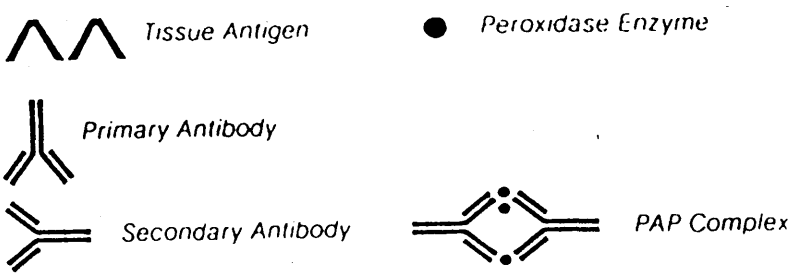


Figure 13 Diagramatic representation of the peroxidase anti-peroxidase staining method.

peroxidase activity was inhibited to prevent background staining by D.A.B..

As the primary antisera used in this study were polyclonal they might have exhibited binding to unrelated antigens, collagen and connective tissue. To prevent this phenomenon, sections were incubated with normal swine serum (N.S.S.), which was the same animal species as that which provided the antiserum for the second layer antibody.

To carry out the primary and subsequent incubations with antisera the slides were placed horizontally with sections uppermost upon a rack within a humid container. The appropriate serum or antiserum dilutions was then dispensed onto the tissue sections (approx 50 µl/slide). Surface tension retains the liquid on the slide. The sections were incubated at room temperature. The containers were either petri dishes or custom made shallow perspex boxes with glass rod racks.

When the sections were washed in Tris Buffered Saline (T.B.S.) they were placed in a slide rack in a needle box containing TBS, a magnetic stirrer ensured that excess antibody was removed during each wash. A Coplin staining jar housed the slides when the endogenous peroxidase was inhibited and also while they were submerged in the D.A.B. solution.

After a brownish colour had developed in the control tissue, the sections were rinsed in tap water, dehydrated, cleared and mounted in a synthetic resin H.S.R.

(iv) Peroxidase Anti-Peroxidase (P.A.P.) Schedule

1. Dewax slides in xylol, alcohol, methylated spirits and running tap water.
2. Remove endogenous peroxidase.
   Slides were immersed in 100 ml methanol containing 0.2 ml HCl and 0.5 ml H_2O_2 for 20 minutes.
3. Wash in tris buffered saline (TBS)
4. Lay slides out in a humid chamber and incubate with normal swine serum (NSS) diluted 1/20 with Tris buffer.
5. Tilt slides to remove NSS then cover with diluted primary antiserum and incubate for 30 minutes.
6. Wash in TBS for 15 minutes.
7. Coat slides in 25 µg/ml swine anti rabbit (S.A.R.) in T.B.S. and leave for 20-30 minutes.
8. Wash slides in T.B.S. for 15 minutes.
9. Incubate slides with rabbit peroxidase anti-peroxidase (P.A.P.) diluted to 1/100 with T.B.S. leave for 20 minutes
10. Wash for 15 minutes in T.B.S.
11. Slides are stained with 30 mg D.A.B in 100 ml of Tris buffer with one drop of H_2O_2. The positive control slide was used to assess the degree of staining. It

should develop a brown colour in approximately 10 minutes.

D.A.B. should be handled with gloved hands in a fume cupboard. Such care must be excercised as D.A.B. is a suspected carcinogen.

12. Wash slides in running tap water.

13. Dehydrate and clear sections:-

Methylated spirits	1 minute
Absolute alcohol	3 minutes
Xylol	5 minutes

14. Mount in H.S.R. and use coverslip of appropriate size.

Buffers

Tris Saline

Saline	450 ml
Tris buffer	50 ml

Tris Buffer

Trizma	12.12 g
Trizma base	2.78 g
Distilled water	2,000 ml

Saline

Sodium chloride	17 g
Distilled water	2,000 ml

(v) Controls for Specificity of Immunoperoxidase Staining

Controls are required to verify the specificity of the immunohistochemical staining procedures as it must be ascertained that the antibody is specific for the antigen being investigated. The following control sections were examined.

1. Non-immune serum as primary antiserum - No staining.
2. Unrelated antiserum as primary antiserum - No staining.
3. Omission of primary antiserum - No staining.
4. Omission of secondary antibody - No staining.
5. Incubation with D.A.B. only - No staining.
6. Incubation of unrelated/uninfected tissue from same species, with primary antiserum - No staining.
7. Incubation of specific antigen with specific antiserum - Staining of specific antigen.
8. Pre-absorption of specific antiserum with specific antigen prior to incubation with known positive tissue - Diminished or no staining of specific antigen.
9. Pre-absorption of specific antiserum with unrelated antigen before incubation with positive control - Staining of specific antigen.

3.6. A Mouse Model of Haematogenous *Candida* Endophthalmitis

The possibility of establishing an animal model for haematogenous *Candida* endophthalmitis analogous to man was examined. Such a model would allow investigation of the pathological changes and pathogenesis associated with the infection.

3.6.1. Fungus

C.albicans Glasgow heroin strain 11 (patient 22, vitrectomy isolate) which had been stored in silica gel was used. When fresh cultures were required, they were grown on glucose peptone agar for 24 hours at 37°C. Inocula for animal challenge experiments were prepared as blastospores in distilled water and were standardised to the appropriate concentrations by counting in an improved Neubauer counting chamber.

3.6.2. Animals

Balb/c mice were used. They were housed in "shoe box" mouse cages and fed on a diet of "Labsure CRMX" and water *ad libertum*.

Balb/c mice do not exhibit retinal degeneration on exposure to light as is the case with some other mouse strains. However, the animals were kept (as far as

possible) in a light regime of 12 hours light at low luminance and 12 hours dark. An excess of light could have damaged the retinae of the mice which would have made the pathology more difficult to interpret.

Mice were employed in preference to rabbits, which have been used in other studies (Hoffman, 1966; Edwards *et al.*, 1975), as they are easier to handle. Moreover they are cheaper to buy and to maintain. The most important factor governing the choice of a murine model was the similarity of the mouse retinal blood supply to that of man. However mice do not possess a macula. The retina of both man and mouse is holangiotic, that is the entire inner retina is supplied with blood vessels. This is in contrast to the rabbit which is a merangiotic animal, its retinal vasculature is confined to an elongated band across the centre of the retina (Duke-Elder, 1958).

Balb/c mice were selected in view of their lack of susceptibility or resistance to candidal infection. The mice used were randomly bred within a closed colony. This ensured a potential for heterogenicity in the expression of the experimental disease.

3.6.3. Infection Protocol

Infections were established by injecting $1x10^6$ blastospores into the tail vein of each mouse; 0.2 ml of a $5x10^6$ suspension was injected with a 30 gauge stainless steel needle.

3.6.4. Determination of Cumulative Mortality

The ability of the *C. albicans* strain H11 to cause systemic infection leading to death, in Balb/c mice, at the concentration used in the mouse model, was determined. Twenty-nine mice were inoculated and maintained under the same conditions as the animals used in the model. The number of deaths occurring on subsequent days was recorded.

3.6.5. Fundus Examination

Some of the experimental mice were examined with an indirect ophthalmoscope during the experimental period. Not all the mice were examined as the intense light beam required for this technique damages the retina. The fundus examination was restricted to four mice.

In order to examine the eyes, the pupils were dilated with 0.5% tropicamide (Minims, Smith and Nephew, Hull) to achieve full dilation the mice were left for 2 minutes before being examined with a 33 diopter Nikon lens and a Keeler indirect ophthalmoscope.

The clinical appearances of the eyes were recorded. This entailed looking for evidence of retinal detachment, segmentation of the blood column, tortuosity of the blood vessels and examining the colour of the choroid.

3.6.6. Fundus Photography

Four days after infection the fundi of the murine eyes were photographed after the method described by Dutton *et al.* (1984). The risk of causing retinal damage precluded mice other than those examined by indirect ophthalmoscopy being photographed.

The eyes were dilated by applying a drop of 0.5% tropicamide. The mice were left for 2 minutes to allow the pupils to dilate fully before the application of one drop of 0.4% benoxinate (Minims) as a local anaesthetic. This ensured the eyes remained open. The mouse to be examined was encouraged to enter a plastic 50 ml syringe case from which the end had been removed. The animal's head protruded from the end but it was unable to turn round (Figure 14). A drop of 2 % methyl cellulose (Dispersa) followed by a circular 22 mm glass coverslip (Chance Propper) were placed on the eye. This allowed the retina to be seen without refractive interference due to the corneal surface.

The photographs were taken using a Zeiss operating microscope (Op-Mi-1) with a beam splitter, a slit lamp attachment, a flashlight, a Zeiss Ikon camera and a 125 mm objective lens. The photographs were taken on Ektachrome 64 ASA film (Kodak) at f18 and at X40 magnification.

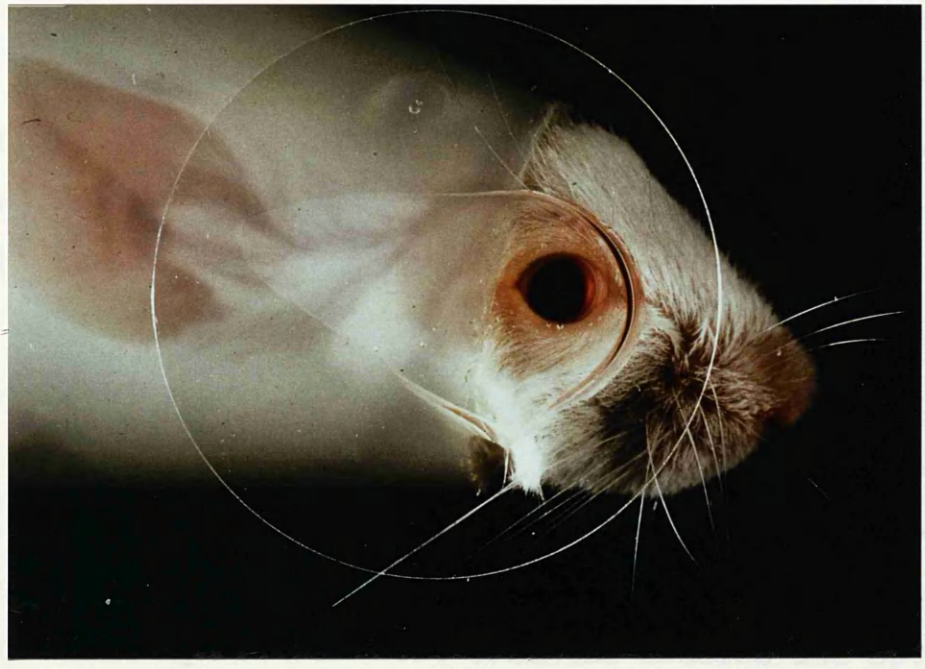


Figure 14 Restraining device for mice, illustrating a mouse with a coverslip in place.

3.6.7. Establishment of Animal Model

Twenty nine mice were infected as described above. The mice were sacrificed at various times after challenge. If any of the mice appeared moribund they were sacrificed; six mice died during the course of the experiment.

After sacrifice by chloroform, the eyes were removed. One eye from each mouse was fixed for histology and the other was cultured. Which eye was taken for histology and which for culture was decided by random sequence.

(i) Histology

The eye chosen for histological examination was carefully removed. The upper and lower eye lids were peeled back and the socket exposed. The eye was held with fine forceps and was cut from the muscles and optic nerve. The eye was immersion-fixed in neutral buffered formalin immediately after it was removed. It was embedded in paraffin and cut as described previously. The sections were placed on poly-L-lysine coated slides in ribbons or individually. Alternate slides were stained by PAS and the others were left as unstained blanks for PAP immunoperoxidase staining.

Some of the mice were selected for a more detailed histopathological examination. These animals were killed, then the skull was split along the saggittal suture. The bone was broken at the nose and the skull cap was removed. The thorax and abdominal cavities were opened. The mice

were immersion-fixed in neutral buffered formalin before being processed. Sections were prepared from brain, liver, skeletal muscle and the heart of these mice. The sections were stained by H & E and by PAS.

(ii) Culture

The kidneys and one eye from each animal were taken for culture to detect the presence of yeast. Culture of the organs would show the presence of any micro-organisms other than the expected *C. albicans*. The eyes were cultured to show eye infections that may not have been detectable on histological examination, to confirm the identity of any yeast present and to establish that any yeast present was viable.

The eyes and kidneys were homogenised with sterile distilled water in a Griffiths tube and cultured on glucose peptone agar. The identity of the isolated yeast was confirmed by germ tube production and chlamydospore tests.

(iii) Latex Particle Agglutination Assay for Mannan Antigenaemia

Blood was taken from some of the mice for serological tests. This blood was removed by heart puncture immediately after death. A *C. albicans* latex agglutination test (Mercia Diagnostics Ltd., Guildford, U.K.) was used to detect mannan antigenaemia in the sera.

Table 10; Details of the number of mice sacrificed at different time intervals post challenge and which eye was examined by histology and which by culture. The table also indicates which mice were selected for detailed histology and which animals were tested for mannan antigenaemia.

Time	Mouse	Eye Histology	Eye Culture	Antigen Assay	Detailed Histology
0	1	R	L		
0	2	L	R		
5 min	3	L	R		
5 min	4	R	L		
5 min	5	L	R		
1 hour	6	L	R		
1 hour	7	R	L		
1 hour	8	L	R		
36 hour	9	L	R	Yes	
36 hour	10	L	R	Yes	
36 hour	11	R	L	Yes	
3 days	12	L	R		Yes
3 days	13	R	L		Yes
3 days	14	L	R		Yes
5 days	15	R	L		Yes
5 days	16	L	R		
5 days	17	R	L		
5 days	18	L	R		Yes
6 days	19	L	R		
6 days	20	R	L		
6 days	21	L	R	Yes	Yes
6 days	22	R	L		Yes
7 days	23	R	L	Yes	Yes
7 days	24	L	R	Yes	Yes
8 days	25	L	R	Yes	Yes
8 days	26	R	L		Yes
8 days	27	L	R		
9 days	28	L	R	Yes	Yes
9 days	29	R	L	Yes	Yes

3.6.8. Differential Localisation of Yeasts Between Mouse Eyes as Determined by Culture.

The *Candida* endophthalmitis infections in the Glaswegian heroin addicts appeared to have a predilection

for the left eye in cases in which unilateral infection was present. Bilateral infection did occur in some patients and often developed if the infection persisted.

To ascertain if this phenomenon was reflected by the animal model, colony counts from cultures of whole eye homogenates were compared. The asymmetric nature of renal candidal infection in mice has been reported (Winblad, 1975).

Three groups of mice were infected with the same *C. albicans* Glasgow H11 strain under the same conditions as described previously. The eyes were removed, homogenised in 2.5 ml sterile distilled water and diluted to 1/10, 1/100, 1/1,000. Four 50 µl amounts of these dilutions were cultured on glucose peptone agar. The number of counts per eye was then calculated.

The minimum and maximum counts, for each mouse, were compared. The differences in the two counts were analysed by a small sample t-test (Bailey, 1981) to discover if any of the differences were significant.

$$t = \frac{x - u}{s\sqrt{n}}$$

The overall results for each group were compared as were the differences in counts per mouse for different times post challenge, in groups 2 and 3.

Group 1 - 8 mice; overall comparison

Group 2 - 10 mice; overall, day 3 and day 5 comparisons

Group 3 - 28 mice; overall, 5 min, 1 hour, 6 hour, 24 hour, 72 hour comparisons.

3.6.9. Determination of *Candida* Antigenaemia in the Mouse Model

(i) Sampling Frequency

Immediately after death blood was removed from 2 groups of mice, by puncturing the heart. The blood was allowed to clot then the serum was separated in a microcentrifuge. *Candida* mannan was assayed in these sera.

(ii) Antigen Detection Methods

Two latex agglutination methods were employed.

1. Immy La Candida (Immuno-Mycologics, Inc., Norman, OK, USA). This test detects mannan antigen. The sensitivity and specificity of this test have not been determined. The test incorporates a proteolytic enzyme step which is designed to dissociate immune complexes and to inactivate interfering factors such as rheumatoid factor.

2. Candida latex (Mercia Diagnostics Ltd., Guildford, U.K.). This test is designed to detect *Candida* mannan down to a level of 50 ng/ml. The test is specific for *C. albicans* serotypes A and B, and *C. glabrata*. The test has a control for rheumatoid factor, but has no dissociation step to release antigens from immune complexes.

In both tests the serum was used neat. Measured amounts of serum and sensitised latex particles were pipetted onto

a dark glass slide in one spot. The slide was gently agitated. If agglutination occurred (clumping of the latex particles) then the result was recorded as positive.

Mice were sacrificed at five minutes, one hour, six hours, 24 hours, 48 hours and 72 hours. Five mice were sacrificed at each time interval apart from at 48 hours, when only four mice were killed.

(iii) Controls for False Positives Results

Eight unchallenged Balb/c mice were sacrificed and the blood was removed and tested for *Candida* antigenaemia with the two latex agglutination kits described above.

3.7. Presumed *Candida* Endophthalmitis in Heroin Abusers: An Epidemiological Study

The aetiology of the *C. albicans* endophthalmitis in Glaswegian heroin addicts was examined from four aspects which appeared to be the most likely sources of the infection. Clinical specimens were taken from the addicts. The injection paraphernalia, a small sample of the street drug, and the diluent the addicts used for their heroin were cultured.

3.7.1. Patient Details

Twenty four heroin addicts who presented at the eye departments of several Glasgow hospitals were suspected of having *Candida* endophthalmitis. Details of these patients are given in Table 11. In an attempt to establish the source of the disease, several samples of injection paraphernalia and patients specimens were cultured on glucose peptone agar containing 50 μg/ml chloramphenicol. Table 12 lists the specimens and paraphrenalia that were obtained from the addicts for culture.

Table 11: Details of patients involved in the Glasgow outbreak. Available information on their age, sex, address, history of heroin abuse and which eye was involved. "-" signifies details not known.

Patient	Age	Sex	Eye	Address	Heroin Abuse
1 G T	29	M	B	Castlemilk	18 months
2 L S	23	M	L	Castlemilk	5 years
3 T S	22	M	R	Castlemilk	2 1/2 years
4 R W	16	M	L	Castlemilk	1 year
5 R M^{c}L	22	M	L	Castlemilk	1 1/2 years
6 G C	22	M	L	Castlemilk	3 years
7 C C	21	F	L	Castlemilk	2 years
8 S K	20	M	L	Castlemilk	6 weeks
9 R R	22	F	L	Castlemilk	-
10 J M^{c}C	27	M	L	Calton	-
11 R J	-	M	-	Pollockshaws	-
12 A T	-	M	-	Castlemilk	-
13 P J	30	M	L	Castlemilk	-
14 J K	19	M	L	Castlemilk	-
15 R W	21	M	L	Easterhouse	-
16 J K S	-	M	-	Castlemilk	-
17 T A	25	M	-	Castlemilk	-
18 G B	33	M	L	Castlemilk	-
19 L B	-	F	-	Castlemilk	-
20 A B	-	M	-	Castlemilk Heroin	-
21 J C	-	M	-	Castlemilk	-
22 G W	26	M	L	Castlemilk	-
23 P M^{c}C	33	F	L	G5	-
24 I R	23	M	L	Barrow/ Furness	-

Table 12: Details of fungal isolates from patients or their injection paraphernalia and the numbers assigned to isolates.

Patient Number	Source	Isolate	Isolate Number
3 T S	Vitrectomy	*C. albicans*	-
5 R McL	Heroin	no fungi	-
	Syringe	*Trichosporon* sp.	-
7 C C	Spoon	No fungi	-
	Syringe	*C. albicans*	1
	Syringe	*A. fumigatus*	-
	Lemon	*C. albicans*	2
9 R R	Scalp	*C. albicans*	4
10 J McC	Vitreous	No fungi	-
	Syringe washing	No fungi	-
	Plunger	*Penicillium* sp.	-
	Syringe	*A. fumigatus*	-
	Needle	No fungi	-
	Lemon	No fungi	-
11 R J	Syringe	*C. parapsilosis*	-
	Syringe	*R. rubra*	-
	Lemon	No fungi	-
12 T A	Lemon	*C. albicans*	3
13 J P	Vitreous	No fungi	-
14 J K	Throat swab	No fungi	-
15 W R	Throat swab	*C. albicans*	5
18 G B	Mouth wash	*C. albicans*	6
	Mouth swab	*C. albicans*	7
19 L B	Mouth swab	*C. albicans*	8
	Mouth wash	*C. albicans*	9
20 A B	Mouth swab	No fungi	-
	Mouth wash	No fungi	-
21 J C	Vitreous	No fungi	-
22 W G	Vitreous	*C. albicans*	10, 11, 12
23 P McC	Mouth wash	*C. albicans*	13, 15
	Faeces	*C. albicans*	14
	Lemon	No fungi	-
24 I R	Faeces	*C. albicans*	16

3.7.2. Biotyping Technique for *C. albicans* Isolates From Heroin Addicts.

The isolates of *C. albicans* cultured from the addicts and their paraphernalia were biotyped by the method of Odds and Abbott (1980; 1983). The method was used to determine if there was any relationship among the isolates by attempting to fingerprint each isolate by its growth pattern on 14 different media. Five of the media differentiated the species and the other nine were used to determine the biotype.

This technique was performed on the heroin isolates using the facilities in Dr. F.C. Odds' laboratory in Leicester. The materials and methods are not described here since the methods employed are given in detail in published papers (Odds and Abbott, 1980; 1983). Essentially, the procedure reveals patterns of assimilation or resistance to chemicals.

The isolates were grown on bromocresol green glucose peptone agar. Three dissimilar colonies from purity plates of each isolate were subcultured for use in the tests. The inocula were prepared from overnight cultures on nitrogen base glucose agar (NBGA) in accordance with the method described by Odds and Abbott (1980) to attain a concentration of yeast of 10^6 cells/ml.

Test petri dishes were poured from freshly prepared agar and employed yeast nitrogen base (YNB) or yeast carbon base

(YCB) as the basal medium. The test media were made in accordance with the modified recipes described by Odds and Abbott (1983) and were dried for the allotted time at 37°C. The proteinase test plates were not dried. Inoculation was made with a Leicester designed multipoint inoculator and the plates were inoculated in the order recommended; the details are given in Table 13. The plates were read at different time intervals depending on the test media concerned and the triplicate results recorded.

Table 13: Biotyping media in the order in which the plates were inoculated and the time after inoculation at which they were read.

Order Inoculated	Agar	Reading (Days)
1	Control	5-7
2	pH 1.55	3-4
3	Proteinase	5-7
4	pH 1.4	3-4
5	Urea	3-4
6	5-FC	5-7
7	Sorbose	5-7
8	Salt tolerance	3-4
9	Citrate	3-4
10	Boric acid	3-4
11	Safranine	2-3
12	Tetrazolium salts	5-7
13	MacConkeys	5-7
14	Cetrimide	3-4
15	Periodate	3-4

The incubation of five control strains on each plate ensured that; the media preparation, drying times, concentration of inocula and incubation period gave consistent results. The biotype consists of a three digit sequence which is consistent for a particular combination of test results for *C. albicans* grown on nine of the precisely controlled agar media. The other media were used to confirm the identification of the yeast.

Table 14; Strain differentiation for designation of biotypes, showing the calculation of biotype number by group.

Group 1		Group 2		Group 3	
Test	Score if +	Test	Score if +	Test	Score if +
pH 1.4	1	Urea	1	Citrate	1
Proteinase	2	Sorbose	2	Boric acid	2
5FC	4	Salt	4	Safranine	4

3.7.3. Inhibition of Yeast Growth by Heroin

The effect of heroin on yeasts was studied in an attempt to elucidate the cause of the Glasgow outbreak. The temporalspatial clustering of the *Candida* endophthalmitis suggested a common source of the infection. The obvious common denominator for all of these patients was heroin, especially as the patients themselves suspected their pusher of adulterating the drug with baker's yeast.

(i) Culture of Street Drug

Some samples of heroin confiscated from Castlemik were provided for culture by the Glasgow Drug Squad. The samples included one sample of dealers drug and six samples of pushers drug (Figure 2). The contents of the bags and the bags themselves were cultured on glucose peptone agar.

(ii) Antifungal Activity of Diamorphine Hydrochloride Sensitivity Plates - Powdered Drug

Agar plates of glucose peptone, yeast morphology and potato dextrose media were seeded with 0.5 ml of a 10^6 suspension of strains of *C. albicans* and *Saccharomyces cerevisiae*. Then 1 mg, 5 mg or 10 mg of diamorphine hydrochloride was placed in the centre. The plates were

prepared in duplicate and one was incubated at 28°C and the other at 37°C. In the instances when an inhibition zone was seen the zone was excised with a size 2 cork borer and further incubated on drug free media.

Yeast isolates Tested:- CA 6, CA 9, SAC 1, SAC 2 and Bakers Yeast (freeze dried shop bought yeast)

Heroin Isolates:- 2,3,4,5,6,7,8,9,10,11,12,13,14,15,16.

Sensitivity Plates - Liquid Drug

Plates seeded with *C. albicans* CA 6 and *S. cerevisiae* SAC 2 had wells removed using a size 2 cork borer. The wells were filled with diamorphine hydrochloride at different concentrations and distilled water to act as a control. The plates were incubated at 37°C and examined at 24 and 48 hours.

Drug concentrations:- 10, 100, 1,000, 10,000 µg/ml

Yeast Isolates:- CA6, CA9, SAC 1, SAC 2.

Incorporation Plates

Glucose peptone agar plates incorporating diamorphine hydrochloride were prepared. The plates were spot inoculated with 20 µl of $1x10^6$ suspension of yeast.

Yeast isolates:- CA 6, CA 9, SAC 1, SAC 2 and Bakers Yeast.

Drug concentrations:- 5, 10, 100 mg/ml.

Broth Dilution Experiments for Minimum Inhibitory Concentrations (MIC)

Tubes of glucose peptone broth containing different concentrations of diamorphine hydrochloride were inoculated with strains of yeast to give a final concentration of $5x10^4$ yeasts/ml in culture. To test the viability of the yeast after different contact times with the drug, 100 µl of the culture medium was removed for further incubation in drug free glucose peptone broth.

Drug concentrations:- 0, 2.5, 5, 10, 100, 250 or 500 mg/ml

Time intervals:- ½, 1, 2, 3, 4, 24 hours

Isolates tested:- CA6, CA9, SAC1, SAC2, Bakers Yeast

Broth Dilution Experiments for IC_{50} Determinations

The quantity of diamorphine hydrochloride required to perform MICs in broth tubes for a range of isolates, necessitated the reduction of the technique. A photometer-read broth microdilution method was used to assess the antifungal properties of the drug (Johnson, Richardson and Warnock, 1984). Test were performed in 96-well microtitre plates (Sterlin Ltd., Middlessex). The strains were tested against dilutions of diamorphine hydrochloride. The drug solutions and inoculum dilutions were prepared in glucose peptone broth and dispensed from an eight channel variable-volume pipette. The final volume in each well was

0.2 ml. Each row of 12 was used to assess one isolate at seven drug concentrations, the first well contained inoculum but no drug and the last well contained drug at the highest concentration but no yeast. The inoculum dilution was 1 x 10^6 cells/ml for every test and incubation was at 37°C. The plates were read at various times depending on the isolate tested.

The optical density (OD) of the microdilution plates were measured by a Dynatech MR 950 microELISA plate reader (Dynatech Laboratories, Billingshurst, U.K.) operating at a wavelength of 570 nm. the results were read and recorded manually.

The contact time with the heroin that leads to yeast death was determined in preliminary experiments. Aliquots of the culture were removed at intervals and cultured in drug free broth for 24 hours in an attempt to quantitate the survival of the yeasts.

Isolates tested:- CA6, CA9, SAC1, SAC2, Bakers Yeast

Drug concentrations:- 10, 20, 30, 40, 50, 100, 200 mg/ml.

Time intervals:- ½, 1, 2, 3, 4, 6, 7½, 24, 48 hours

The IC_{50} was determined for patient isolates after 24 hours incubation in diamorphine hydrochloride at various concentrations in glucose peptone broth. The IC_{50} was

calculated as the lowest concentration at which the OD was <30% of that of the positive control.

Isolates from Heroin Addicts:- 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15.

Drug Concentrations:- 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50 mg/ml.

Time Interval:- 24 hours.

3.7.4. Growth of Fungi in Lemon Juice

The drug addicts from Castlemilk diluted their heroin in the juice from "Jif" plastic lemons. The possibility that *Candida* could survive or grow in this juice was investigated.

Twenty seven "Jif" lemons were cultured for fungi. Seven were from friends and colleagues who had kept them in their cupboards for several months, five were donated by addicts. The remaining 15 were purchased from shops in the Castlemilk area of Glasgow.

(i) Survival of *C. albicans* in "Jif" Lemon Juice

C. albicans strain H 11 was inoculated into "Jif" lemon juice:- new juice, steamed new juice and juice past its "best by" date. 100 µl of a 1.2 X 10^7 yeast suspension in 2.5 ml of juice.

100 µl amounts were removed and cultured on glucose peptone agar plates at one hour intervals up to six hours and at 12 and 14 hours. The numbers of yeasts were high so dilution plates were employed. A glucose peptone broth was used as a control.

(ii) Determination of Sulphur Dioxide Content

Sulphites are permitted preservatives for certain foods including lemon juice. The amount of preservative is calculated in parts per million (ppm) of sulphur dioxide (SO_2).

Sulphurous acid inhibits the growth of moulds, yeast and bacteria. The browning of fruit and vegetables due to natural enzymatic activity is prevented by SO_2. SO_2 also prevents the breakdown of ascorbic acid but it causes the destruction of vitamin B_1. Since SO_2 is a gas there is a tendency for it to be lost if the packaging is not completely airtight. Once a container is opened the level of SO_2 falls rapidly. Moreover, a proportion of the gas will have formed complexes with constituents of the food and will no longer have any antimicrobial properties. The legislation, however is based on the total level of SO_2 in the foodstuff. The permitted level for lemon juice is 350 ppm (Preservative in Food Regulations, 1979). Colemans, the manufacturers of "Jif " lemons, say they buy lemon juice from more than one country, dilute it by one third in tap water, incorporate SO_2 as a preservative and then flash sterilise it at 200°C for 10 seconds before putting the juice into containers.

There are two principal methods for determining SO_2 content. The iodine titration method which is simpler and

quicker but less accurate than the distillation method. The more precise distillation method was the one employed in this study. The SO_2 in the sample is distilled slowly in the presence of an inert atmosphere. The gas is trapped in an oxidising agent which converts the sulphurous acid to sulphuric acid. The amount of sulphuric acid is determined by titration.

Distillation Method (Egan, 1981)

1. 15 ml of 3% H_2O_2 was pipetted into a Q & Q conical flask.
2. 5 ml of H_2O_2 was pipetted into a Q & Q U-tube.
3. 20 ml of H_2O_2 was pipetted into a conical flask for the blank titration.
4. 350 ml of deionised water was measured into a round bottomed flask.

The unit was assembled as in Figure 15. The condenser was cooled by tap water and the flow rate of the nitrogen gas was set at nine bubbles a minute. The nitrogen tube was removed and the weighed sample of lemon juice was introduced into the apparatus and 20 ml of concentrated hydrochloric acid (HCl) were added. The nitrogen tube was replaced.

The sample was distilled for 30 minutes. The conical flask and U-tube were disconnected and the liquid from the U-tube was washed into the conical flask.

Three drops of 0.4% bromophenol blue were added to the flask before the sample was titrated against 0.05 N sodium hydroxide (NaOH). The blank was titrated against NaOH to detect any free acid.

The concentration of SO_2 was determined as:-

1 ml 0.05 N Na OH = 1.6 mg SO_2 (mg/kg = ppm).

The following samples of lemon juice were tested for the presence of SO_2 by the method described above.

Table 15: Lemon juice samples tested. Details of the "best by" date, the source of the lemon if known, and if steamed how long it was steamed for.

Best By Date	Condition
Before Date	2 Weeks old
Before Date	2 Weeks old
New	Steamed 5 minutes
New	Steamed 15 minutes
New	Steamed 30 minutes
Beyond Date (5 months)	Lemon 17*
Beyond Date (7 Months)	Lemon 18*

*denotes lemons purchased in the Castlemilk area of Glasgow

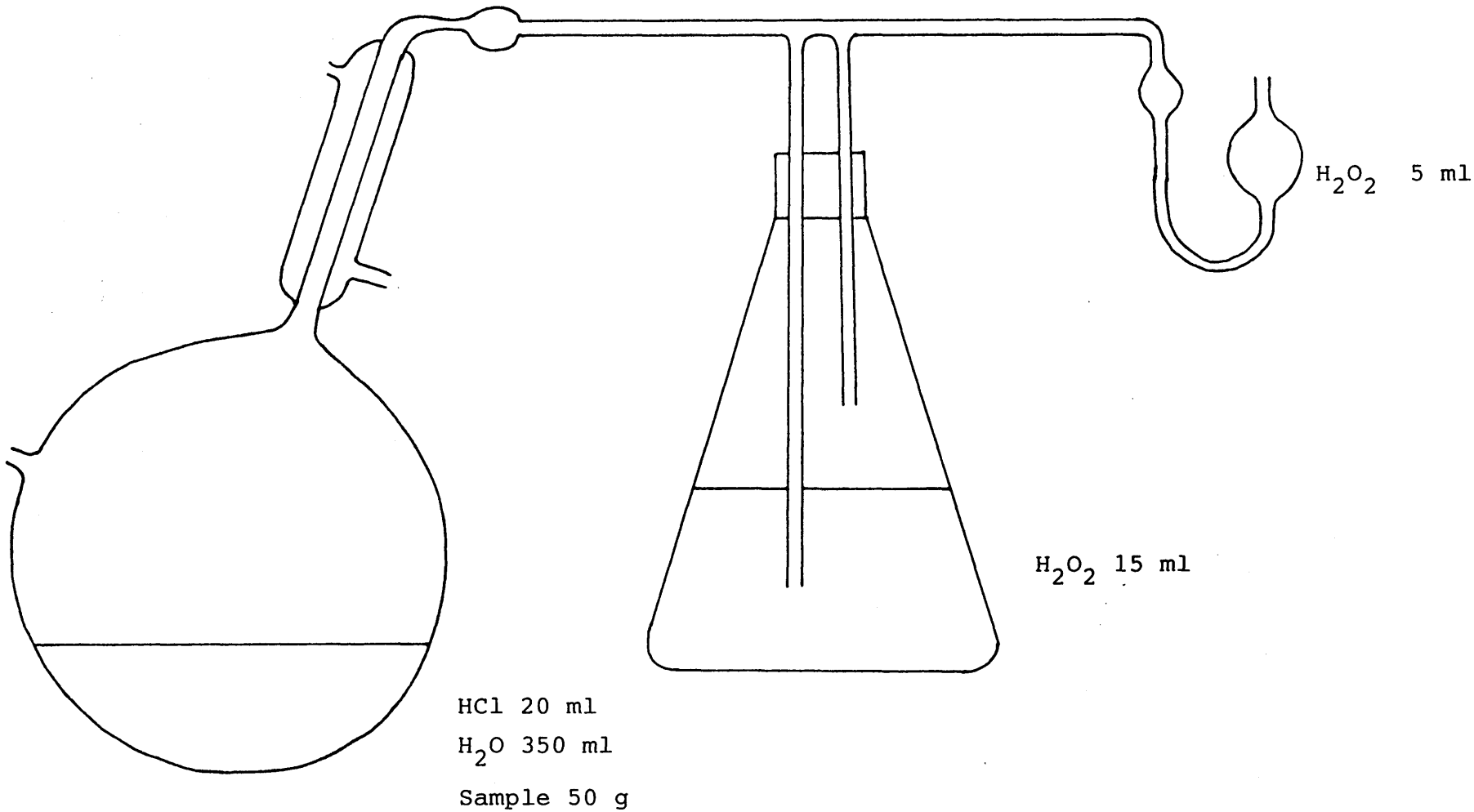


Figure 15 Distillation apparatus for the measurement of SO_2 .

4. RESULTS

4.1. Strength and Specificity of Antisera

4.1.1. Counterimmunoelectrophoresis

(i) Strength of Antisera

To determine the strength of the sera used in this study each antiserum was titred against a corresponding antigen type on an agarose gel in a CIE system as described in section 3.3.1. The results are shown in Table 16.

Table 16: Titre of laboratory prepared and commercial antisera as determined by CIE.

Source	Antiserum	Antigen	Titre
Rabbit 1	*A. fumigatus*	GAF 5	1:8
Rabbit 2	*R. oryzae*	Rh.sp.1	1:8
Rabbit 3	*C. albicans*	MRL	1:8
Rabbit 4	*C. albicans*	MRL	1:16
Rabbit 6	*A. fumigatus*	GAF 5	1:8
Inst. Past.	*A. fumigatus*	Commercial	1:8
Inst. Past.	*A. flavus*	Commercial	1:8
Inst. Past.	*A. nidulans*	Commercial	1:8
Inst. Past.	*A. niger*	Commercial	1:8
Inst. Past.	*A. terreus*	Commercial	1:8

(ii) Sensitivity and Specificity of Antisera

To demonstrate the ability of the antisera to react with a wide range of homologous antigens the antisera were electrophoresed in CIE with several antigens of the fungal species they were raised against. The presence (+) or absence (-) of cross-reactivity is shown in Table 17.

Table 17: Results of CIE demonstrating interactions of different *Aspergillus*, *Candida* and *Rhizopus* antigens with homologous antisera and indicating possible cross-reactions with heterologous antisera.

Antiserum	Antigen	Antigen	Result
A. fumigatus	*A. fumigatus*	Indian	+
A. fumigatus	*A. fumigatus*	GAF 5	+
A. fumigatus	*A. fumigatus*	GAF 83	+
A. fumigatus	*A. fumigatus*	GAF 84	+
A. fumigatus	*A. fumigatus*	GAF 85	+
A. fumigatus	*A. fumigatus*	GAF 86	+
A. fumigatus	*A. fumigatus*	GAF 86B	+
A. fumigatus	*A. flavus*	GAFl 2	−
A. fumigatus	*A. clavatus*	GAC 2	−
A. fumigatus	*A. nidulans*	ANi 1	−
A. fumigatus	*A. niger*	ANig 4	−
A. fumigatus	*A. terreus*	ATe 8	+
A. fumigatus	*C. albicans*	Mercia	−
A. fumigatus	*C. albicans*	MRL	−
A. fumigatus	*C. parap.*	MRL	−
A. fumigatus	*R. oryzae*	Rh 1	−
A. flavus	*A. fumigatus*	Indian	+
A. flavus	*A. flavus*	GAFl 1	+
A. flavus	*A. clavatus*	GAC 2	+
A. flavus	*A. nidulans*	GANi 1	−
A. flavus	*A. niger*	GANig 4	−
A. flavus	*A. terreus*	GATe 8	−
A. nidulans	*A. fumigatus*	Indian	−
A. nidulans	*A. flavus*	GAFl 1	−
A. nidulans	*A. clavatus*	GAC 2	−
A. nidulans	*A. nidulans*	GANi 1	+
A. nidulans	*A. niger*	GANig 4	−
A. nidulans	*A. terreus*	GATe 8	−
A. niger	*A. fumigatus*	Indian	+
A. niger	*A. flavus*	GAFl 1	+
A. niger	*A. clavatus*	GAC 2	+
A. niger	*A. nidulans*	GANi 1	+
A. niger	*A. niger*	GANig 4	+
A. niger	*A. terreus*	GATe 8	−
A. terreus	*A. fumigatus*	Indian	−
A. terreus	*A. flavus*	GAF 1	−
A. terreus	*A. clavatus*	GAC 2	−
A. terreus	*A. nidulans*	GANi 1	−
A. terreus	*A. niger*	GANig 4	−
A. terreus	*A. terreus*	GATe 8	+

Table 17 continued

Antiserum	Antigen	Antigen	Result
C. albicans	*C. albicans*	Mercia	+
C. albicans	*C. albicans*	MRL	+
C. albicans	*C. albicans*	GCA003	+
C. albicans	*C. albicans*	GCA406	+
C. albicans	*C. parap.*	MRL	+
C. albicans	*A. fumigatus*	Indian	-
C. albicans	*R. oryzae*	Rh 1	-
R. oryzae	*R. oryzae*	Rh 1	+
R. oryzae	*A. fumigatus*	Indian	-
R. oryzae	*C. albicans*	MRL	-

The *A. fumigatus* antisera reacted well against all the homologous antigen strains tested. There was no evidence of cross-reactions with *C. albicans*, *C. parapsilosis* or *R. oryzae* antigens. The other aspergilli varied in the response. No cross-reaction was evident with *A. clavatus*, *A. flavus*, *A. nidulans* and *A. niger* but precipitin lines were seen on gels electrophoresed with *A. terreus* antigen.

The *A. flavus* antiserum reacted not only with the *A. flavus* antigen but with the *A. fumigatus* and *A. clavatus* antigens. The *A. nidulans* antiserum showed no cross-reactions when tested with antigens from the other aspergilli. *A. niger* antiserum cross-reacted with antigens of all the aspergilli tested with the exception of *A. terreus*. The *A. terreus* antiserum only showed a response to the antigen from that species. The summarised results of the CIE experiments are given in Table 18.

Antibodies	Antigens								
	C.A.	C.P.	R.O.	A.C.	A.F.	A.Fl.	A.Ni.	A.Nig.	A.T.
C.A.	+	+	-		-	ND	ND	ND	ND
R.O.	-	ND	+	ND	-	ND	ND	ND	ND
A.F.	-	-	-	-	+	-	*	-	+
A.Fl.	ND	ND	ND	+	+	+	-	-	-
A.Ni.	ND	ND	ND	-	-	-	+	-	-
A.Nig.	ND	ND	ND	+	+	+	+	+	-
A.T.	ND	ND	ND	-	-	-	-	-	+

Table 18: Summary of CIE cross-reactivity results.

4.1.2. Double Diffusion Experiments to Demonstrate Cross-reactions and Lines of Identity With Antigens and Heterologous Antisera.

Cross-reactions between antigens with homologous and heterologous antisera were determined by double diffusion. The results are presented in Tables 19 and 20.

Table 19: Results of double diffusion experiments to determine cross-reactions between antisera and their homologous and heterologous antigens.

Antiserum	Antigen	Antigen	Result
A. fumigatus	*A. fumigatus*	MRL	+
C. albicans	*A. fumigatus*	MRL	-
R. oryzae	*A. fumigatus*	MRL	-
A. fumigatus	*A. fumigatus*	GAF 83	+
C. albicans	*A. fumigatus*	GAF 83	-
R. oryzae	*A. fumigatus*	GAF 83	-
A. fumigatus	*A. fumigatus*	GAF 84	+
C. albicans	*A. fumigatus*	GAF 84	-
R. oryzae	*A. fumigatus*	GAF 84	-
A. fumigatus	*C. albicans*	MRL	-
C. albicans	*C. albicans*	MRL	+
R. oryzae	*C. albicans*	MRL	-
A. fumigatus	*C. albicans*	GCA003	-
C. albicans	*C. albicans*	GCA003	+
R. oryzae	*C. albicans*	GCA003	-
A. fumigatus	*C. albicans*	C406	-
C. albicans	*C. albicans*	C406	+
R. oryzae	*C. albicans*	C406	-
A. fumigatus	*R. oryzae*	Rh. 1	-
C. albicans	*R. oryzae*	Rh. 1	-
R. oryzae	*R. oryzae*	Rh. 1	+

There were no lines of identity indicating cross-reactions among antigens of *A. fumigatus*, *C. albicans* and

R. oryzae and the heterologous antisera, although there were precipitin lines formed with the homologous antisera.

Table 20: Cross-reactivity results among *A. fumigatus*, *C. albicans* and *R. oryzae* as determined by double diffusion.

	Antigens 10 mg/ml		
Antibody	C. albicans	A. fumigatus	R. oryzae
C. abicans	+	-	-
A. fumigatus	-	+	-
R. oryzae	-	-	+

4.1.3. Experiments to Determine Strength and Specificity of Antibody with ELISA.

ELISA experiments were performed to ascertain the strength of the antisera with relation to homologous antisera and the amount of cross-reactivity with heterologous antisera. Dose response curves of antisera activity were plotted for each antigen antiserum combination. The discriminatory point between positive and negative values was set by taking the minimum positive response value as being $\chi 3$ the mean absorbance of the negative control (de Savigny and Voller, 1980). Any value greater than $\chi 3$ that of the negative controls at serum dilution 10^{-1} was regarded as being positive.

Tables 21 and 22 list the detailed results of the ELISA experiments comparing cross-reactions of antisera with various antisera. These results are summarised in Table 23. Examples of the dose response graphs drawn for *A. fumigatus*, *C. albicans* and *R. oryzae* are shown in Figures 16, 17 and 18.

Table 21: Details of ELISA experiments to detect reactions of various antigens at different concentrations with homologous and heterologous antiserum from laboratory and commercial sources.

Antigen	Antigen Number	Antiserum	Results
C. albicans	MRL 3153 (5 mg/ml)	*C. albicans* (R3)	+
C. albicans	MRL 3153 (5 mg/ml)	*C. albicans* (R4)	+
C. albicans	MRL 3153 (5 mg/ml)	*A. fumigatus* (R1)	-
C. albicans	MRL 3153 (5 mg/ml)	*A. fumigatus* (R6)	-
C. albicans	MRL 3153 (5 mg/ml)	*A. flavus* (PI)	-
C. albicans	MRL 3153 (5 mg/ml)	*A. nidulans* (PI)	-
C. albicans	MRL 3153 (5 mg/ml)	*A. niger* (PI)	-
C. albicans	MRL 3153 (5 mg/ml)	*A. terreus* (PI)	-
C. albicans	MRL 3153 (5 mg/ml)	*R. oryzae* (R2)	-
C. albicans	MRL 3153 (30 mg/ml)	*C. albicans* (R3)	+
C. albicans	MRL 3153 (30 mg/ml)	*C. albicans* (R4)	+
C. albicans	MRL 3153 (30 mg/ml)	*A. fumigatus* (R1)	-
C. albicans	MRL 3153 (30 mg/ml)	*A. flavus* (PI)	+
C. albicans	MRL 3153 (30 mg/ml)	*A. nidulans* (PI)	+
C. albicans	MRL 3153 (30 mg/ml)	*A. niger* (PI)	+
C. albicans	MRL 3153 (30 mg/ml)	*A. terreus* (PI)	-
C. albicans	MRL 3153 (30 mg/ml)	*R. oryzae* (R2)	-
C. albicans	mannan (30 mg/ml)	*C. albicans* (R3)	+
C. albicans	mannan (30 mg/ml)	*C. albicans* (R4)	+
C. albicans	mannan (30 mg/ml)	*A. fumigatus* (R1)	-
C. albicans	mannan (30 mg/ml)	*A. fumigatus* (R6)	-
C. albicans	mannan (30 mg/ml)	*A. flavus* (PI)	+
C. albicans	mannan (30 mg/ml)	*A. nidulans* (PI)	+
C. albicans	mannan (30 mg/ml)	*A. niger* (PI)	+
C. albicans	mannan (30 mg/ml)	*A. terreus (PI)*	-
C. albicans	mannan (30 mg/ml)	*R. oryzae*(R2)	-
C. parap.	MRL (5 mg/ml)	*C. albicans* (R3)	+
C. parap.	MRL (5 mg/ml)	*C. albicans* (R4)	+

Table 21 continued

Antigen	Antigen Number	Antiserum	Results
C. parap.	MRL (5 mg/ml)	*A. fumigatus* (R1)	-
C. parap.	MRL (5 mg/ml)	*R. oryzae* (R2)	-
A. clavatus	GAC 2 (30 mg/ml)	*A. fumigatus* (R1)	+
A. clavatus	GAC 2 (30 mg/ml)	*A. flavus* (PI)	+
A. clavatus	GAC 2 (30 mg/ml)	*A. nidulans* (PI)	+
A. clavatus	GAC 2 (30 mg/ml)	*A. niger* (PI)	+
A. clavatus	GAC 2 (30 mg/ml)	*A. terreus* (PI)	+
A. fumigatus	Indian	*C. albicans* (R3)	-
A. fumigatus	Indian	*C. albicans (R4)*	-
A. fumigatus	Indian	*A. fumigatus* (R1)	+
A. fumigatus	Indian	*R. oryzae* (R2)	-
A. fumigatus	AF 5 (5 mg/ml)	*C. albicans* (R3)	-
A. fumigatus	AF 5 (5 mg/ml)	*C. albicans* (R4)	-
A. fumigatus	AF 5 (5 mg/ml)	*A. fumigatus* (R1)	+
A. fumigatus	AF 5 (5 mg/ml)	*A. fumigatus* (R6)	+
A. fumigatus	AF 5 (5 mg/ml)	*A. flavus* (PI)	+
A. fumigatus	AF 5 (5 mg/ml)	*A. nidulans* (PI)	-
A. fumigatus	AF 5 (5 mg/ml)	*A. niger* (PI)	+
A. fumigatus	AF 5 (5 mg/ml)	*A. terreus* (PI)	-
A. fumigatus	AF 5 (5 mg/ml)	*R. oryzae (R2)*	-
A. fumigatus	AF 5 (30 mg/ml)	*C. albicans* (R3)	-
A. fumigatus	AF 5 (30 mg/ml)	*C. albicans* (R4)	-
A. fumigatus	AF 5 (30 mg/ml)	*A. fumigatus* (R1)	+
A. fumigatus	AF 5 (30 mg/ml)	*A. flavus* (PI)	+
A. fumigatus	AF 5 (30 mg/ml)	*A. nidulans* (PI)	-
A. fumigatus	AF 5 (30 mg/ml)	*A. niger* (PI)	+
A. fumigatus	AF 5 (30 mg/ml)	*A. terreus* (PI)	-
A. fumigatus	AF 5 (30 mg/ml)	*R. oryzae* (R2)	-
A. fumigatus	MRL (1.5 mg/ml)	*C. albicans* (R3)	-
A. fumigatus	MRL (1.5 mg/ml)	*C. albicans* (R4)	-
A. fumigatus	MRL (1.5 mg/ml)	*A. fumigatus* (R1)	+
A. fumigatus	MRL (1.5 mg/ml)	*A. flavus* (PI)	+
A. fumigatus	MRL (1.5 mg/ml)	*A. nidulans* (PI)	-
A. fumigatus	MRL (1.5 mg/ml)	*A. niger* (PI)	+
A. fumigatus	MRL (1.5 mg/ml)	*A. terreus* (PI)	-
A. fumigatus	MRL (1.5 mg/ml)	*R. oryzae*(R2)	-
A. fumigatus	MRL (30 mg/ml)	*A. fumigatus* (R1)	+
A. fumigatus	MRL (30 mg/ml)	*A. flavus* (PI)	+
A. fumigatus	MRL (30 mg/ml)	*A. nidulans* (PI)	-
A. fumigatus	MRL (30 mg/ml)	*A. niger* (PI)	+
A. fumigatus	MRL (30 mg/ml)	*A. terreus* (PI)	+
A. flavus	GA FL 1 (3 mg/ml)	*C. albicans* (R3)	-
A. flavus	GA FL 1 (3 mg/ml)	*C. albicans* (R4)	-

Table 21 continued

Antigen	Antigen Number	Antiserum	Results
A. flavus	GA FL 1 (3 mg/ml)	*A. fumigatus* (R1)	-
A. flavus	GA FL 1 (3 mg/ml)	*A. fumigatus* (R6)	-
A. flavus	GA FL 1 (3 mg/ml)	*A. flavus* (PI)	+
A. flavus	GA FL 1 (3 mg/ml)	*A. nidulans* (PI)	-
A. flavus	GA FL 1 (3 mg/ml)	*A. niger* (PI)	+
A. flavus	GA FL 1 (3 mg/ml)	*A. terreus* (PI)	+
A. flavus	GA FL 1 (3 mg/ml)	*R. oryzae* (R2)	-
A. flavus	GA FL 1 (30 mg/ml)	*C. albicans* (R3)	+
A. flavus	GA FL 1 (30 mg/ml)	*C. albicans* (R4)	+
A. flavus	GA FL 1 (30 mg/ml)	*A. fumigatus* (R1)	+
A. flavus	GA FL 1 (30 mg/ml)	*A. fumigatus* (R6)	-
A. flavus	GA FL 1 (30 mg/ml)	*A. flavus* (PI)	+
A. flavus	GA FL 1 (30 mg/ml)	*A. nidulans* (PI)	+
A. flavus	GA FL 1 (30 mg/ml)	*A. niger* (PI)	+
A. flavus	GA FL 1 (30 mg/ml)	*A. terreus* (PI)	+
A. flavus	GA FL 1 (30 mg/ml)	*R. oryzae* (R2)	-
A. nidulans	GA Nid 1 (30 mg/ml)	*C. albicans* (R3)	-
A. nidulans	GA Nid 1 (30 mg/ml)	*C. albicans* (R4)	-
A. nidulans	GA Nid 1 (30 mg/ml)	*A. fumigatus* (R1)	-
A. nidulans	GA Nid 1 (30 mg/ml)	*A. flavus* (PI)	-
A. nidulans	GA Nid 1 (30 mg/ml)	*A. nidulans* (PI)	+
A. nidulans	GA Nid 1 (30 mg/ml)	*A. niger* (PI)	-
A. nidulans	GA Nid 1 (30 mg/ml)	*A. terreus* (PI)	-
A. nidulans	GA Nid 1 (30 mg/ml)	*R. oryzae* (R2)	-
A. niger	GA N 2 (3 mg/ml)	*C. albicans* (R3)	-
A. niger	GA N 2 (3 mg/ml)	*C. albicans* (R4)	-
A. niger	GA N 2 (3 mg/ml)	*A. fumigatus* (R1)	-
A. niger	GA N 2 (3 mg/ml)	*A. flavus* (PI)	+
A. niger	GA N 2 (3 mg/ml)	*A. nidulans* (PI)	+
A. niger	GA N 2 (3 mg/ml)	*A. niger* (PI)	+
A. niger	GA N 2 (3 mg/ml)	*A. terreus* (PI)	+
A. niger	GA N 2 (3 mg/ml)	*R. oryzae* (R2)	-
A. niger	GA N 2 (30 mg/ml)	*C. albicans* (R3)	+
A. niger	GA N 2 (30 mg/ml)	*C. albicans* (R4)	+
A. niger	GA N 2 (30 mg/ml)	*A. fumigatus* (R1)	-
A. niger	GA N 2 (30 mg/ml)	*A. flavus* (PI)	+
A. niger	GA N 2 (30 mg/ml)	*A. nidulans* (PI)	+
A. niger	GA N 2 (30 mg/ml)	*A. niger* (PI)	+
A. niger	GA N 2 (30 mg/ml)	*A. terreus* (PI)	+
A. niger	GA N 2 (30 mg/ml)	*R. oryzae* (R2)	-
A. terreus	GA T 2 (30 mg/ml)	*C. albicans* (R3)	+
A. terreus	GA T 2 (30 mg/ml)	*C. albicans* (R4)	-
A. terreus	GA T 2 (30 mg/ml)	*A. fumigatus* (R1)	+
A. terreus	GA T 2 (30 mg/ml)	*A. fumigatus* (R6)	-
A. terreus	GA T 2 (30 mg/ml)	*A. flavus* (PI)	+

Table 21 continued

Antigen	Antigen Number	Antiserum	Results
A. terreus	GA T 2 (30 mg/ml)	*A. nidulans* (PI)	+
A. terreus	GA T 2 (30 mg/ml)	*A. niger* (PI)	+
A. terreus	GA T 2 (30 mg/ml)	*A. terreus* (PI)	+
A. terreus	GA T 2 (30 mg/ml)	*R. oryzae* (R2)	-
R. oryzae	G Rh1 (30 mg/ml)	*C. albicans* (R3)	-
R. oryzae	G Rh1 (30 mg/ml)	*C. albicans* (R4)	-
R. oryzae	G Rh1 (30 mg/ml)	*A. fumigatus* (R1)	-
R. oryzae	G Rh1 (30 mg/ml)	*A. flavus* (PI)	-
R. oryzae	G Rh1 (30 mg/ml)	*A. nidulans* (PI)	-
R. oryzae	G Rh1 (30 mg/ml)	*A. niger* (PI)	-
R. oryzae	G Rh1 (30 mg/ml)	*A. terreus* (PI)	-
R. oryzae	G Rh1 (30 mg/ml)	*R. oryzae* (R2)	+

The ELISA cross-reactivity results are summarised in Table 22.

The *C. albicans* antisera had a high affinity for both *C. albicans* antigens tested at both concentrations. They also reacted against the *C. parapsilosis* antigen. The antisera did not cross-react with any of the *Aspergillus* antigens at the lower concentration. However, if the concentration of the coating antigen was increased then the sera bound to *A. flavus* and *A. niger*. There was no reaction against the *R. oryzae* antigen.

The antisera raised against *R. oryzae* demonstrated no cross-reactions with any of the *Candida* or *Aspergillus* antigens even at the highest concentrations tested. The serum only reacted with the antigen it was raised against.

The *A. fumigatus* antiserum did not bind to either the *Candida* or the *R. oryzae* antigens. However, it did react against all the *A. fumigatus* antigens it was tested

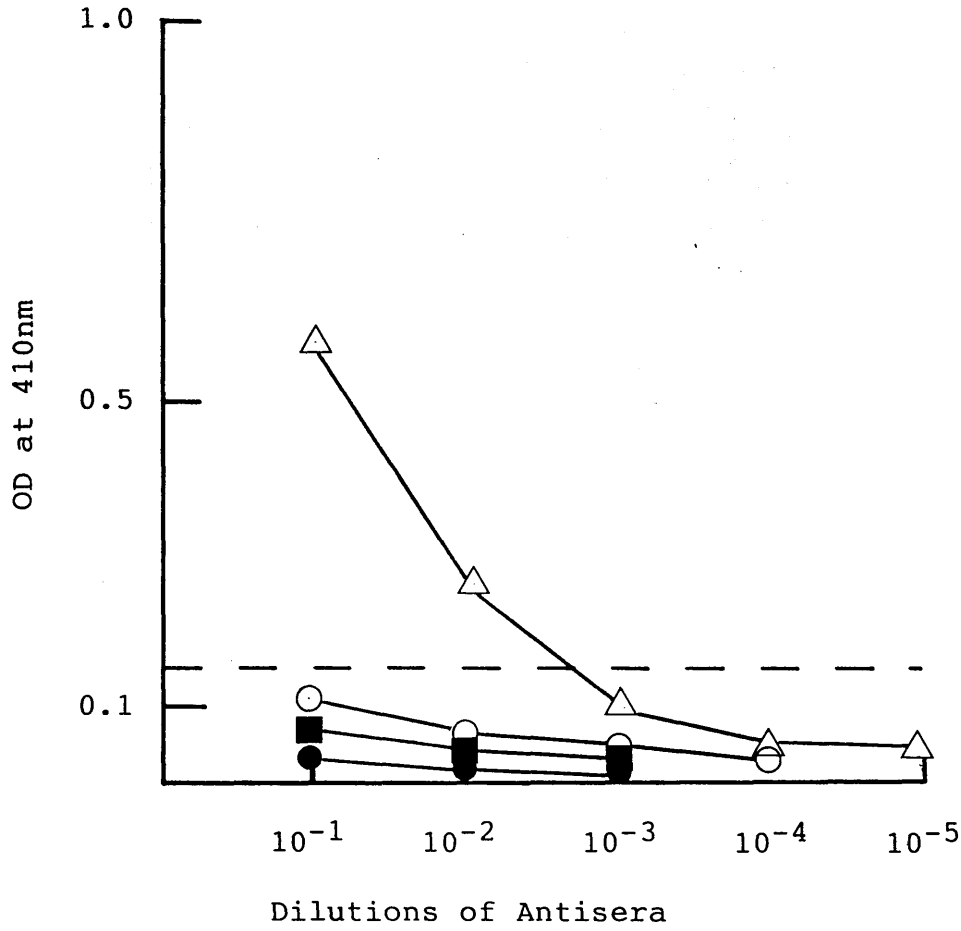


Figure 16 Reactivity of A.fumigatus (△), C.albicans (○), R.oryzae (■) antisera and normal rabbit serum (●) against A.fumigatus CF antigen in ELISA. The positive/ negative discrimination was set at three times the optical density (OD) of the normal rabbit serum at 10^{-1} and is indicated by the dotted line.

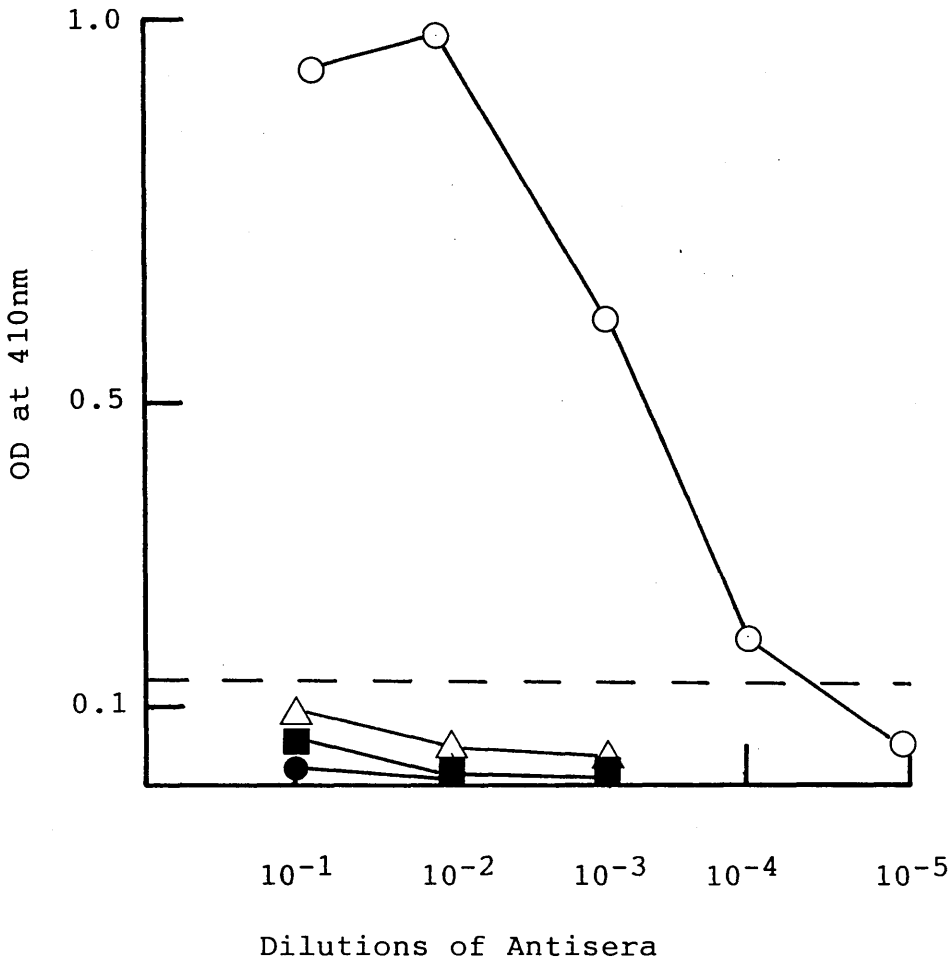


Figure 17 Reactivity of A.fumigatus (△), C.albicans (○), R.oryzae (■) antisera and normal rabbit serum (●) against C.albicans MRL 5mg/ml antigen in ELISA, The positive/ negative discrimination was set at three times the optical density (OD) of the normal rabbit serum at 10^{-1} and is indicated by the dotted line.

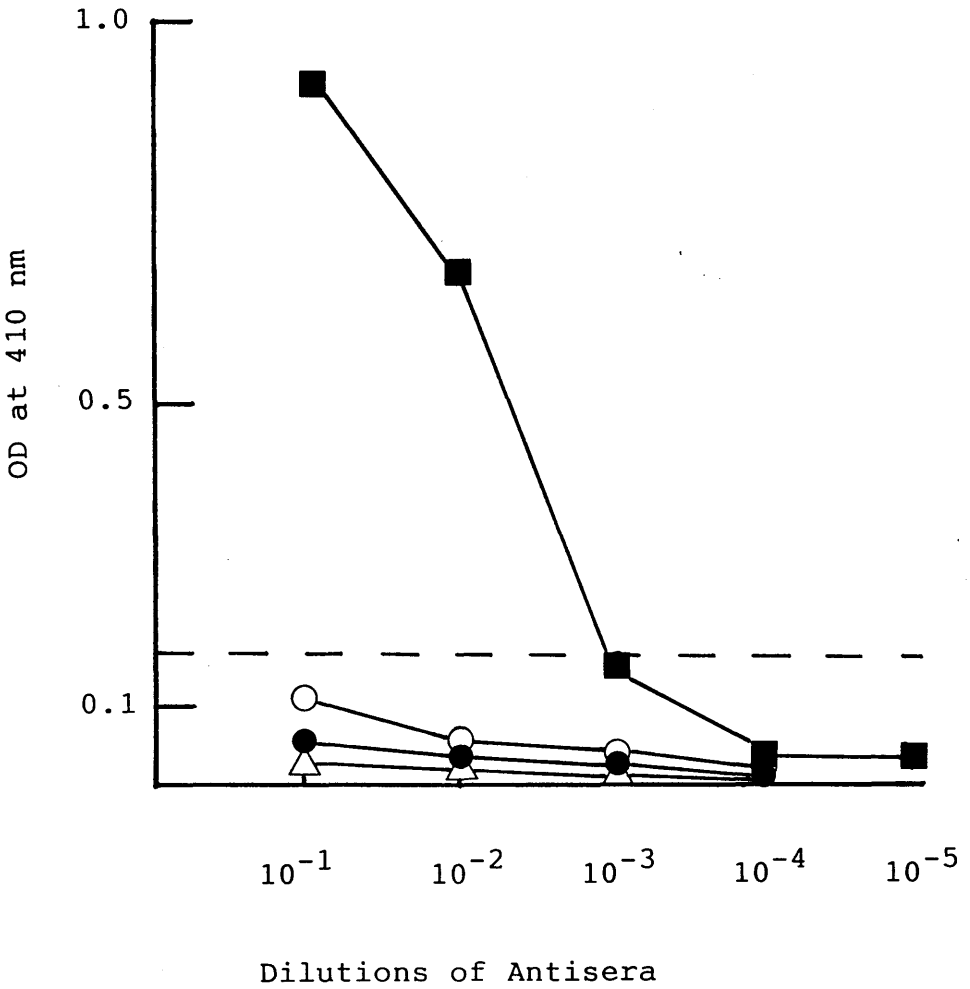


Figure 18 Reactivity of A.fumigatus (△), C.albicans (○), R.oryzae (■) antisera and normal rabbit serum (●) against R.oryzae antigen in ELISA. The positive/negative discrimination was set at three times the optical density (OD) of the normal rabbit serum at 10^{-1} and is indicated by the dotted line.

Antigens

Primary Antibody	C.A. MRL 5	C.A. MRL 30	C.A. Man 30	C.P. MRL 5	Rh Rh 30	A.C. A.C. 30	A.F. Ind 12	A.F. A.F. 5	A.F. A.F. 30	A.Fl. MRL 15	A.Fl. MRL 30	A.Fl. A.Fl. 3	A.Fl. A.Fl. 30	A.Ni. A.Ni. 30	A.Nig. A.Nig. 3	A.Nig. A.Nig. 30	A.T. A.T. 30
R3 C.A.	+	+	+	+	-	ND	-	-	-	-	ND	-	+	-	-	+	-
R4 C.A.	+	+	+	+	-	ND	-	-	-	-	ND	-	+	-	-	+	-
R2 R.O.	-	-	-	-	+	ND	-	-	-	-	ND	-	-	-	-	-	-
R1 A.F.	-	-	-	-	-	+	+	+	+	+	+	-	-	-	-	-	+
R6 A.F.	-	ND	-	ND	ND	ND	ND	+	ND	ND	ND	-	-	ND	ND	ND	-
P.I. A.Fl.	-	+	+	ND	+	+	ND	+	+	+	+	+	+	-	+	+	+
P.I. A.Ni.	+	+	+	ND	-	+	ND	-	-	-	-	-	+	+	+	+	+
P.I. A.Nig.	-	+	+	ND	-	+	ND	+	+	+	+	+	+	-	+	+	+
P.I. A.T.	-	-	-	ND	-	+	ND	-	-	-	-	+	+	-	+	+	+

Table 22: ELISA cross-reactivity results.

		SOLID PHASE COATING ANTIGEN								
		C.A.	C.P.	R.O.	A.C.	A.F.	A.Fl.	A.Ni.	A.Nig.	A.T.
Primary Antibody	C.A.	+	+	-	ND	-	-*	-	-*	+
	R.O.	-	-	+	ND	-	-	-	-	-
	A.F.	-	-	-	+	+	-*	-	-	+
	A.Fl.	-*	ND	-	+	+	+	-	+	+
	A.Ni.	+	ND	-		-	-*	+	+	+
	A.Nig.	-*	ND	-	+	+	+	-	+	+
	A.T.	-	ND	-	+	-	+	-	+	+

Table 23: ELISA cross-reactivity results

* If concentration of coating antigen was increased, some cross-reaction was evident.

against. It also reacted against *A. clavatus* and *A. terreus* at the 30 mg/ml concentration of antigen. The antiserum did not cross-react with *A. niger* or A. nidulans at the same concentration, nor did it react with the *A. flavus* antigen at 3 mg/ml.

The commercial *Aspergillus* antisera showed a greater amount of cross-reactivity than the laboratory raised antisera. *A. flavus* cross-reacted with all the antigens it was tested against with the exception of *A. nidulans* and a *C. albicans* antigen at 5 mg/ml. The *A. nidulans* antiserum did not show any reaction with *R. oryzae*, *A. fumigatus* or the lower concentration of the *A. flavus* antigen but bound to *C. albicans*, *A. clavatus*, *A. niger*, *A. terreus* and thehigher concentration of the *A. flavus* antigen.
Table 23 shows a summary of the ELISA cross-reactivity results

4.1.4. Streptavidin-Biotin Enzyme Linked Immunosorbent Antigen Assay for Testing Antiserum Specificity.

A streptavidin-biotin ELISA system employing an anti *A. fumigatus* human capture antibody and a rabbit secondary antibody was used to detect cross-reactions of *A. fumigatus* antisera with antigens of other aspergilli.

No cross-reactions were seen with antigens of *A. clavatus*, *A. flavus*, *A. nidulans*, *A. niger* or *A. terreus* when the antigens were diluted in human sera at levels of 100 µg to 10 ng/ml (Figure 19).

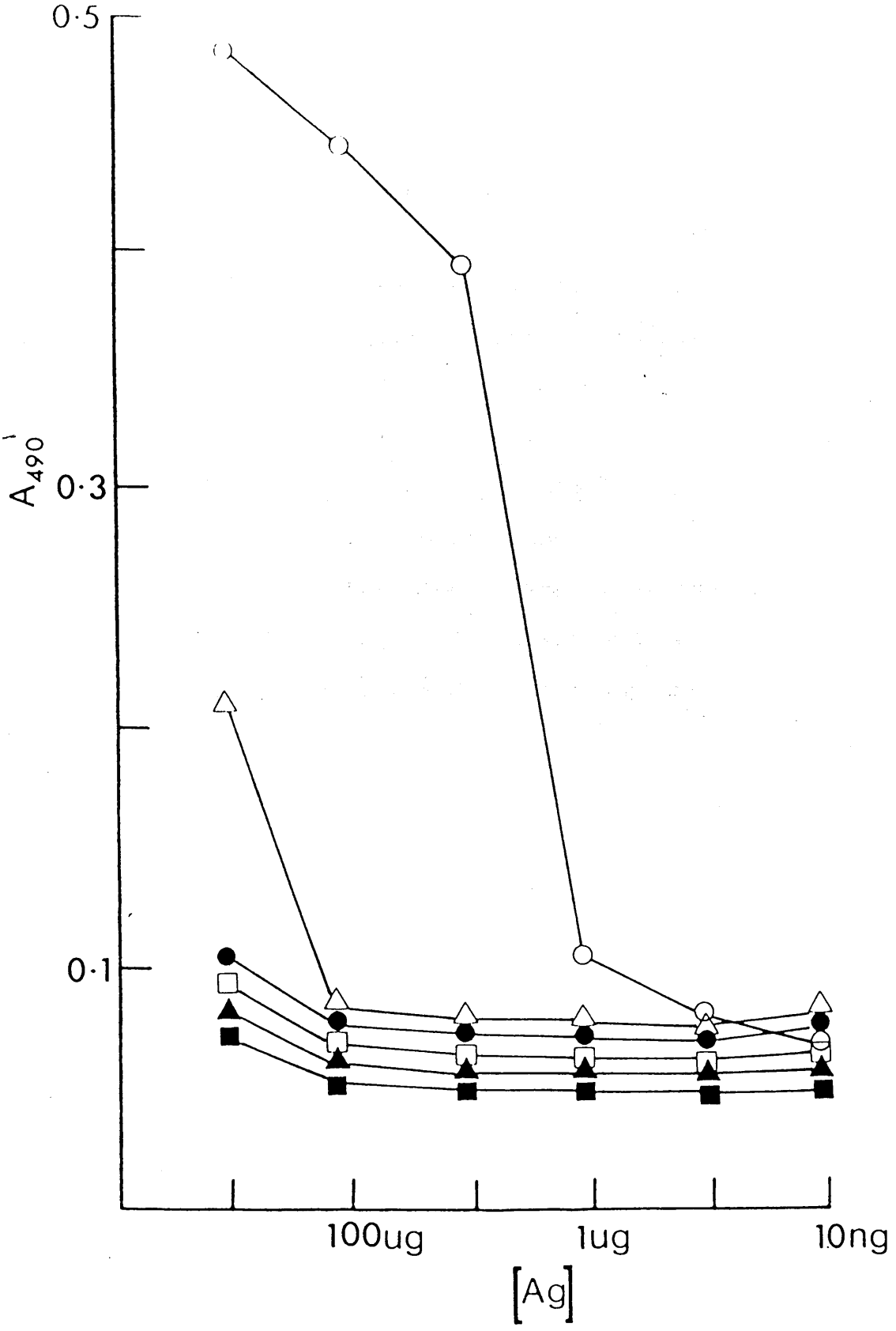


Figure 19 Dose-response curves for cytoplasmic antigens of A.fumigatus (○), A.flavus (□), A.niger (△), A.terreus (●) and A.nidulans (■) diluted in normal human serum detected by a human anti-A.fumigatus capture antibody - rabbit anti-A.fumigatus second antibody assay format.

4.2. Agar Block Technique for the Evaluation of the Peroxidase Anti-Peroxidase Staining Technique.

In order to determine the specificity of the peroxidase anti-peroxidase (PAP) technique, frozen sections from agar blocks inoculated with different species of fungi were stained using homologous and heterologous antiserum. The agar block method was successful in simulating fungi penetrating tissue in three dimensions. The results of staining sections from the blocks, employing the PAP technique are given in Table 24 and are summarised in Table 25. Positive staining and no staining are signified by "+" and "−" .

Table 24: Results of staining of agar blocks with the PAP technique. *C. parap.* is an abbreviation for *C. parapsilosis*.

Agar Block Inoculated With	Primary Antibody	Source	Result
C. albicans	*C. albicans*	R3 & R4	+
C. albicans	*A. fumigatus*	R1	−
C. albicans	*A. flavus*	P I	−
C. albicans	*A. nidulans*	P I	−
C. albicans	*A. niger*	P I	−
C. albicans	*A. terreus*	P I	−
C. albicans	*R. oryzae*	R2	−
C. albicans	Negative Serum	Rabbit	−
C. parap.	*C. albicans*	R3 & R4	−
C. parap.	*A. fumigatus*	R1	−
C. parap.	*A. flavus*	P I	−
C. parap.	*A. nidulans*	P I	−
C. parap.	*A. niger*	P I	−
C. parap.	*A. terreus*	P I	−
C. parap.	*R. oryzae*	R2	−
C. parap.	Negative serum	Rabbit	−

Table 24 continued.

Agar Block Inoculated with	Primary Antibody	Source	Result
A. fumigatus	*C. albicans*	R3 & R4	-
A. fumigatus	*A. fumigatus*	R1	+
A. fumigatus	*A. flavus*	P I	+
A. fumigatus	*A. nidulans*	P I	-
A. fumigatus	*A. niger*	P I	+
A. fumigatus	*A. terreus*	P I	+
A. fumigatus	*R. oryzae*	R2	-
A. fumigatus	Negative Serum	Rabbit	-
A. flavus	*C. albicans*	R3 & R4	-
A. flavus	*A. fumigatus*	R 1	-
A. flavus	*A. flavus*	P I	+
A. flavus	*A. nidulans*	P I	+
A. flavus	*A. niger*	P I	+
A. flavus	*A. terreus*	P I	+
A. flavus	*R. oryzae*	R2	-
A. flavus	Negative Serum	Rabbit	-
A. nidulans	*C. albicans*	R3 & R4	-
A. nidulans	*A. fumigatus*	R1	-
A. nidulans	*A. flavus*	P I	+
A. nidulans	*A. nidulans*	P I	+
A. nidulans	*A. niger*	P I	+
A. nidulans	*A. terreus*	P I	+
A. nidulans	*R. oryzae*	R2	-
A. nidulans	Negative Serum	Rabbit	-
A. niger	*C. albicans*	R3 & R4	-
A. niger	*A. fumigatus*	R1	-
A. niger	*A. flavus*	P I	+
A. niger	*A. nidulans*	P I	+
A. niger	*A. niger*	P I	+
A. niger	*A. terreus*	P I	+
A. niger	*R. oryzae*	R2	-
A. niger	Negative Serum	Rabbit	-
A. terreus	*C. albicans*	R3 & R4	-
A. terreus	*A. fumigatus*	R1	-
A. terreus	*A. flavus*	P I	+
A. terreus	*A. nidulans*	P I	-
A. terreus	*A. niger*	P I	+
A. terreus	*A. terreus*	P I	+
A. terreus	*R. oryzae*	R2	-
A. terreus	Negative Serum	Rabbit	-
R. oryzae	*C. albicans*	R3 & R4	-
R. oryzae	*A. fumigatus*	R1	-
R. oryzae	*A. flavus*	P I	+
R. oryzae	*A. nidulans*	P I	+

Table 24 continued.

Agar Block Inoculated With	Primary Antibody	Source	Result
R. oryzae	*A. niger*	P I	+
R. oryzae	*A. terreus*	P I	-
R. oryzae	*R. oryzae*	R2	+
R. oryzae	Negative Serum	Rabbit	-
M. hiemalis	*C. albicans*	R3 & R4	-
M. hiemalis	*A. fumigatus*	R1	-
M. hiemalis	*A. flavus*	P I	+
M. hiemalis	*A. nidulans*	P I	+
M. hiemalis	*A. niger*	P I	+
M. hiemalis	*A. terreus*	P I	-
M. hiemalis	*R. oryzae*	R2	+
M. hiemalis	Negative Serum	Rabbit	-
A. glauca	*C. albicans*	R3 & R4	-
A. glauca	*A. fumigatus*	R1	-
A. glauca	*A. flavus*	P I	-
A. glauca	*A. nidulans*	P I	-
A. glauca	*A. niger*	P I	-
A. glauca	*A. terreus*	P I	-
A. glauca	*R. oryzae*	R2	-
A. glauca	Negative Serum	Rabbit	-

The cross-reactivity results are summarised in Table 25. The *C. albicans* antisera stained the sections from the *C. albicans* block (Figure 20). However, it did not appear to stain the sections from the *C. parapsilosis* block. None of the sections from heterologous blocks stained when the *C. albicans* antisera was employed as the first antibody.

The *R. oryzae* antiserum did not react with any of the sections from the *Aspergillus* blocks nor the *C. albicans* sections it was tested against. The *R. oryzae* antiserum stained only when used as the first antibody for the *R.*

oryzae (Figure 21) and *Mucor hiemalis* sections. It did not stain sections containing *Absidia glauca*.

The *A. fumigatus* antiserum when employed as the primary antibody only stained the sections from the agar inoculated with *A. fumigatus* (Figure 22). This antiserum did not stain any of the other *Aspergillus* sections nor did it stain any of the mucoraceous fungi or *Candida*.

Candida sections were not stained when the *A. flavus* antisera was used as the first antibody in the PAP technique. However, the antiserum showed positive staining in sections from blocks inoculated with all the other *Aspergillus* species tested and with *R. oryzae* and *M. hiemalis*. The *A. flavus* antiserum did not react with the *A. glauca* sections.

A. nidulans antiserum did not stain any *Candida* or *A. glauca* sections. The antiserum did not stain *A. fumigatus* or *A. terreus*. but did stain sections from *A. flavus* and *A. niger* blocks in addition to the sections from the *A. nidulans* block.

Cross-reactions were evident when the antiserum raised against *A. niger* was used as the primary antibody in PAP staining. The antiserum did not stain the *Candida* or the *A. glauca* sections. However, the *R. oryzae, M. hiemalis* and all the *Aspergillus* sections which the serum was tested against showed positive staining (Figure 23).

The *A. terreus* antiserum did not stain sections from blocks inoculated with fungi from genera other than

		AGAR BLOCKS INOCULATED WITH:									
		C.A.	C.P.	A.F.	A.Fl.	A.Ni.	A.Nig.	A.T.	R.O.	M.H.	A.G.
PRIMARY ANTIBODY	C.A.	+	-	-	-	-	-	-	-	-	-
	R.O.	-	-	-	-	-	-	-	+	+	-
	A.F.	-	-	+	-	-	-	-	-	-	-
	A.Fl.	-	-	+	+	+	+	+	+	+	-
	A.Ni.	-	-	-	+	+	+	-	+	+	-
	A.Nig.	-	-	+	+	+	+	+	+	+	-
	A.T.	-	-	+	+	+	+	+	-	-	-
	Negative Control	-	-	-	-	-	-	-	-	-	-

TABLE 25 : Agar blocks cross-reactivity results.

Aspergillus. However, the *A. terreus* antiserum did cross-react with every *Aspergillus* species it was tested against in the PAP immunoperoxidase staining procedure.

Examples of the PAP staining of sections from agar blocks inoculated with fungi are illustrated in Figures 20, 21, 22 and 23.

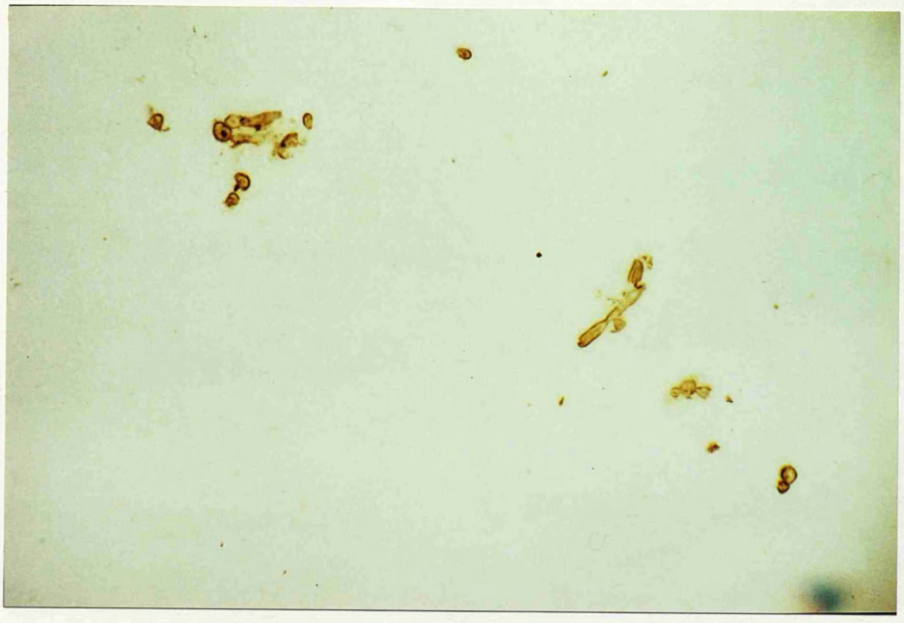


Figure 20 Section from an agar block inoculated with C.albicans stained by the PAP method using C.albicans antiserum as the primary antibody.

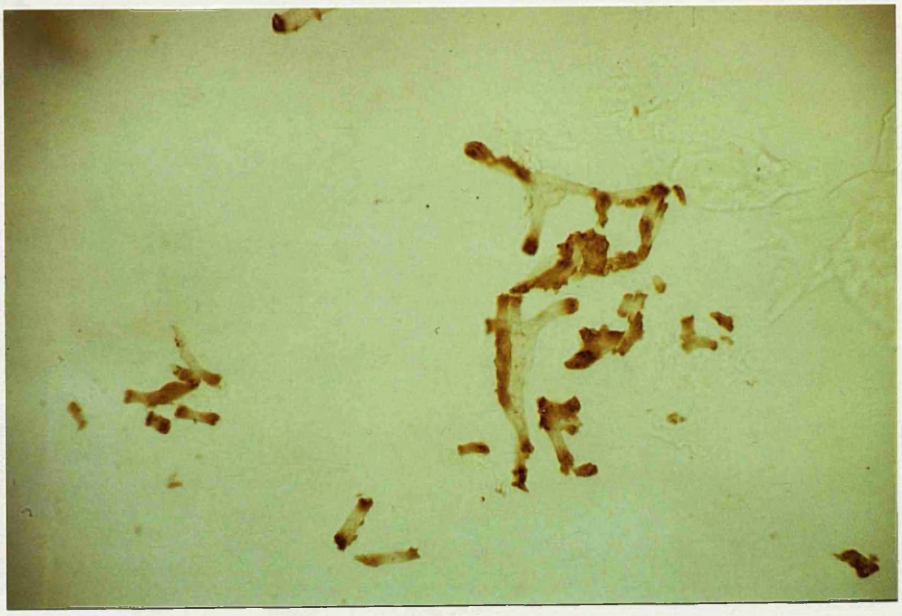


Figure 21 Section from an agar block inoculated with R.oryzae stained by the PAP method using R.oryzae antiserum as the primary antibody.

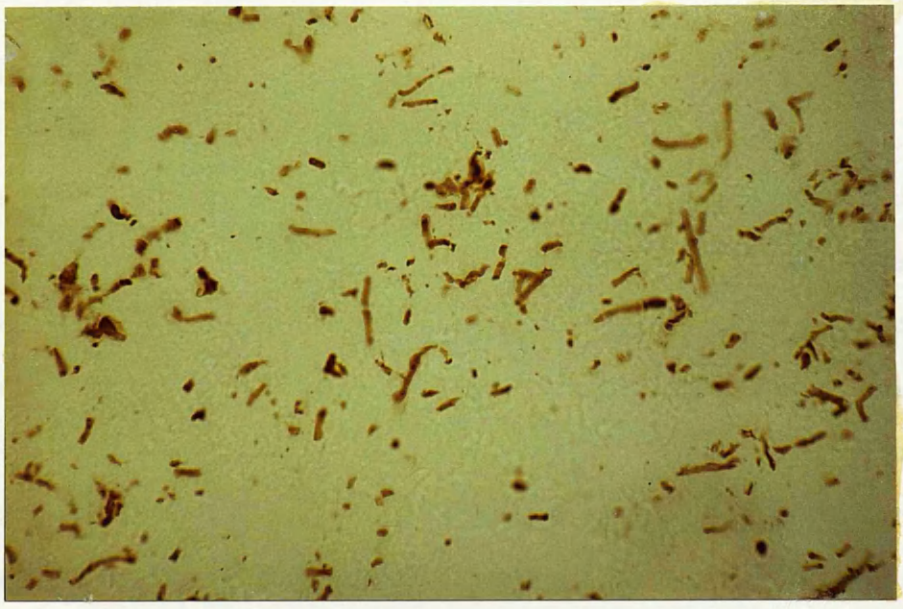


Figure 22 Section from an agar block inoculated with A.fumigatus stained by the PAP method using A.fumigatus antiserum as the primary antibody.

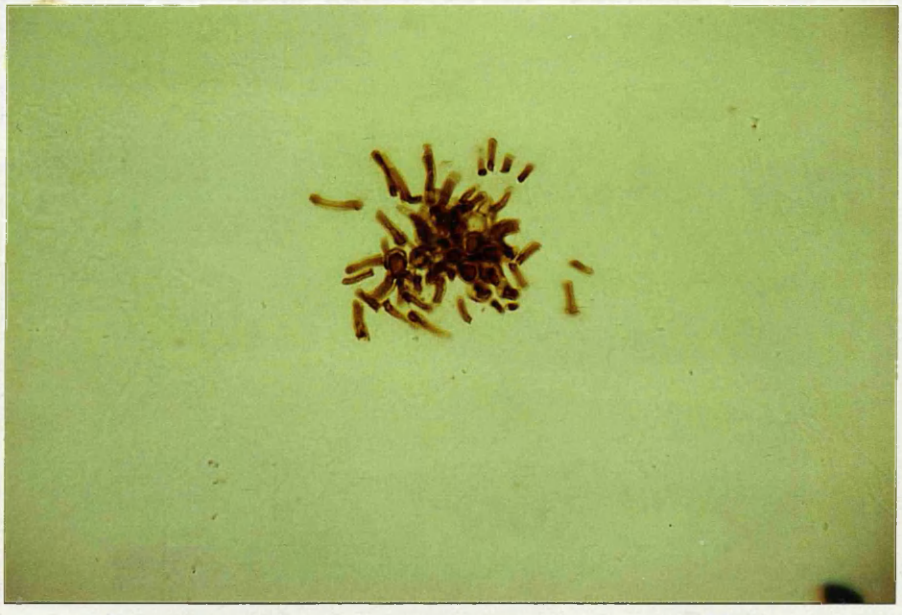


Figure 23 Section from an agar block inoculated with A.niger stained by the PAP method using A.niger antiserum as the primary antibody.

4.3. Peroxidase Anti-Peroxidase Staining of Tissues

Tissue samples from several cases of opportunistic fungal infections were stained by the PAP immunoperoxidase method. Homologous and heterologous antisera were employed to determine the degree of cross-reactivity that occurred.

Details of diseases, organs, which antiserum was used as the primary antibody in the staining method and the results of the staining are given in Table 26.

Table 26: Sections from clinical material stained by the PAP immunoperoxidase method with details of the organ involved, the suspected aetiologic agent and the antiserum used as the primary antibody. A/loma denotes aspergilloma, *A. fum.* denotes *A. fumigatus* and *C.parap.* denotes *C. parapsilosis.*

Antiserum	Disease	Organ	Result
A. fumigatus	Aspergillosis (*A. fum.*)	Lung	+
A. fumigatus	Aspergillosis (*A. fum.*)	A/loma	+
A. fumigatus	Aspergillosis (*A. fum.*)	Brain	+
A. fumigatus	Aspergillosis (*A. fum.*)	Trachea	+
A. fumigatus	Aspergillosis	Paranasal sinuses	+
A. fumigatus	Aspergillosis	Kidney	+
A. fumigatus	Aspergillosis	Heart	+
A. fumigatus	Aspergillosis (*A. flavus*)	Lung	-
A. fumigatus	Candidosis (*C. albicans*)	Liver	-
A. fumigatus	Candidosis (*C. albicans*)	Kidney (mouse)	-
A. fumigatus	Candidosis	Liver	-
A. fumigatus	Candidosis	Brain	-
A. fumigatus	Candidosis	Laryngeal biopsy	-
A. fumigatus	Candidosis (*C. parap.*)	Lung	-
A. fumigatus	Candidosis (*C. parap.*)	Kidney	-
A. fumigatus	Candidosis (*C. parap.*)	Adrenal	-

Table 26 continued

Antiserum	Disease	Organ	Result
A. fumigatus	Mucormycosis (*R. oryzae*)	Soft palate	-
A. fumigatus	Mucormycosis	Brain	-
A. fumigatus	Mucormycosis	Kidney	-
A. fumigatus	Mucormycosis	Spleen	-
A. flavus	Aspergillosis (*A. fum.*)	Lung	+
A. flavus	Aspergillosis (*A. flavus*)	Lung	+
C. albicans	Candidosis (*C. albicans*)	Liver	+
C. albicans	Candidosis (*C. albicans*)	Kidney (mouse)	+
C. albicans	Candidosis	Liver	+
C. albicans	Candidosis	Brain	+
C. albicans	Candidosis	Laryngeal biopsy	+
C. albicans	Candidosis (*C. parap.*)	Lung	-
C. albicans	Candidosis (*C. parap.*)	Kidney	-
C. albicans	Candidosis (*C. parap.*)	Adrenal	-
C. albicans	Aspergillosis (*A. fum.*)	Lung	-
C. albicans	Aspergillosis (*A. fum.*)	A/loma	+
C. albicans	Aspergillosis	Lung	-
C. albicans	Aspergillosis	Trachea	-
C. albicans	Aspergillosis	Paranasal sinuses	-
C. albicans	Aspergillosis	Kidney	-
C. albicans	Aspergillosis	Heart	-
C. albicans	Aspergillosis (*A. flavus*)	Lung	-
C. albicans	Mucormycosis (*R. oryzae*)	Soft palate	-
C. albicans	Mucormycosis	Brain	-
C. albicans	Mucormycosis	Kidney	-
C. albicans	Mucormycosis	Spleen	-
R. oryzae	Mucormycosis (*R. oryzae*)	Soft palate	+
R. oryzae	Mucormycosis	Brain	+
R. oryzae	Mucormycosis	Kidney	+
R. oryzae	Mucormycosis	Spleen	+
R. oryzae	Aspergillosis (*A. fum.*)	Lung	-
R. oryzae	Aspergillosis (*A. fum.*)	A/loma	-
R. oryzae	Aspergillosis	Lung	-
R. oryzae	Aspergillosis	Trachea	-
R. oryzae	Aspergillosis	Paranasal sinuses	-
R. oryzae	Aspergillosis	Kidney	-
R. oryzae	Aspergillosis	Heart	-
R. oryzae	Aspergillosis (*A. flavus*)	Lung	-

Table 26 continued

Antiserum	Disease	Organ	Result
R. oryzae	Candidosis (*C. albicans*)	Liver	-
R. oryzae	Candidosis (*C. albicans*)	Kidney (mouse)	-
R. oryzae	Candidosis	Liver	-
R. oryzae	Candidosis	Brain	-
R. oryzae	Candidosis	Laryngeal biopsy	-
R. oryzae	Candidosis (*C. parap.*)	Lung	-
R. oryzae	Candidosis (*C. parap.*)	Kidney	-
R. oryzae	Candidosis (*C. parap.*)	Adrenal	-

As indicated in Table 26 the laboratory raised *A. fumigatus* antisera, when used as primary antibody, stained *A. fumigatus* in sections from all the infected organs tested (Figures 24 and 25). In addition to staining *A. fumigatus* from culture proven cases, the method confirmed the infection in cases where the aetiologic fungus was not isolated in culture. The *A. fumigatus* antisera did not stain sections from cases of *A. flavus* aspergillosis. No unstained sections from cases of aspergillosis caused by other *Aspergillus* species were available. The *A. fumigatus* antisera did not stain sections from cases of candidosis caused by either *C. albicans* or *C. parapsilosis*, nor any of the sections from patients with mucormycosis.

However, the commercial *A. flavus* antiserum not only stained the sections from *A. flavus* cases of aspergillosis but also sections of lung from non-*A. flavus* cases.

The laboratory raised *C. albicans* antisera strongly stained cases of infections attributable to *C. albicans* (Figure 26) but it was less successful in staining cases

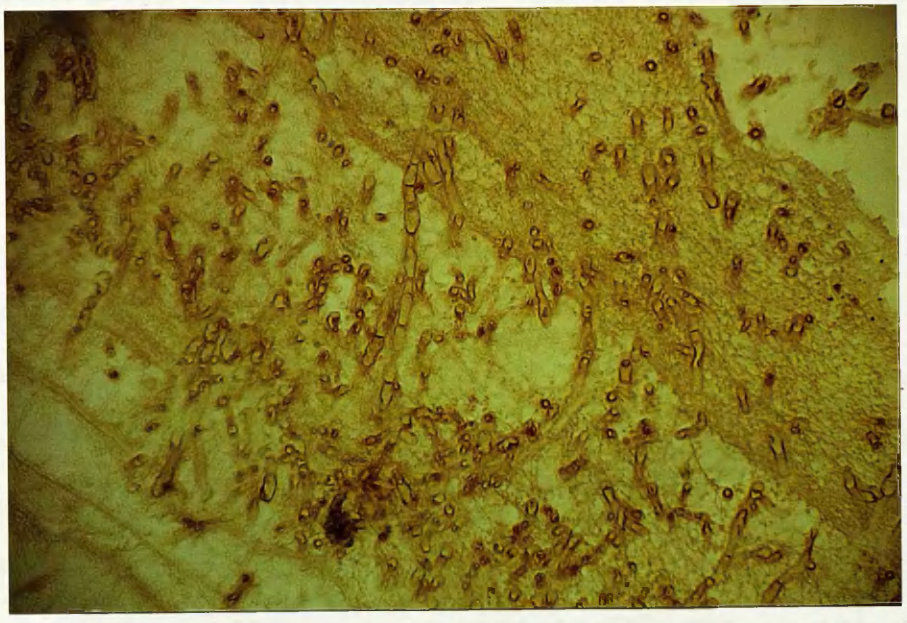


Figure 24 A.fumigatus infection of lung stained by the PAP method using A.fumigatus as the primary antibody.

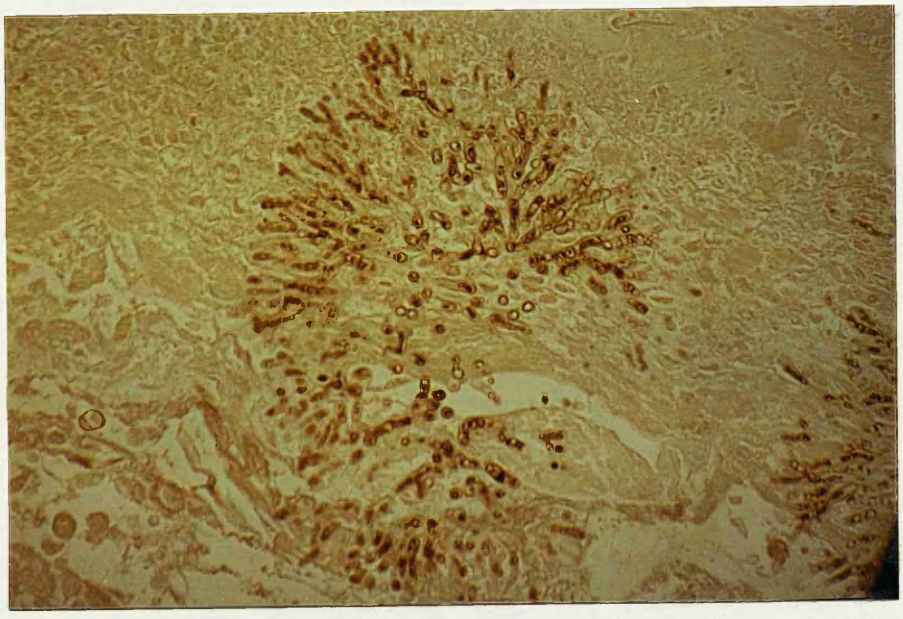


Figure 25 A.fumigatus infection of trachea stained by the PAP method using A.fumigatus as the primary antibody.

infected by *C. parapsilosis*. The *C. albicans* antisera did not stain tissue sections from patients with aspergillosis or mucormycosis.

The *R. oryzae* antiserum only stained fungus in tissue from patients with mucormycosis (Figure 27). It stained the fungi in all the sections, although in two of the cases the mucoraceous fungus implicated had not been isolated. The amount of cross-reaction with this antiserum appeared to be minimal as it did not stain the fungi in sections from patients with aspergillosis or candidosis. An example of the lack of staining by this antibody when employed in staining heterologous fungi is shown in Figure 28.

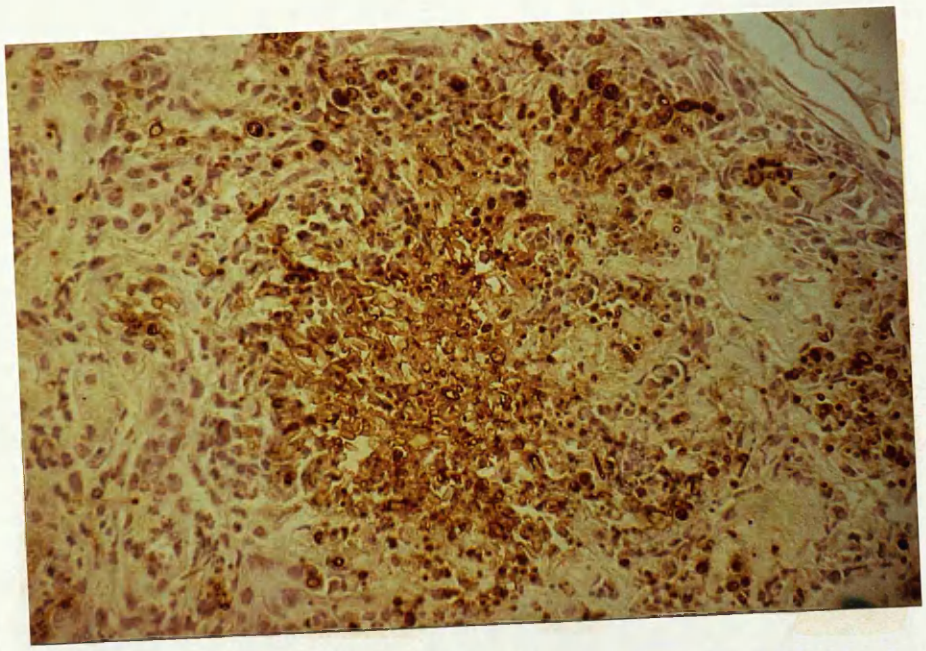


Figure 26 C.albicans infection of mouse kidney stained by the PAP method using C.albicans as the primary antibody. The section was weakly counterstained with haematoxylin.

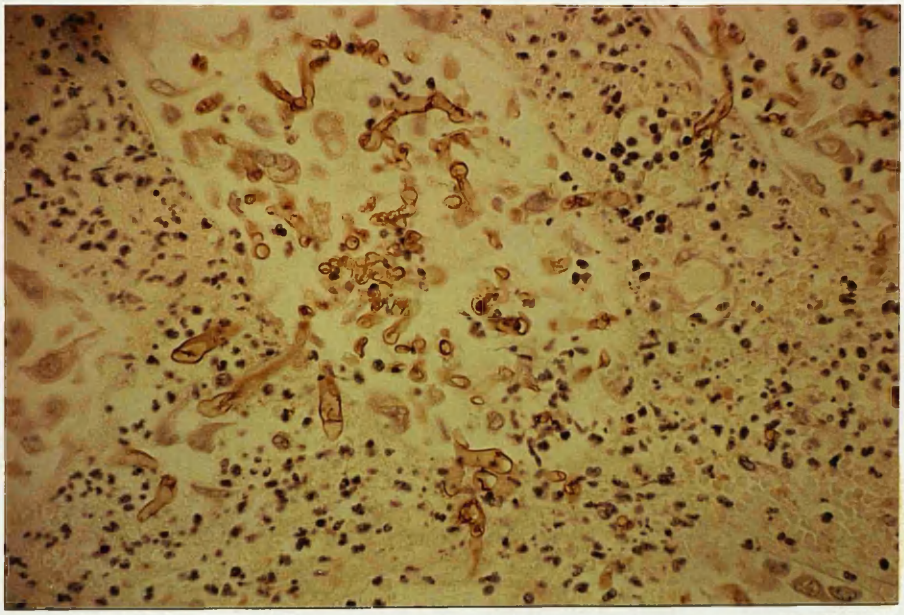


Figure 27 Muceraceous fungi in brain stained by the PAP method using R.oryzae antiserum as the primary antibody. The section was weakly counterstained with haematoxylin.

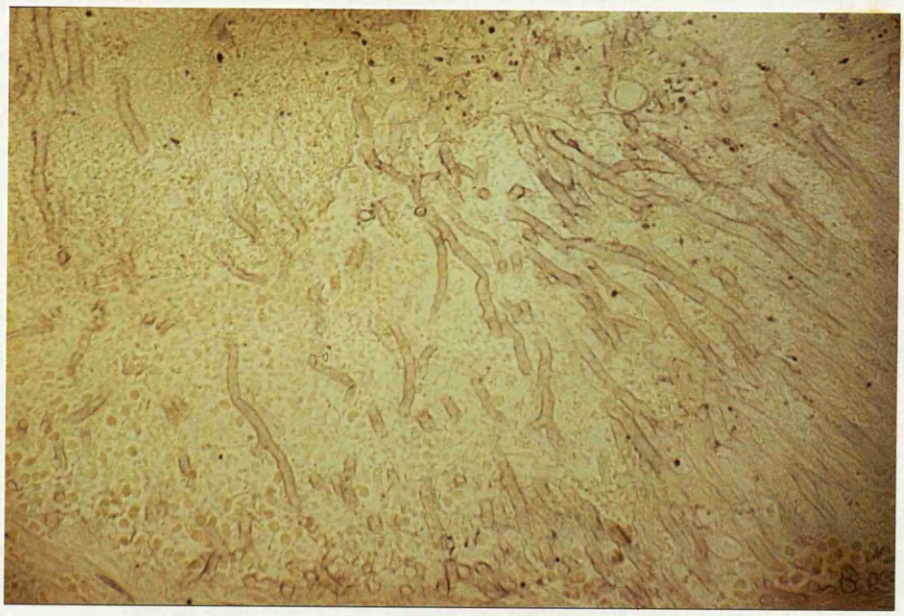


Figure 28 A.flavus infection of lung that did not stain by the PAP method using R.orzyae as the primary antibody. The section was weakly counterstained with haematoxylin which demonstrates the presence of the fungal elements.

4.4. A Mouse Model of Haematogenous *Candida* Endophthalmitis

4.4.1. Cumulative Mortality

The H 11 strain of *C. albicans* was injected into 29 mice at the concentration to be used in the mouse model and the cumulative mortality was recorded. The results are given in Table 27. Figure 29 shows the graph of cumulative mortality.

Table 27: Results of the cumulative mortality of 29 mice inoculated with *C. albicans* strain H 11 at a concentration of $1x10^6$ per animal.

Days Post challenge	Number of mice died	Cumulative mortality
1	0	0
2	2	2
3	3	5
4	6	11
5	9	20
6	6	26
7	1	27
8	2	29

The mouse survival time was eight days, with the most mice dying on the fifth day.

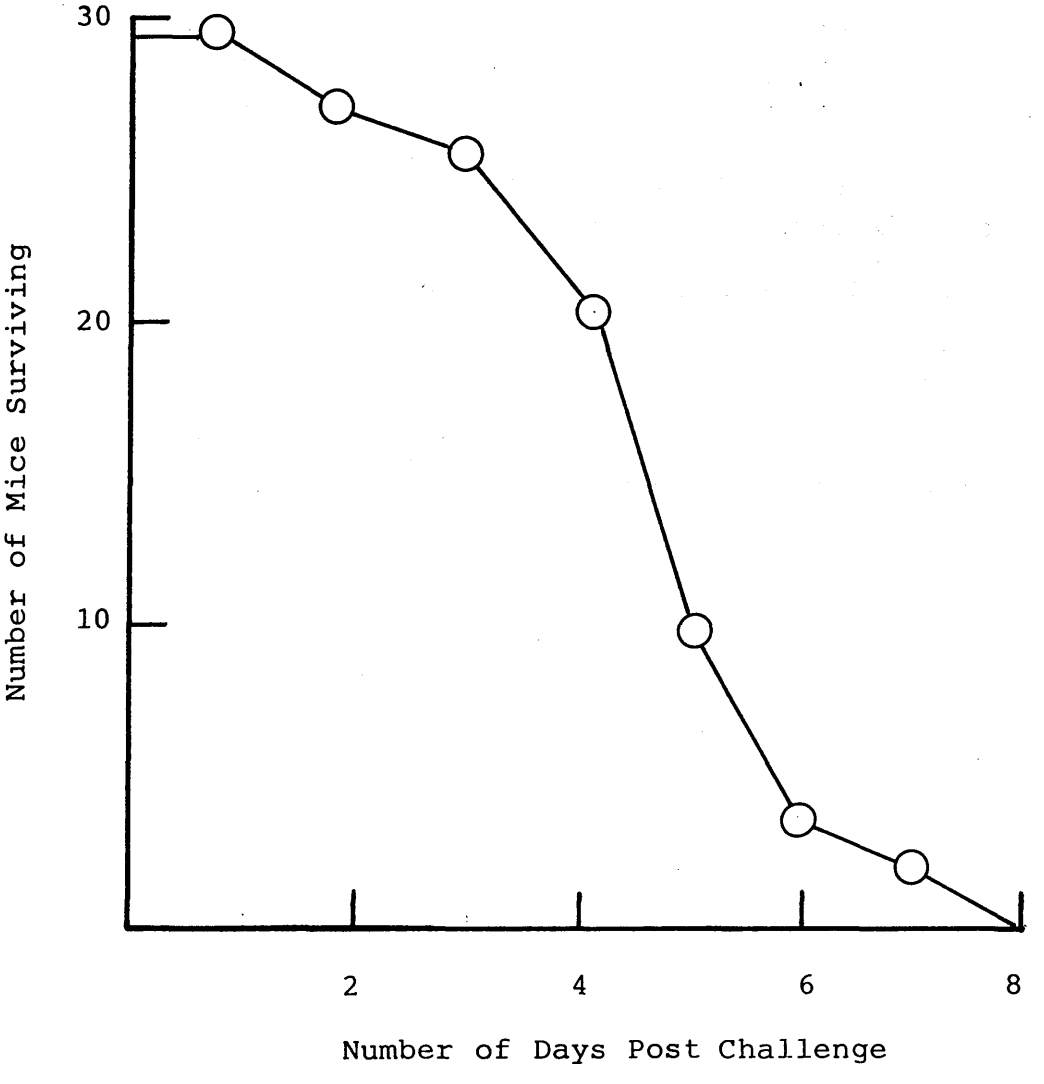


Figure 29 Cumulative mortality curve for 29 mice inoculated with 1 x 10^6 blastospores of C.albicans stain H11.

4.4.2. Fundus Examination and Photography

The mice that were examined by indirect ophthalmoscopy had a clinical record taken of the appearance of their retinae during the experimental period.

(i) 36 hours post infection

All the retinae were normal.

(ii) 3 days post infection

Mouse 1. Both eyes showed evidence of detachment of the retina. There was diffuse elevation of the retina as evidenced by an increase in parallax between the retinal and choroidal vessels and by blurring of the choroidal vessels. Segmentation (Figure 30) and tortuosity of the retinal vessels were seen. Segmentation is narrowing of the vessels, probably due to perivascular inflammatory cell infiltration, this could be an indication of a focus of infection. Tortuosity is caused by elevation and distortion of the vessels as a result of an underlying inflammation and retinal detachment.

Mouse 2. There was parallax between the retinal and choroidal vessels with some evidence of vein dilation. Dilated capillary loops were visible and there was tortuosity of some vessels (Figure 31).

Mouse 3. The right eye of this animal was normal but the left eye had a deep intra-retinal focal lesion superonasal to the disc.

Mouse 4. There was evidence of elevation of the retina in both eyes and dilated veins were visible.

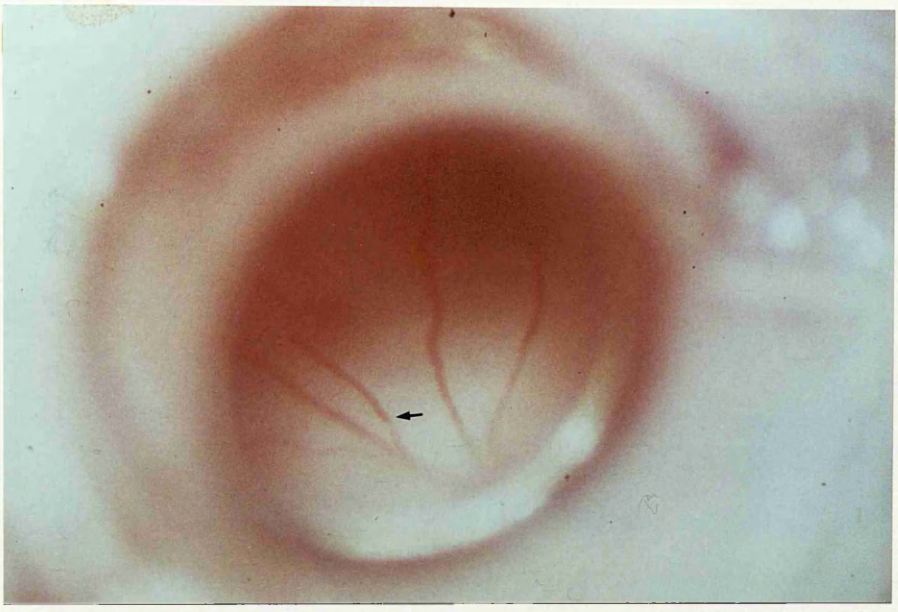


Figure 30 Fundus photograph of a mouse eye showing segmentation of a blood vessel.

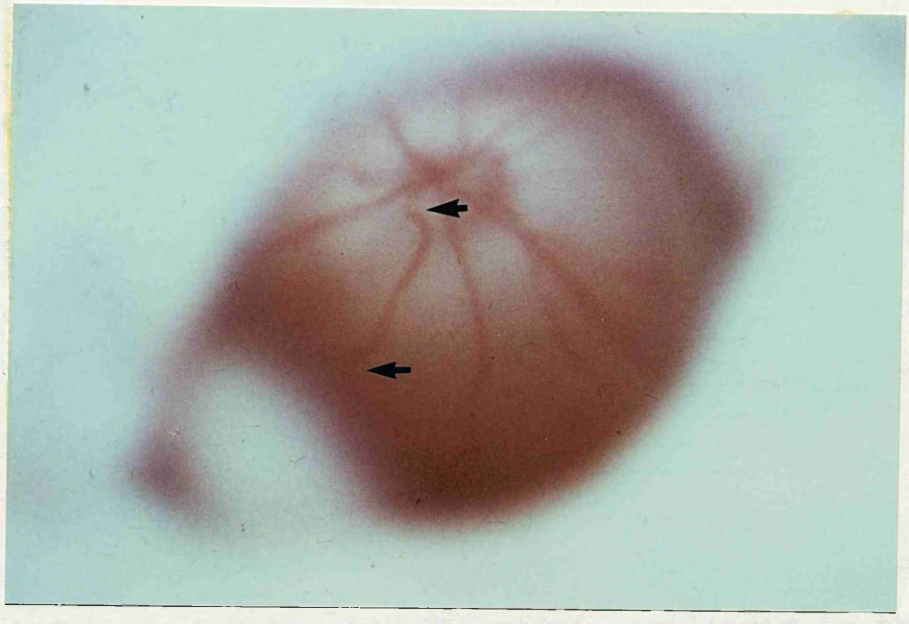


Figure 31 Fundus photograph of a mouse eye showing tortuosity of the blood vessels.

(iii) 6 days post infection

Mouse 1. Mouse died during the night and was therefore not available for examination.

Mouse 2. There was marked segmentation of the blood column within the arterioles, the retina remained elevated above the choroid as evidenced by parallax.

Mouse 3. Both eyes in this mouse showed attenuated arterioles, there was no segmentation but the retinae appeared elevated as evidenced by parallax.

Mouse 4. There was segmentation of the vessels of both eyes and the right eye showed some choroidal pallor.

(iv) 8 days post infection

Mice 1, 2 and 3 were dead.

Mouse 4. The left eye appeared normal. The right eye showed segmentation of the vessels.

4.4.3. Establishment of Mouse Model for *Candida* Endophthalmitis

(i) Histological Examination of Eye

Start of Experiment:- Two control mice were sacrificed before the mice were infected to show the appearance of the normal retina of a Balb/c mouse that has been stained by the PAS method (Figure 32).

5 minutes post challenge:- The mice that were sacrificed after 5 minutes demonstrated the presence of *C. albicans* in the choroid. The choriocapillaris is the part

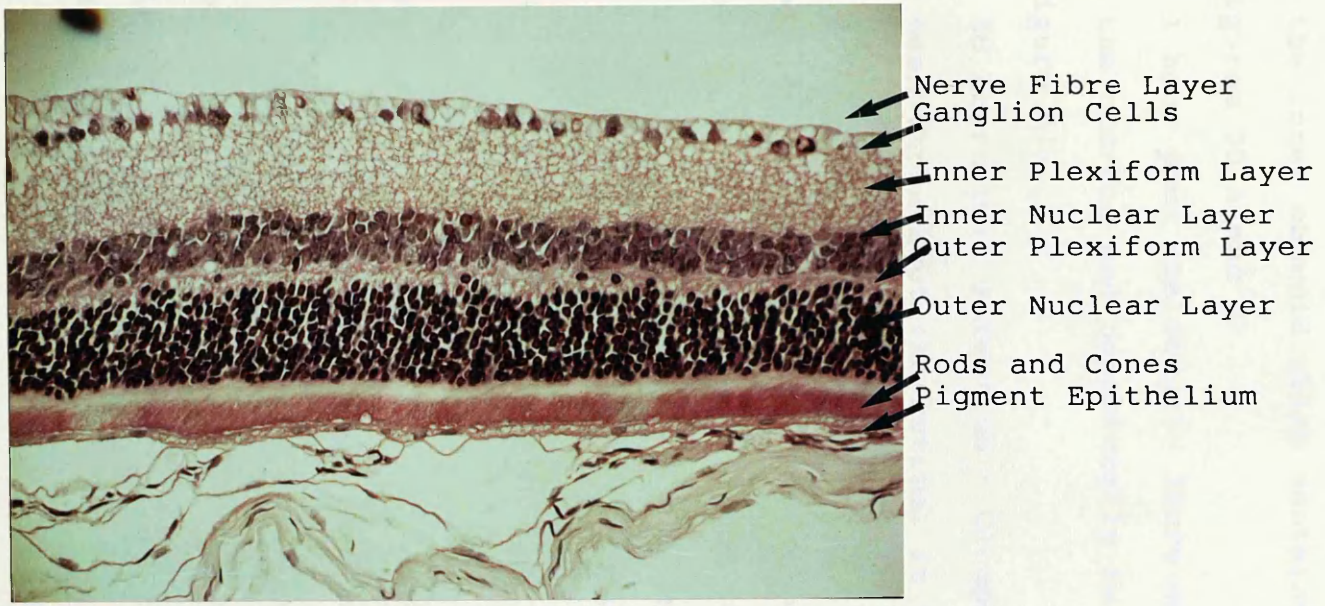


Figure 32 PAS stained section from a normal retina of a Balb C mouse.

of the inner choroid which contains the capillaries. (Figures 33 A and B).

1 hour post challenge:- There were yeast cells visible in the choroid and occasionally in the nuclear layers (Figure 34).

36 hours post infection:- Clumps of yeast cells could be seen throughout the retina, in the nuclear layers and in the ganglion cell layer. There was some evidence of pseudomycelial formation and in some cases local disturbances of the nucleated cells (Figures 35 A and B).

3-6 days post infection:- There was development of foci of *C. albicans* infection in the retinae of animals that were sacrificed during this time interval (Figure 36).

The yeast could be seen in the nerve fibre layer, ganglion cell layer and inner plexiform layer. There was protrusion of cells into the vitreous giving the retina an elevated appearance. The fungus became surrounded by inflammatory cells and resulted in the disruption of the cellular configuration of the retina behind and adjacent to the focus of infection (Figure 37).

The inflammatory nodule projected into the vitreous and the yeast cells and pseudomycelium were visible (Figure 38). The nodule appeared to be an outgrowth of a combination of yeasts and inflammatory cells extending into the vitreous cavity. The mononuclear cells were probably derived from the adjacent vasculature and were

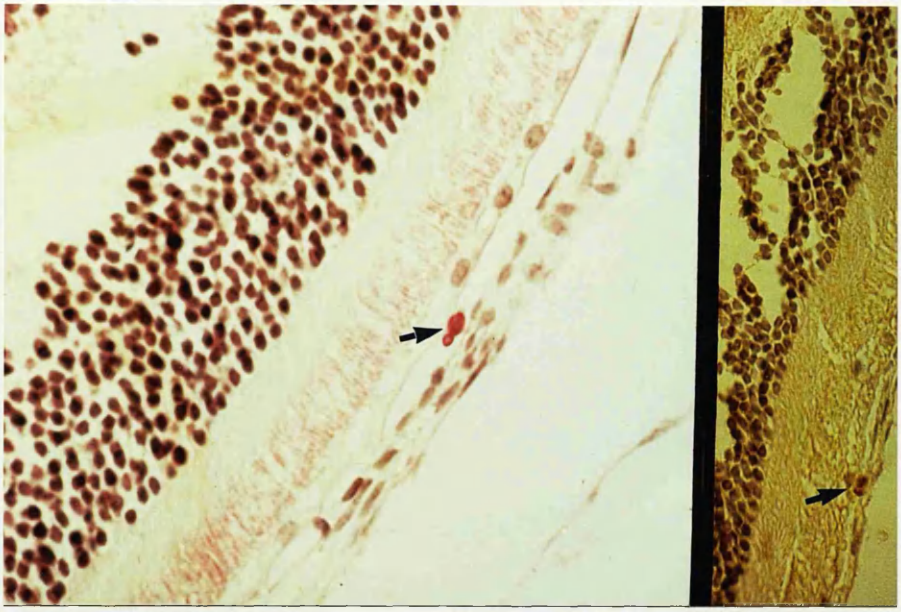


Figure 33 Mouse retina 5 minutes post challenge, stained by A) PAS and B) PAP, yeast cells are present in the choriocapillaris.

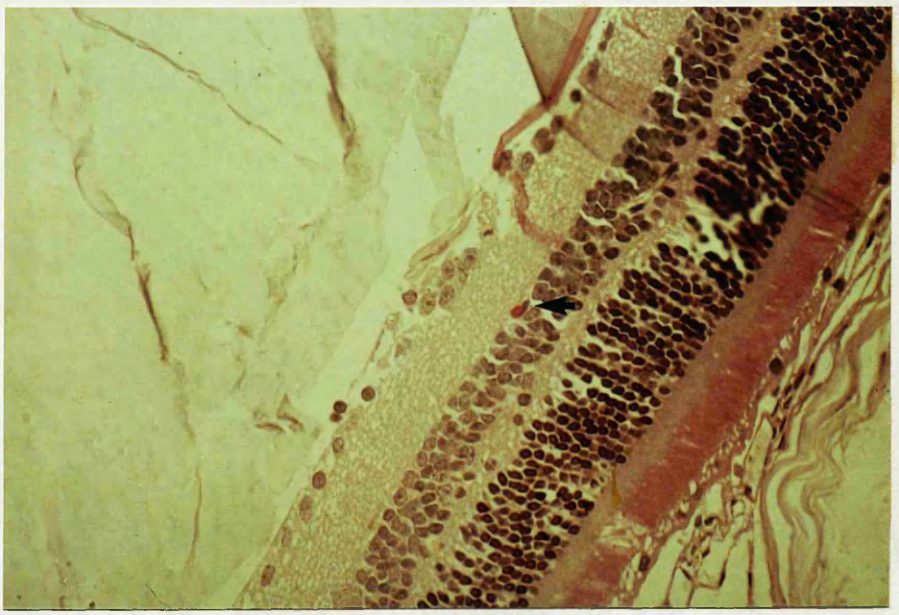


Figure 34 Mouse retina 1 hour post challenge, yeast can be seen in the inner nuclear layer.

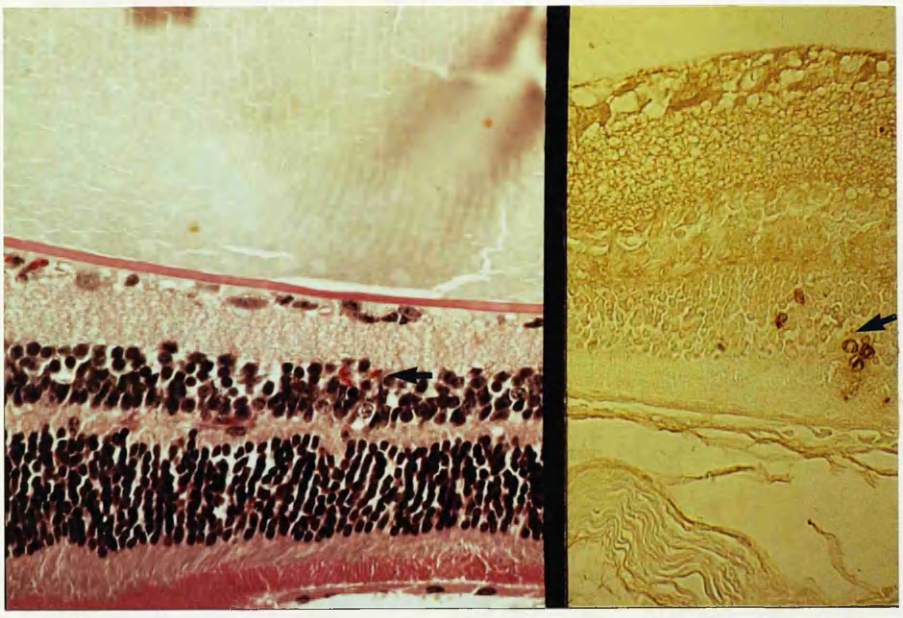


Figure 35 Mouse retina 36 hour post challenge stained by A) PAS and B) PAP. Yeast is throughout the retina in the nuclear layers and ganglion cell layer. There is evidence of pseudomycelium formation and disturbance of the nuclear cell layers.

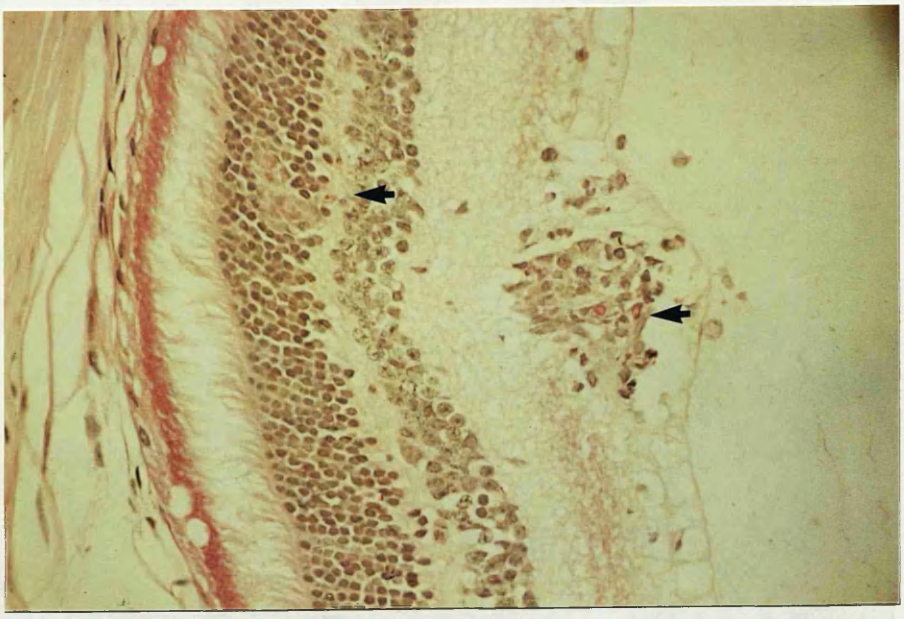


Figure 36 Mouse retina showing the development of a focus of infection.

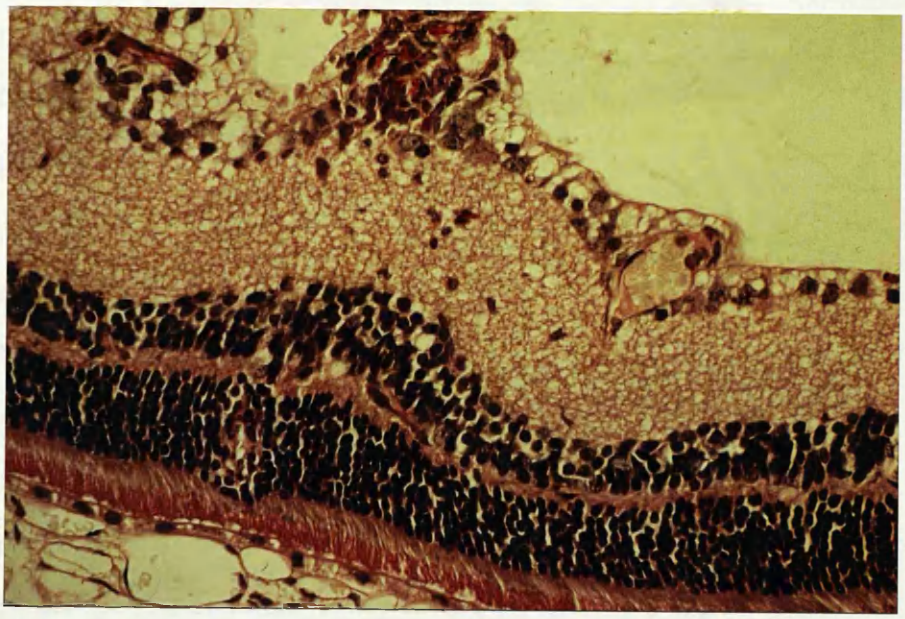


Figure 37 Mouse retina demonstrating the disruption of the retinal layers adjacent to the focus of C.albicans infection.

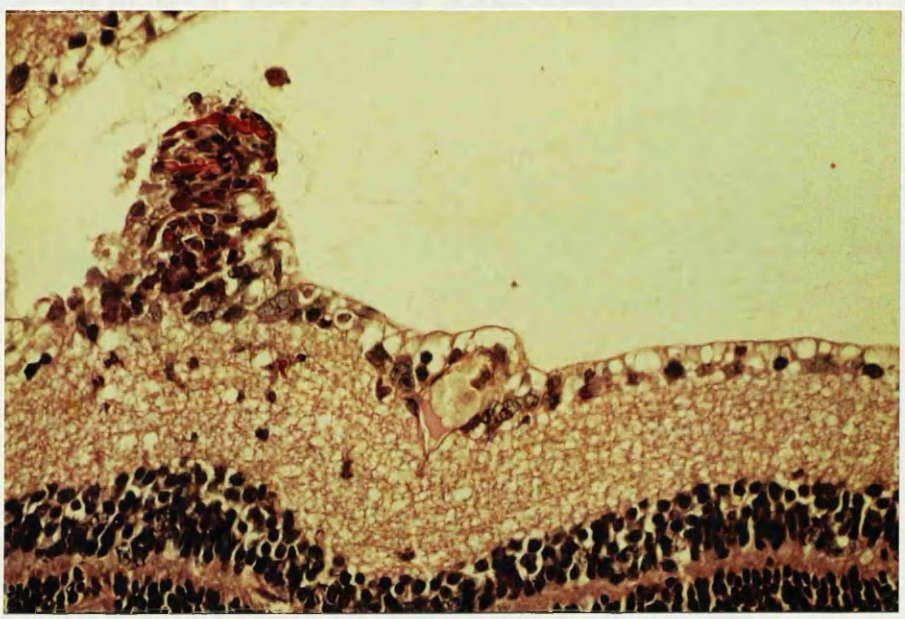


Figure 38 Mouse retina showing a focus of C.albicans infection. The outgrowth contains yeasts, pseudomycelium and inflammatory cells.

seen in close proximity to blood vessels. Inflammatory cells were also seen in the inner plexiform layer.

Overall there was localisation of the inflammatory response to the *Candida* infection. It was a feature of the disease that there were no inflammatory cells in the subretinal space or underlying choroid.

There were differences in the number of sections cut from each eye block. In an attempt to quantify the number of foci of infection in the mice at different time intervals of the experiment an index of the number of lesions per section was calculated for each time of sacrifice. The results of this quantitative procedure are given in Table 28.

Table 28: Quantitative analysis of fungal lesions in the eyes of mice inoculated with *C. albicans* and sacrificed at time intervals during the experiment.

Time	Number of mice	Foci	Foci/ mouse	Sections	Foci/ section
0	1	0	0	20	0
5 min	4	34	8.5	49	0.69
1 hour	2	8	4	24	0.33
36 hours	3	28	9.3	36	0.78
3 days	2	18	9	19	0.95
5 days	5	0	0	44	0
6 days	3	12	4	18	0.67
7 days	2	0	0	18	0
8 days	3	0	0	27	0
9 days	2	0	0	18	0

There were no yeasts present in the mouse eyes taken at the start of the experiment before the mice were infected. There was a high number of *Candida* cells visible in the retina only five minutes after infection. The number of yeasts visible decreased after one hour then increased again at 36 hours. The numbers peaked at three days, had decreased by six days and on days seven, eight and nine; no yeasts were visible on the sections.

(ii) Histological Examination of Brain, Heart, Liver and Skeletal Muscle.

The pathology of the infection in these mice is detailed in Table 29. The mice used for detailed histology showed particular involvement of the brain (Figure 39), liver, heart (Figure 40) and skeletal muscle (Figure 41) ranked in that order. Other organs were examined but all were found to be normal insofar as there was no evidence of abscess formation or of fungi. However, the lungs of many animals were haemorrhagic.

The cerebral lesions appeared to be haematogenous microabcesses and consisted of focal encephalitis; in some animals there was associated meningitis and/or ventriculitis. Initially the lesions appear as necrotic foci surrounded by polymorphs and sometimes also eosinophils. As the lesions developed monocytes/macrophages were apparent and by nine days there was early indication of granuloma formation.

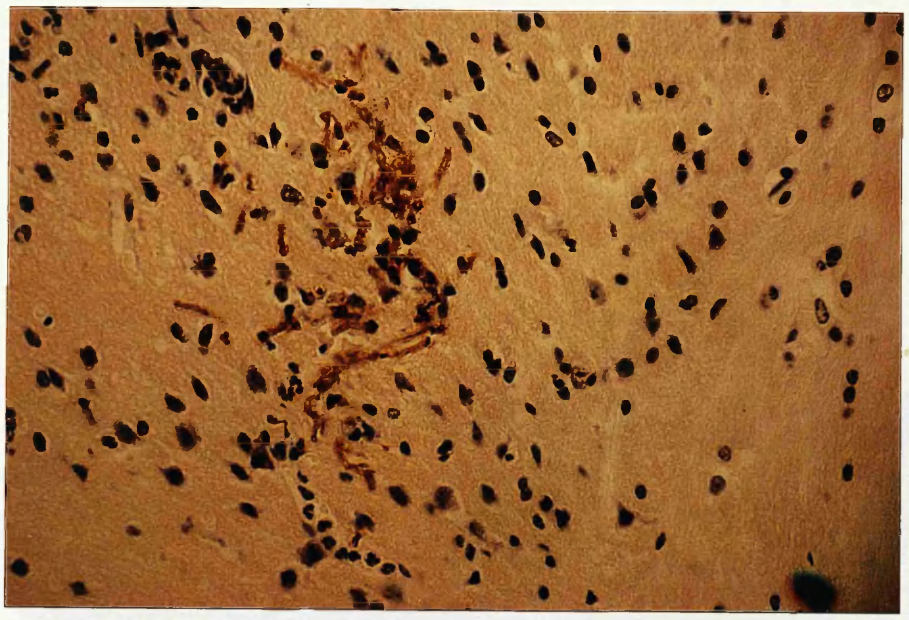


Figure 39 Section of mouse brain showing C.albicans stained by PAP. The section has been weakly counterstained with haematoxylin.

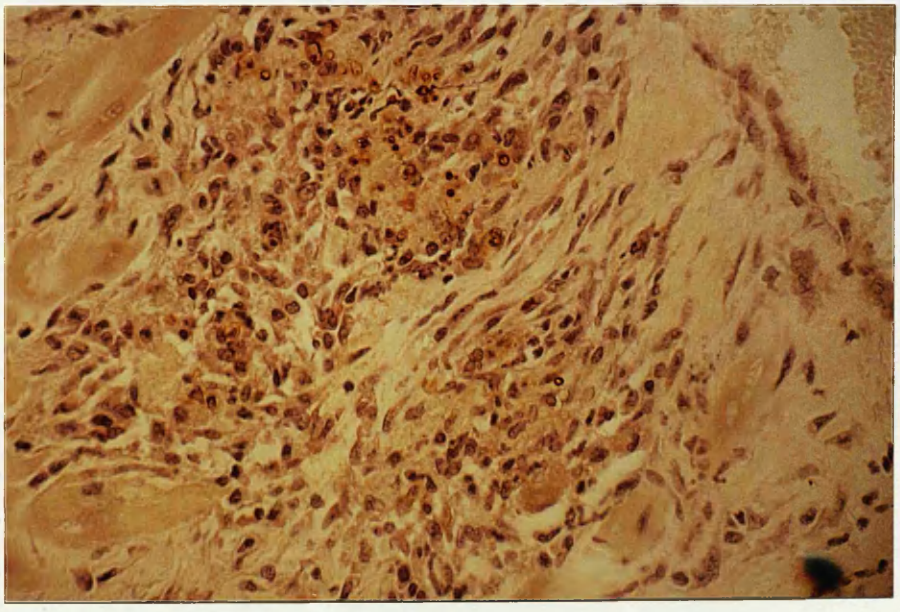


Figure 40 Section of mouse heart showing C.albicans stained by PAP. The section has been weakly counterstained with haematoxylin.

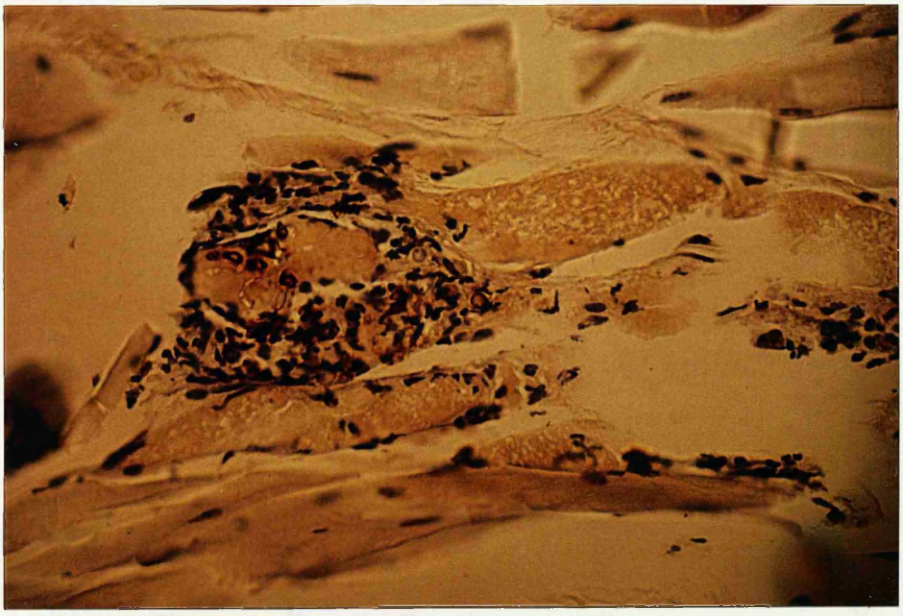


Figure 41 Section of skeletal muscle of mouse showing C.albicans stained by PAP. The section has been weakly counterstained with haematoxylin.

Mouse Number	Survival (days)	Brain	Liver	Muscle	Heart	Others
12	3	++(F)	+	+(F)	+(F)	Pancreas (F)
13	3	+(F) ventriculitis	+	+	+	-
14	3	++++(F)	+	+	+	-
15	3	++++(F) choroid plexus	-	-	-	-
18	6	++++(F) meningitis ventriculitis	-	+	-	-
21	6	+(F)	+	+	+	-
22	6	+++(F)	-	-	+	-
23	7	+(F)	-	-	-	-
24	7	++(F)	-	-	-	-
25	8	++(F)	+	-	-	-
26	8	++(F)	+	-	-	-
28	9	+	-	-	-	-
29	9	+	-	-	-	-

Table 29 : Pathology Candida albicans Infected Mice

F = fungi + - ++++ grading system of severity

There were many more lesions in the animals killed within six days than thereafter although fungi could be demonstrated in the brain sections up to eight days. There was a discrepancy between the number of lesions in the brain compared with other organs. It was usually possible to detect fungi in the brain lesions but this was not always the case in other organs.

(iii) Culture of Eye and Kidney

The eyes that were taken from the mice for culture all yielded *C. albicans* apart from the control animal sacrificed before injection and the right eye from one mouse (number 6) sacrificed one hour post challenge. These eyes were culture negative.

The fungus was present in the eyes and was demonstrated by culture within five minutes after injection and remained present and viable in the eyes of mice for the duration of the experiment.

When the kidneys of the mice were cultured a similar picture emerged. The kidneys from the control mouse and a mouse sacrificed one hour post challenge (number 6) did not grow *C. albicans* when cultured. All the other kidneys yielded *C. albicans*.

No attempt was made at this stage to quantify the amount of viable yeast present in the eyes or the kidneys.

(iv) Latex Particle Agglutination for Mannan Antigenaemia

Blood was taken from some of the mice for *Candida* serology. the results are given in Table 30.

Table 30: Results of tests for *Candida* mannan antigenaemia, using Mercia Diagnostic latex agglutination kit, on mice infected with *C. albicans* H11.

Time Post Infection	Mouse Ref Number	Result
36 hours	9	-
36 hours	10	-
36 hours	11	-
6 days	21	+
7 days	23	+
7 days	24	+
8 days	25	+
9 days	28	+
9 days	29	-

The mice showed no evidence of having *Candida* mannan in their blood at 36 hours post infection but at 6 days all the mice tested were positive. The mice remained positive until nine days post challenge when one of the mice gave a negative result.

4.4.4. Differential Localisation of Yeast Between Mouse Eyes as Determined by Culture.

To determine if the *Candida* endophthalmitis was bilateral in the animal model, quantitative counts were made of the viable yeasts present in the eyes. The results were analysed to determine if there was an asymmetric

distribution of the organism between the eyes. The results are given in Tables 31, 32, and 33.

Table 31: Differential localisation of viable *C. albicans* between the eyes of mice infected with *C. albicans*. Group 1.

Time Interval	Mouse Number	L eye	R eye	Difference
6 days	6	1000	1400	400
6 days	19	350	350	0
7 days	7	562	137	425
7 days	9	350	140	210
7 days	10	-	110	110
15 days	5	50	13	37
22 days	3	-	-	0
25 days	11	-	110	110

The values for the overall experiment were analysed as there were insufficient numbers of mice to test the significance of the difference in culture numbers at each time interval.

t = 2.69, there was a significant difference between the distribution of the yeast between the two eyes of each mouse at a 5 % level.

Table 32: Differential localisation of viable *C. albicans* between the eyes of mice infected with *C. albicans* Group 2.

Time Interval	Mouse Number	L eye	R eye	Difference
2 days	23	2358	11034	8676
3 days	21	8100	14175	6075
3 days	22	7791	12150	4359
3 days	25	1788	1880	92
5 days	6	1404	1125	279
5 days	8	1062	1188	126
5 days	12	8280	180	8280
5 days	17	2106	2907	801
5 days	19	19350	6100	13250
6 days	14	882	1062	240

Overall t = 2.84 with 9 degrees of freedom, therefore there was a significant difference between the eyes at 5% significance level.

Day three t = 1.97 with 2 degrees of freedom and there was no significance between the two eyes of the mice sacrificed on this day.

Day five t = 1.7 with 4 degrees of freedom therefore there was no significant difference between the distribution between the eyes.

Table 33: The differential location of viable *C. albicans* between the eyes of mice in group 3 at different time intervals after inoculation with *C. albicans*.

Time Interval	Mouse Number	L eye	R eye	Difference
5 min	26	1062	900	162
5 min	27	918	504	414
5 min	28	756	954	198
5 min	29	918	504	414
5 min	30	756	954	198
1 hour	1	684	932	248
1 hour	2	1170	206	964
1 hour	3	782	504	278
1 hour	4	612	234	378
1 hour	5	1080	792	288
6 hours	16	1062	1360	298
6 hours	17	1484	990	494
6 hours	18	1050	486	564
6 hours	19	288	756	468
6 hours	20	2016	1350	666
24 hours	6	0	0	0
24 hours	7	90	82	8
24 hours	8	218	414	196
24 hours	9	108	162	54
24 hours	10	144	198	54
48 hours	11	1360	750	610
48 hours	12	1160	970	190
48 hours	13	0	0	0
48 hours	14	380	380	0
48 hours	15	1140	40	1100
72 hours	21	1570	460	1110
72 hours	22	630	650	20
72 hours	23	140	940	800
72 hours	24	100	200	100
72 hours	25	570	720	150

Overall t=6.18 with 27 degrees of freedom, therefore there is a significant difference in the numbers of yeasts between the two eyes at a 5% significance level.

At 5 minutes post infection t=4.9 with 4 degrees of freedom which is significant at the 5% level.

One hour post infection t=3.19 for 4 degrees of freedom, therefore there was a significant difference between the eyes at a 5% level.
Six hours post infection t=8.2 with four degrees of freedom which is significant at the 5% level.
At 24 hours t=1.77 with 5 degrees of freedom and shows no significant difference.
At 48 hours t=1.79 with 4 degrees of freedom and shows no significant difference at the 5% level.
72 hours post infection t=2.23 with 4 degrees of freedom which is not significant at the 5 % level.

The results from the three groups of mice were examined collectively. The results are given in Tables 34 and 35.

Table 34: The significance of the difference between the localisation of yeast in mice eyes at different time intervals after challenge with *C. albicans*.

Time	Group	t value	Significance at 5%
5 min.	3	4.9	Yes
1 hour	3	3.19	Yes
6 hours	3	8.2	Yes
24 hours	3	1.77	No
48 hours	3	1.79	No
3 days	3	2.23	No
3 days	2	1.97	No
5 days	2	1.7	No

Table 35: Means of quantitative counts of *C. albicans* cultured from both eyes of mice sacrificed at different times post challenge.

Time post Challenge	Mean Number of Colonies per Eye
5 minutes	1645
1 hour	1399
6 hours	3050
24 hours	345
48 hours	3754
3 days	6483
5 days	8740
6 days	1681
7 days	433
15 days	63
22 days	0
25 days	0

4.4.5. Determination of *Candida* Antigenaemia in the Mouse Model

Blood was taken from 29 mice sacrificed at different times after inoculation with *C. albicans* H 11. The sera from the mice were tested for *Candida* antigenaemia using two latex agglutination kits. The results obtained using the two kits were compared and the results are given in Table 36.

Table 36: Results of latex tests for *Candida* antigenaemia for the sera from mice in a model for *C. albicans* endophthalmitis. ND denotes not done.

Time Post Challenge	Number of Mice Tested	Mercia +	Mercia −	Immy +	Immy −	
5 min	5	0	5	2	3	
1 hour	5	0	5	4	1	
6 hours	5	0	5	5	0	
24 hours	5	1	4	5	0	
48 hours	4	3	1	3	0	(1 ND)
72 hours	5	4	1	5	0	

The Mercia kit did not indicate the presence of mannan in the mouse sera until 24 hours post challenge nor did it show all the mice to be positive at any time. The Immy test gave a positive result for 2 out of 5 mice after only five minutes post inoculation and showed all mice to be positive after six hours.

In tests for false positive results with latex agglutination kits.

The reagents were tested against sera from uninoculated control mice to discover if any false positive results occurred. The results are detailed in Table 37.

Table 37: Results of test for false positive latex agglutination.

Mouse Number	Weight (g)	Mercia Latex	Immy Latex
1	17.9	-	+
2	22.0	-	+
3	17.1	-	+
4	20.4	-	-
5	18.4	-	-
6	19.9	-	+
7	21.2	-	+
8	18.6	-	-

With the control animals the Mercia kit gave 100 % negative results. However, the Immy kit gave 62.5% false positive results.

4.5. Epidemiological Study of Presumed *Candida* Endophthalmitis In Heroin Abusers

Various aspects of the aetiology of an outbreak of *Candida* endophthalmitis in Glasgow heroin addicts were examined. The most likely sources of the yeast appeared to be; the addicts' commensal flora, the injection paraphernalia, the street drug or the diluent employed in prepar ing the drug for injection.

4.5.1. Culture of Specimens and Paraphernalia from Glaswegian Heroin Abusers.

Several pieces of injection paraphernalia and clinical specimens from Glaswegian heroin addicts with presumed *Candida* endophthalmitis were cultured in an attempt to discover the possible source of infection. The details of the items and samples cultured are given in Table 12.

There were 33 samples cultured from 17 patients. Five were vitrectomy specimens from five different patients, one was a scalp biopsy, there were nine throat swabs and mouth washes from six addicts and two faecal samples from different patients. Four of the addicts donated their syringes, plungers and needles for culture. Five plastic lemons, the juice of which was used as a diluent, were provided by different addicts; one spoon and one sample of heroin were also obtained.

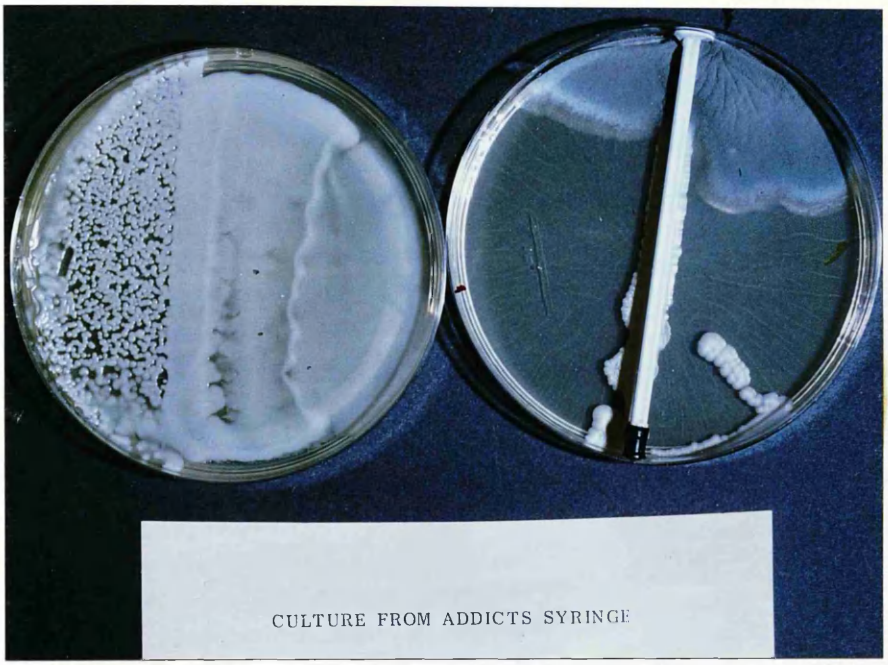


Figure 42 C.parapsilosis cultured from addict's syringe.

Figure 43 Culture of addict's syringe which yielded C.albicans.

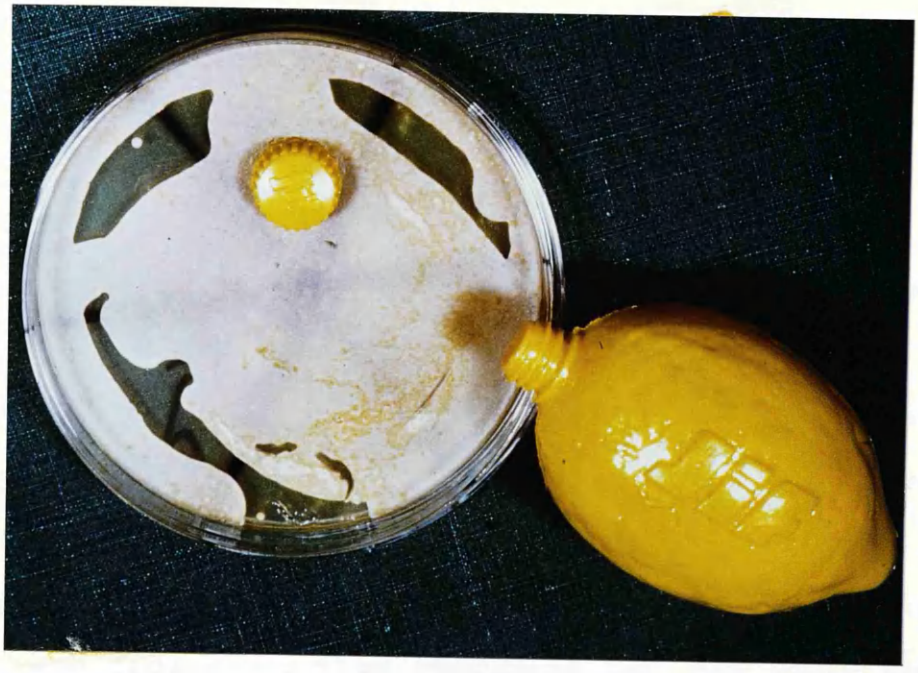


Figure 44 C.albicans culture from a "Jif" lemon provided by an addict.

Figure 45 Culture of C.albicans from an addict's "Jif" lemon.

Thirteen of the samples did not grow yeasts or filamentous fungi when cultured. A *Penicillium sp.* was isolated from one syringe. Two sets of injection paraphernalia grew *A. fumigatus*. A *Trichosporon sp.* and a *Rhodotorula rubra* were isolated from two different syringes. *C. parapsilosis* was isolated twice, once from a syringe (Figure 42) of one addict and once from a faecal sample of another.

Thirteen of the samples yielded *C. albicans* on culture. These were from vitrectomy specimens (2), the scalp biopsy (1), the throat swabs and mouth washes (6), and the faecal samples (1), a syringe (1) (Figure 43) and plastic "Jif" lemons (2) (Figures 44 and 45).

4.5.2. Biotyping of *C. albicans* Isolates From Heroin Addicts.

The isolates of *C. albicans* cultured from clinical specimens and injection paraphernalia were assigned a number and preserved in silica gel and sterile distilled water. The *C. albicans* isolates were fingerprinted by the Leicester biotyping method. The results of growth on the first five agars confirmed the indentity of the yeasts while the growth patterns on the other nine agars gave the biotype number as explained in Table 14. Table 38 gives the results of growth on the plates which were inoculated in duplicate with three colonies of each isolate. Table 39

	Isolate number:	pH1.5 A	pH1.5 B	tetra A	tetra B	MacCo A	MacCo B	NaIO, A	NaIO, B	cetri A	cetri B	pH1.4 A	pH1.4 B	prot. A	prot. B	5FC A	5FC B	urea A	urea B	sorb. A	sorb. B	salt A	salt B	citr. A	citr. B	bora. A	bora. B	safr. A	safr. B	code
1	73/005*	+	+	-	-	-	-	(+)	-	+	+	-	-	+	+	-	-	+	(+)	-	-	+	(+)	+	(+)	-	-	+	-	
2	73/026*	+	+	-	-	-	-	+	+	+	+	(+)	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	(+)	(+)	
3	73/071*	-	-	-	-	-	-	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	
4	74/074*	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	+	+	-	-	+	+	+	+	-	-	-	-	
5	75/043*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	
6	H2 A	+	+	-	-	-	-	+	+	-	(+)	+	+	-	-	-	-	+	+	-	-	+	+	+	+	+	+	-	-	
7	H2 B	+	+	-	-	-	-	+	+	(+)	-	+	(+)	-	-	-	-	+	+	-	-	+	+	+	+	+	+	-	-	153
8	H2 C	+	+	-	-	-	-	+	+	-	-	+	+	-	-	-	-	+	+	-	-	+	+	+	+	+	+	-	-	153
9	H4 A	+	+	-	-	-	-	+	+	-	-	-	-	(+)	(+)	-	-	+	+	-	-	+	+	+	+	+	+	-	-	153
10	H4 B	+	+	-	-	-	-	+	+	-	-	-	-	(+)	(+)	-	-	+	+	-	-	+	+	+	+	+	+	-	-	253
11	H4 C	+	+	-	-	-	-	+	+	-	(+)	-	-	(+)	(+)	-	-	+	+	-	-	+	(+)	+	+	+	+	-	-	21/53
12	H5 A	+	+	-	-	-	-	(+)	-	(+)	+	-	-	+	+	-	-	+	+	-	-	-	(+)	-	+	+	+	-	-	21/53
13	H5 B	+	+	-	-	-	-	-	-	+	+	+	+	-	-	-	-	+	+	-	-	+	(+)	+	+	+	+	-	-	253
14	H5 C	+	+	-	-	-	-	(+)	-	+	+	+	+	-	-	-	-	+	+	-	-	+	+	+	+	+	+	-	-	153
15	H6 A	+	+	-	-	-	-	+	+	(+)	(+)	+	+	-	-	-	-	+	+	-	-	+	(+)	+	+	+	+	-	-	153
16	H6 B	+	+	-	-	-	-	+	+	+	+	+	+	-	-	-	-	+	+	-	-	+	+	+	+	(+)	+	-	-	153
17	H6 C	+	(+)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	(+)	-	-	-	153
18	H6 C	+	+	-	-	-	-	+	+	(+)	(+)	+	+	-	-	-	-	+	+	-	-	(+)	(+)	+	+	+	+	-	-	153
19	H7 A	+	+	-	-	-	-	+	(+)	+	+	+	+	+	+	-	-	+	+	-	-	+	+	+	+	+	+	-	-	153
20	H7 B	+	+	-	-	-	-	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	+	+	+	(+)	-	363
21	H7 C	+	(+)	-	-	-	-	+	(+)	+	+	+	+	-	-	-	-	-	+	+	-	+	+	+	+	+	+	-	-	153
22	H8 A	+	+	-	-	-	-	+	-	+	+	+	(+)	-	-	-	-	+	+	-	-	+	+	+	-	-	(+)	-	-	153
23	H8 B	+	+	-	-	-	-	+	(+)	+	+	+	+	-	-	-	-	+	+	-	-	+	+	+	+	+	+	(+)	(+)	157
24	H8 C	+	+	-	-	-	-	+	+	(+)	+	+	+	-	-	-	-	+	+	-	-	+	+	+	+	+	+	-	(+)	153
25	H9 A	+	+	-	-	-	-	(+)	-	+	+	+	+	-	-	-	-	+	+	-	-	+	+	+	+	+	+	(+)	-	153
26	H9 B	+	+	-	-	-	-	-	-	+	+	+	+	-	-	-	-	+	+	-	-	+	+	+	+	+	+	(+)	(+)	153/7
27	H9 C	+	+	-	-	-	-	(+)	-	+	+	+	+	-	(+)	-	-	+	+	-	-	+	+	+	+	+	+	-	(+)	153
28	H10 A	+	+	-	-	-	-	(+)	+	+	+	-	-	+	+	-	-	+	+	-	-	-	-	-	-	-	-	+	+	214
29	H10 B	+	+	-	-	-	-	+	+	+	+	+	+	-	-	-	-	+	+	-	-	+	+	+	+	(+)	(+)	+	+	157
30	H10 C	+	+	-	-	-	-	+	+	+	+	-	-	+	+	-	-	+	+	-	-	-	-	(+)	-	-	-	+	+	214
31	H11 A	+	+	-	-	-	-	+	+	+	+	+	(+)	-	-	-	-	+	+	-	-	(+)	-	+	+	-	-	+	+	155
32	H11 B	+	+	-	-	-	-	+	+	+	+	+	+	-	-	-	-	+	+	(+)	-	+	+	+	+	-	-	+	+	155
33	H11 C	+	+	-	-	-	-	+	+	+	+	+	(+)	+	(+)	-	-	+	+	-	(+)	(+)	-	+	+	-	-	+	+	1/355
34	H12 A	+	+	-	-	-	-	+	+	+	+	+	+	-	-	-	-	+	+	-	-	-	(+)	+	+	-	-	(+)	+	115
35	H12 B	+	+	-	-	-	-	+	+	+	+	(+)	(+)	+	+	-	-	+	+	-	-	+	-	+	+	-	-	+	+	11/55
36	H12 C	+	+	-	-	-	-	+	+	+	+	+	+	-	-	-	-	+	+	-	-	+	-	+	+	-	-	(+)	+	11/55
37	H13 A	+	+	-	-	-	-	(+)	(+)	+	+	(+)	(+)	-	-	-	+	+	+	-	-	+	+	+	+	-	-	+	+	4/555
38	H13 B	+	+	-	-	-	-	-	-	+	+	-	-	-	-	+	+	+	+	-	-	(+)	(+)	+	-	-	-	(+)	(+)	455
39	H13 C	+	+	-	-	-	-	-	-	+	+	-	-	-	-	+	+	+	+	-	-	(+)	(+)	+	+	-	-	(+)	(+)	455
40	H14 A	+	+	-	-	-	-	-	-	+	+	-	-	-	-	+	+	+	+	-	-	+	+	+	+	-	(+)	+	+	455
41	H14 B	+	+	-	-	-	-	-	-	+	+	(+)	-	-	-	+	+	+	+	-	-	+	+	+	+	-	(+)	+	+	455
42	H14 C	+	+	-	-	-	-	-	-	+	+	-	(+)	-	-	+	+	+	+	-	-	+	+	+	+	-	-	+	+	455
43	H15 A	+	+	-	-	-	-	-	-	+	+	-	+	-	-	+	+	+	+	-	-	+	+	+	+	(+)	-	+	+	455
44	H15 B	+	+	-	-	-	-	-	-	+	+	(+)	-	-	-	+	+	+	+	-	-	+	+	+	+	-	-	(+)	+	455
45	H15 C	+	+	-	-	-	-	-	-	+	+	-	-	-	-	+	+	+	+	-	-	+	+	+	+	-	-	(+)	(+)	451/5
46																														

Table 38: Results of growth of *C. albicans* isolates on biotyping media.

* Denotes indicator strains ( ) Denotes variable results

details a summary of the results and the biotype number for the isolates tested by this method.

Table 39: Biotypes of *C. albicans* isolated from heroin addicts and their injection paraphernalia.

Isolate Number	Addict Number	Source	Biotype Number
2	7	Lemon	1 5 3
4	9	Scalp	2 1/5 3
5	15	Throat Swab	1 5 3
6	18	Mouthwash	1 5 3
7	18	Mouth Swab	1 1/5 3
8	19	Mouth Swab	1 5 3/7
10	22	Vitreous	2 1 4
11	22	Vitreous (pellet)	1 5 5
12	22	Vitreous (pellet)	1 5 5
13	23	Mouthwash	4 5 5
14	23	Faeces	4 5 5
15	23	Mouthwash	4 5 5

The biotype results are internally consistent, apart from addict 22 who yielded isolates H10, H11 and H12. Biotype 153 was found to be the most common as it accounted for five of the isolates from five different addicts.

4.5.3. Inhibition of Yeast by Diamorphine Hydrochloride

The role of street heroin in the outbreak of presumed *Candida* endophthalmitis was investigated. Samples of dealers and pushers heroin were cultured, and the growth of yeast in the presence of diamorphine hydrochloride was examined.

(i) Culture of Street Drug

Six samples of pushers drug and a sample of dealers drug, supplied by the Glasgow Drug Squad, were cultured on glucose peptone agar. The results are given in Table 40.

Table 40: Results of culture of samples of heroin supplied by the Glasgow Drug Squad.

Sample	Organisms Recovered-Drug	Organisms Recovered-Bag
Dealer	-	-
Pusher 1	-	*A. fumigatus*
Pusher 2	-	-
Pusher 3	*A. fumigatus*	-
Pusher 4	-	Bacteria
Pusher 5	-	*A. fumigatus*
Pusher 6	-	-

C. albicans was not isolated from any of the samples or their containers. *A. fumigatus* was isolated from the drug on one occasion and was grown from the bags twice. Bacteria were grown from one of the packets.

(ii) Antifungal Activity of Diamorphine Hydrochloride Sensitivity Plates - Powdered Drug.

The results of powdered diamorphine hydrochloride on the growth of yeast are given in Table 41. The results were consistent regardless of which agar media was used.

Table 41: Results of sensitivity tests of yeast isolates to powdered diamorphine hydrochloride, including tests for fungicidal activity by incubation of core of agar from within the inhibition zone. "+" indicates the presence of a zone or growth of yeast from the zone.

Yeast	Drug Weight	Incubation Temperature	Zone	Growth of Zone
SAC 1	1 mg	28°C	+	+
SAC 1	1 mg	37°C	+	+
SAC 1	5 mg	28°C	+	-
SAC 1	5 mg	37°C	+	-
SAC 1	10 mg	28°C	+	-
SAC 1	10 mg	37°C	+	-
SAC 2	1 mg	28°C	+	+
SAC 2	1 mg	37°C	+	+
SAC 2	5 mg	28°C	+	-
SAC 2	5 mg	37°C	+	-
SAC 2	10 mg	28°C	+	-
SAC 2	10 mg	37°C	+	-
CA 6	1 mg	28°C	+	+
CA 6	1 mg	37°C	+	+
CA 6	5 mg	28°C	+	-
CA 6	5 mg	37°C	+	+
CA 6	10 mg	28°C	+	-
CA 6	10 mg	37°C	+	-
CA 9	1 mg	28°C	+	+
CA 9	1 mg	37°C	+	+
CA 9	5 mg	28°C	+	+
CA 9	5 mg	37°C	+	-
CA 9	10 mg	28°C	+	-
CA 9	10 mg	37°C	+	-
B Y	1 mg	28°C	+	+
B Y	1 mg	37°C	+	+
B Y	5 mg	28°C	+	-

Table 41 continued

Yeast	Drug Weight	Incubation Temperature	Zone	Growth of Zone
B Y	5 mg	37°C	+	-
B Y	10 mg	28°C	+	-
B Y	10 mg	37°C	+	-
H 2	5 mg	37°C	+	-
H 2	10 mg	37°C	+	-
H 3	5 mg	37°C	+	-
H 3	10 mg	37°C	+	-
H 4	5 mg	37°C	+	+
H 4	10 mg	37°C	+	-
H 5	5 mg	37°C	+	-
H 5	10 mg	37°C	+	-
H 6	5 mg	37°C	+	-
H 6	10 mg	37°C	+	-
H 7	5 mg	37°C	+	-
H 7	10 mg	37°C	+	-
H 8	5 mg	37°C	+	-
H 8	10 mg	37°C	+	-
H 9	5 mg	37°C	+	-
H 9	10 mg	37°C	+	-
H 10	5 mg	37°C	+	-
H 10	10 mg	37°C	+	-
H 11	5 mg	37°C	+	-
H 11	10 mg	37°C	+	-
H 12	5 mg	37°C	+	-
H 13	10 mg	37°C	+	-
H 14	5 mg	37°C	+	-
H 14	10 mg	37°C	+	-
H 15	5 mg	37°C	+	+
H 15	10 mg	37°C	+	-

All the yeast isolates tested were sensitive to diamorphine hydrochloride regardless of the temperature of incubation or the media used (Figures 46 and 47). The results were the same if actively growing or stationary phase yeasts were tested. There was some evidence of fungicidal activity. On the occasions when the excised zones showed evidence of yeast growth, the inhibition zone was smaller than the cork borer and carry over of viable yeast probably occurred.

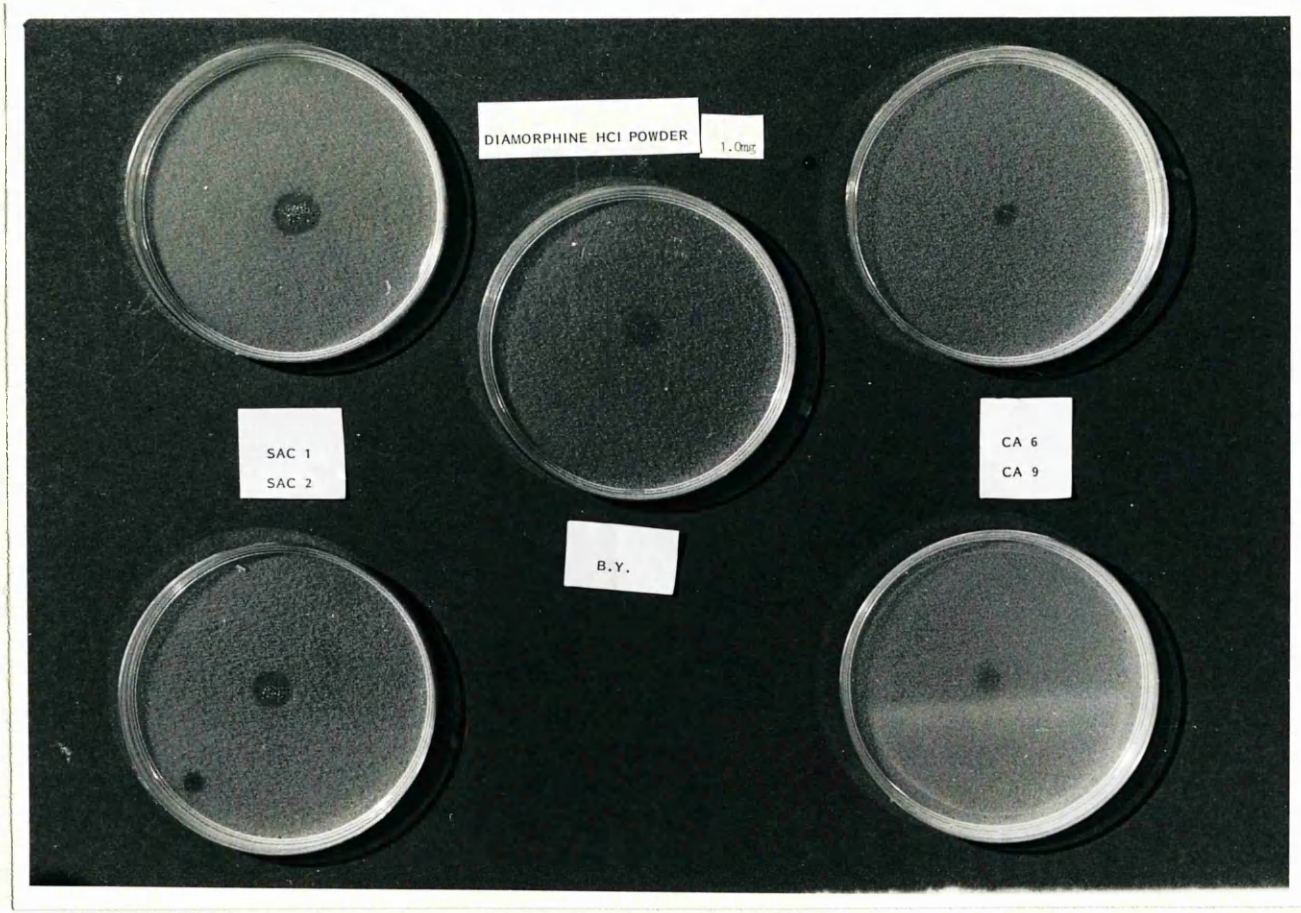


Figure 46 Inhibition zones formed by the application of 1.0mg diamorphine hydrochloride to agar seeded with different yeasts.

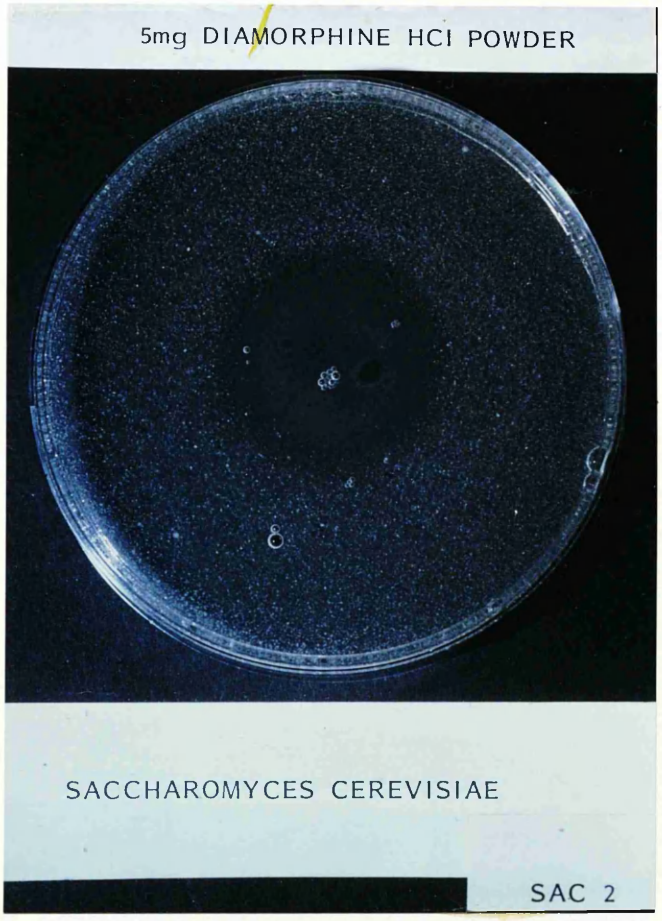


Figure 47 Inhibition zone formed by the application of 5 mg diamorphine hydrochloride to agar seeded with S.cerevisiae.

Sensitivity Plates - Liquid Drug

The results of plates seeded with yeast containing wells filled with various concentrations of diamorphine hydrochloride are given in Table 42.

Table 42: Growth of yeast around wells containing diamorphine hydrochloride.

Yeast	Drug Concentration (µg/ml)	Growth
SAC 1	10	+
SAC 1	100	+
SAC 1	1000	+
SAC 1	10000	+
SAC 2	10	+
SAC 2	100	+
SAC 2	1000	+
SAC 2	10000	+
CA 6	10	+
CA 6	100	+
CA 6	1000	+
CA 6	10000	+
CA 9	10	+
CA 9	100	+
CA 9	1000	+
CA 9	10000	+

None of the isolates tested, even the highest concentrations, showed sensitivity to the drug. There were no zones of inhibition surrounding the wells.

Incorporation Plates

Agar plates incorporating diamorphine hydrochloride were spot inoculated with yeasts. The results of growth of the yeasts on these plates are given in Table 43.

Table 43: Growth of yeasts on agar incorporating different concentrations of diamorphine hydrochloride.

Yeast	Drug Concentration (mg/ml)	Growth
CA 6	5	+
CA 6	10	+
CA 6	100	-
CA 9	5	+
CA 9	10	+
CA 9	100	-
SAC 1	5	+
SAC 1	10	+
SAC 1	100	-
SAC 2	5	+
SAC 2	10	+
SAC 2	100	-
B Y	5	+
B Y	10	+
B Y	100	-

All the isolates grew on agar containing 5 mg/ml and 10 mg/ml of diamorphine hydrochloride. However, none of the yeasts grew on the agar containing 100 mg/ml.

Broth Dilution Experiments - Minimum Inhibitory Concentrations (MIC).

The contact time leading to the death of yeasts was determined for different concentrations of diamorphine hydrochloride. The results are detailed in Table 44. The killing times for the isolates when tested by this method were for both the *C. albicans* strains; more than 24 hours for up to 10 mg/ml, 3 - 4 hours up to 250 mg/ml decreasing to 2 - 3 hours for CA9 at 500 mg/ml. The killing times for the *S. cerevisiae* strains varied with the concentration of

Table 44 Presence of growth (+) of yeast isolates after various contact times with diamorphine hydrochloride.

Yeast Isolate	Drug Conc. mg/ml.	Time (hours)					
		½	1	2	3	4	24
CA6	0	+	+	+	+	+	+
	2.5	+	+	+	+	+	+
	5	+	+	+	+	+	+
	10	+	+	+	+	+	+
	100	+	+	-	+	-	-
	250	+	+	+	+	-	-
	500	+	+	+	+	-	-
CA9	0	+	+	+	+	+	+
	2.5	+	+	+	+	+	+
	5	+	+	+	+	+	+
	10	+	+	+	+	+	+
	100	+	+	+	+	-	-
	250	+	+	+	+	-	-
	500	+	+	+	-	-	-
SAC1	0	+	+	+	+	+	+
	2.5	+	+	+	+	+	+
	5	+	+	+	+	+	+
	10	+	+	+	+	+	+
	100	+	+	+	-	-	-
	250	+	+	+	-	-	-
	500	-	-	-	-	-	-
SAC2	0	+	+	+	+	+	+
	2.5	+	+	+	+	+	+
	5	+	+	+	+	+	+
	10	+	+	+	+	-	-
	100	+	-	-	-	-	-
	250	+	-	-	-	-	-
	500	-	-	-	-	-	-
BY	0	+	+	+	+	+	+
	2.5	+	+	+	+	+	+
	10	+	+	+	+	+	+
	100	+	+	+	+	-	-
	250	+	+	+	-	-	-
	500	+	+	-	-	-	-

the diamorphine hydrochloride. SAC 1 was the least sensitive, with a killing time of more than 24 hours for 10 mg/ml and less, for 100 and 250 mg/ml the killing time was 2 - 3 hours but 500 mg/ml killed the yeast within 30 minutes. SAC 2 was killed after 1 hour in contact with diamorphine hydrochloride at 100 and 250 mg/ml, 500 mg/ml killed the yeast within 30 minutes. The shop bought bakers yeast was the most robust, it was killed only after 4 hours exposure to the drug at 100 mg/ml, 250 mg/ml killed the strain after 3 hours and 500 mg/ml was fungicidal after 2 hours.

The MICS for the yeasts used in this study were determined by a broth dilution method. Table 45 lists the results.

Table 45: Growth of yeast strains after 24 hours in culture media containing diamorphine hydrochloride.

	Yeast Isolates				
Drug Conc. mg/ml	CA6	CA9	SAC1	SAC2	BY
0	+	+	+	+	+
10	+	+	+	+	+
20	+	+	+	+	+
25	+	+	-	-	+
30	+	+	-	-	-
40	+	+	-	-	-
50	-	-	-	-	-

The range of MICs for the *C. albicans* strains is 40 - 50 mg/ml which is higher than that of the *S. cerevisiae* strains which had MICs of the order of 20 - 25 mg/ml. The

S. cerevisiae purchased from the supermarket as bakers yeast had a MIC in the range 25 -30 mg/ml.

Broth Dilution Experiments - IC_{30} Determinations.

The length of time that killed the yeast was determined for different concentrations of diamorphine hydrochloride.

The optical densities after 24 hours incubation were measured for the yeasts transferred to drug free media after various times in contact with the drug. Graphs of these optical densities (ODs) after different contact times were plotted for each drug concentration. Figure 48 illustrates the ODs for the isolates tested with 100 mg/ml diamorphine hydrochloride.

Table 46 details the highest concentration at which yeasts were able to grow in drug free media, after being transferred, at various times, from media containing diamorphine hydrochloride. The MICs for the isolates tested this way were:-

CA 6 40 mg/ml	CA 9 40 mg/ml
SAC 1 20 mg/ml	SAC 2 20 mg/ml

Figure 49 shows graphs of some IC_{30} determinations for isolates from heroin addicts.

Table 46: Details of the highest concentration at which growth of yeast occurred after various contact times with diamorphine hydrochloride.

	Yeast Isolates			
Contact Time (Hours)	CA 6	CA 9	SAC 1	SAC 2
½	100	200	100	100
1	75	100	75	75
2	75	100	20	50
3	75	100	20	40
4	75	100	30	30
5	75	100	30	30
6	50	75	20	20
8	50	75	20	10

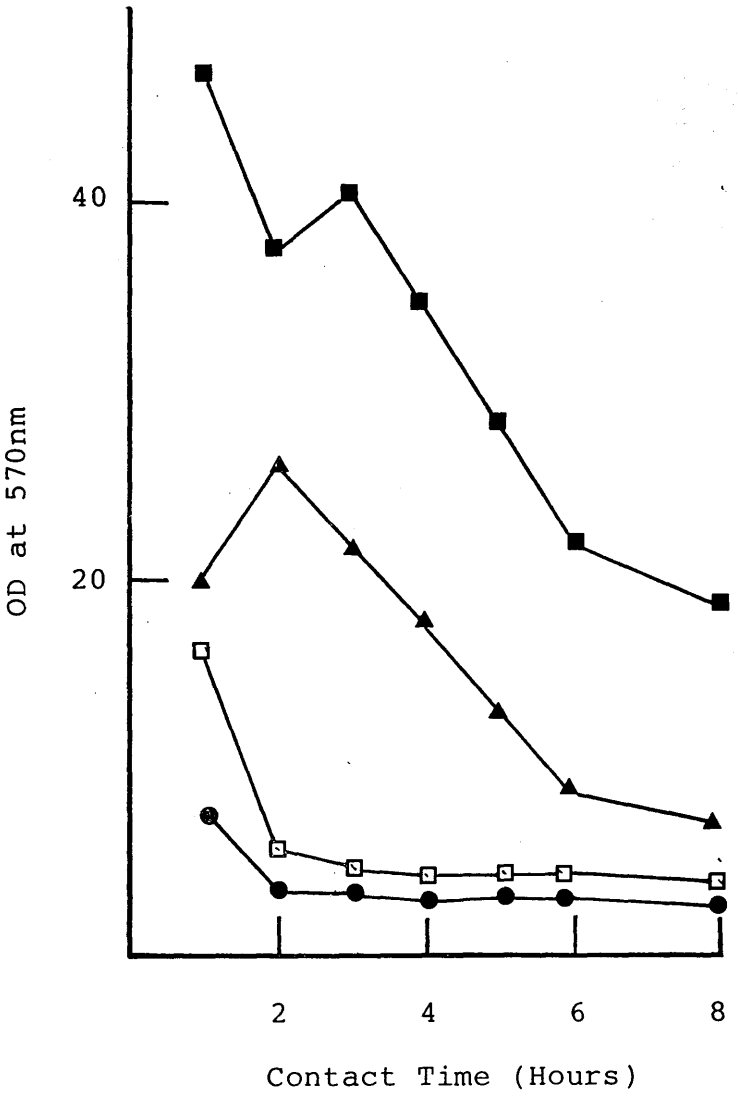
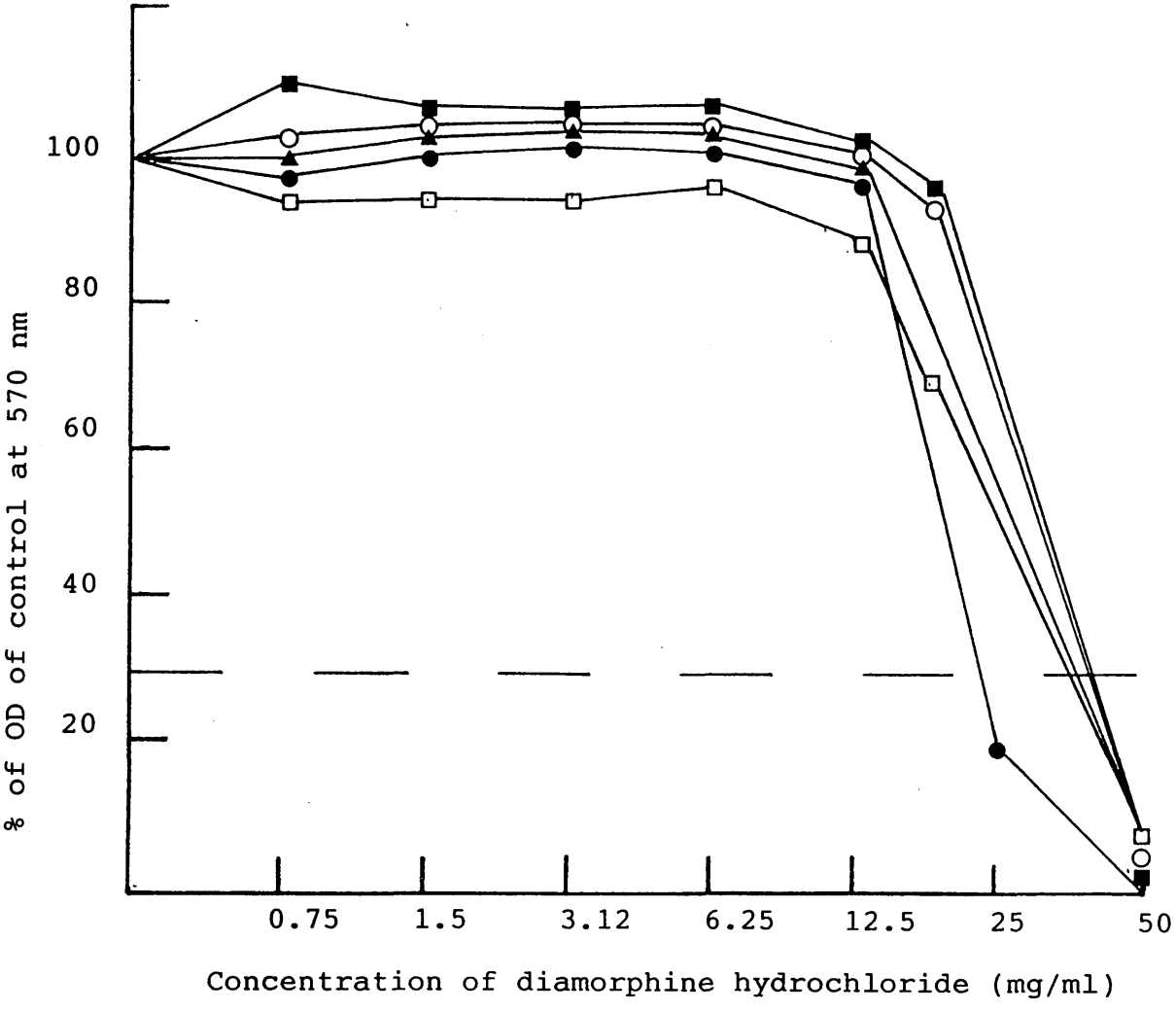


Figure 48 Survival of C.albicans CA6 (▲), CA9 (■) and S.cervisiae SAC1 (□), SAC2 (●) in diamorphine hydrochloride at 100mg/ml.



(●) Isolate 2 lemon
(○) Isolate 4 scalp
(▲) Isolate 6 mouthwash
(□) Isolate 11 vitreous
(■) Isolate 13 faeces

Figure 49 IC_{30} determinations for addicts isolates of C.albicans.

4.5.4. Growth of Fungi in Lemon Juice.

The juice from twenty seven plastic "Jif" lemons was cultured to detect the presence of fungi. Six of the seven lemons donated by friends and colleagues were culture negative. The seventh, which was more than a year old, grew a yeast. The yeast was not one of the common pathogenic species and eluded attempts at identification by the API 20C system and conventional fermentation and assimilation methods.

Nine of the lemons purchased in shops in the Castlemilk locality were within their "best by" dates, two were at their "best by "dates and one was beyond its "best by " date, the juices from these lemons were all negative on culture. Three of the lemons had no date tags and appeared to be rather old, moreover the lemons showed signs of leakage. These three lemons were purchased at the Centra food store in the middle of the Castlemilk housing estate. When cultured, the juice from two were negative but the the juice from the third grew *A. niger*.

Five plastic lemons from addicts were cultured. Three were culture negative but the other two yielded *C. albicans*. One of these *C. albicans* isolates was investigated further by the Leicester biotyping method.

(i) Survival of *C. albicans* in Lemon Juice.

The length of time *C. albicans* remains viable in contact with juice from plastic lemons was investigated.

New juice, old juice and new juice which had been steamed were inoculated with *C. albicans* and the survival was studied. The results are given in Tables 47 and 48.

Table 47: Survival (+) of *C. albicans* in old (O), new (N) and steamed lemon juice after different contact times. Glucose peptone broth was employed as a control (C).

Contact Time (Hours)	Juice C	O	N	Steamed Time ¼	½	1	2	3	4	5	6	7	8
1	+	+	+	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+	+	+	+	+	+
6	+	+	-	+	+	+	+	+	+	+	+	+	+
12	+	+	-	+	+	+	+	+	+	+	+	+	+
24	+	+	-	+	+	+	+	+	+	+	+	+	+

C. albicans could be grown in old and steamed lemon juice but was killed after five hours in new juice. Even a short steaming (15 minutes) appeared to allow the yeast to grow in the lemon juice.

Figure 50 shows the petri dish cultures of the dilutions of the surviving yeasts. The survival curve of the *C. albicans* in the three types of lemon juice is illustrated in Figure 51. The graph shows the number of viable *C. albicans* present in the juice, after 12 hours incubation. The number of viable yeasts present in the old and steamed lemon juice were comparable but after 12 hours incubation the yeast appeared to grow slightly better in

Contact time (Hours)	Old	New	Time steamed (hours)									
			¼	½	1	2	3	4	5	6	7	8
1	7.5	15	8	11	8	11.5	11.5	12	13	19.5	8	5.5
2	33	5.5	7	10	11.5	9.5	11.5	8.5	9	27.5	13	15.5
3	14.5	6	9	12.5	5.5	12	9.5	7.5	11	10.5	9	13.5
4	14	1	11	7.5	8.5	7.5	10.5	16	9.5	12	7	12
5	12	8.5	12.5	9	11	13.5	10.5	13.5	12.5	15.5	3	10.5
6	17	0	9.5	17	9.5	19	11	12.5	12.5	14	9.5	11
12	150	0	74	71.5	67.5	62	63	43	42	27.5	23.5	21.5
24	59,000	0	65,000	34,500	23.500	70,500	22,000	8,000	28,500	21,000	23,000	15,000

Table 48: Number of C.albicans per 50 µl of lemon juice.

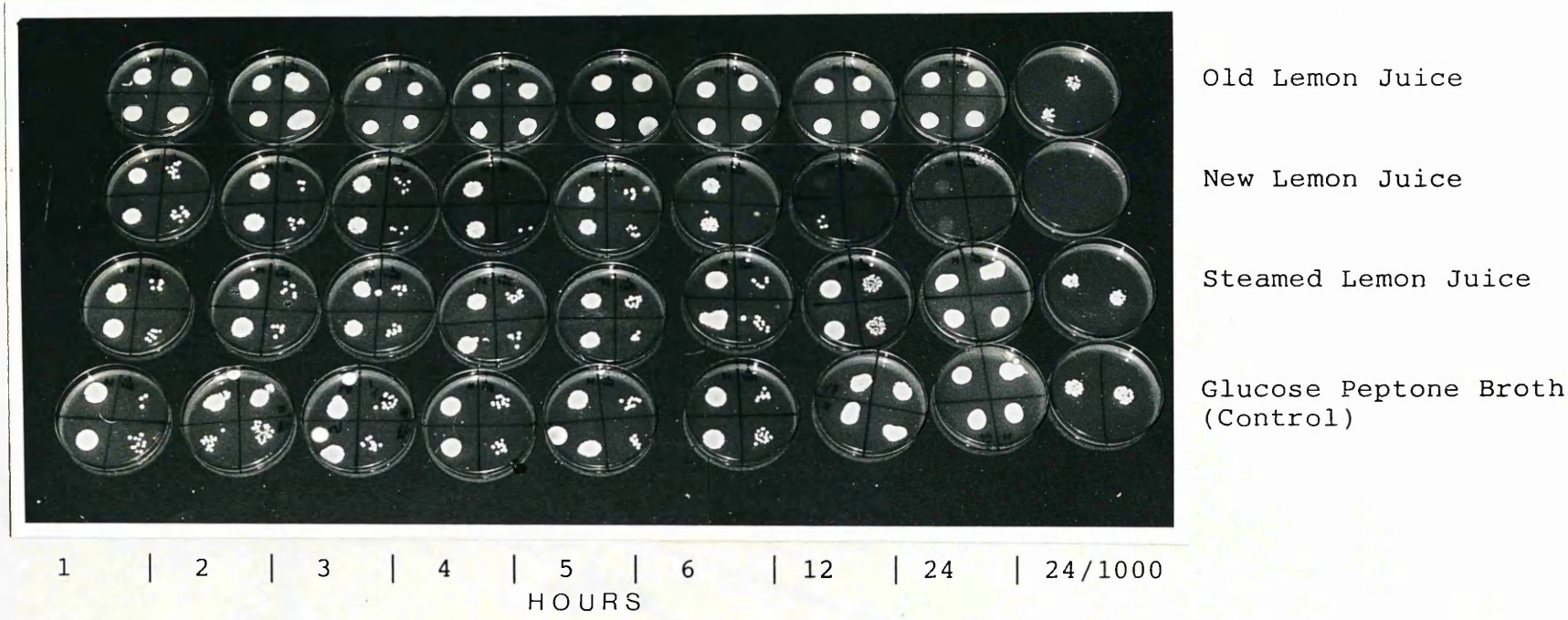


Figure 50 Cultures of C.albicans surviving in "Jif" lemon juice for different times. Juice was cultured undiluted and at a 1/100 dilution. At 24 hours the juice was also plated at a 1/1000 dilution.

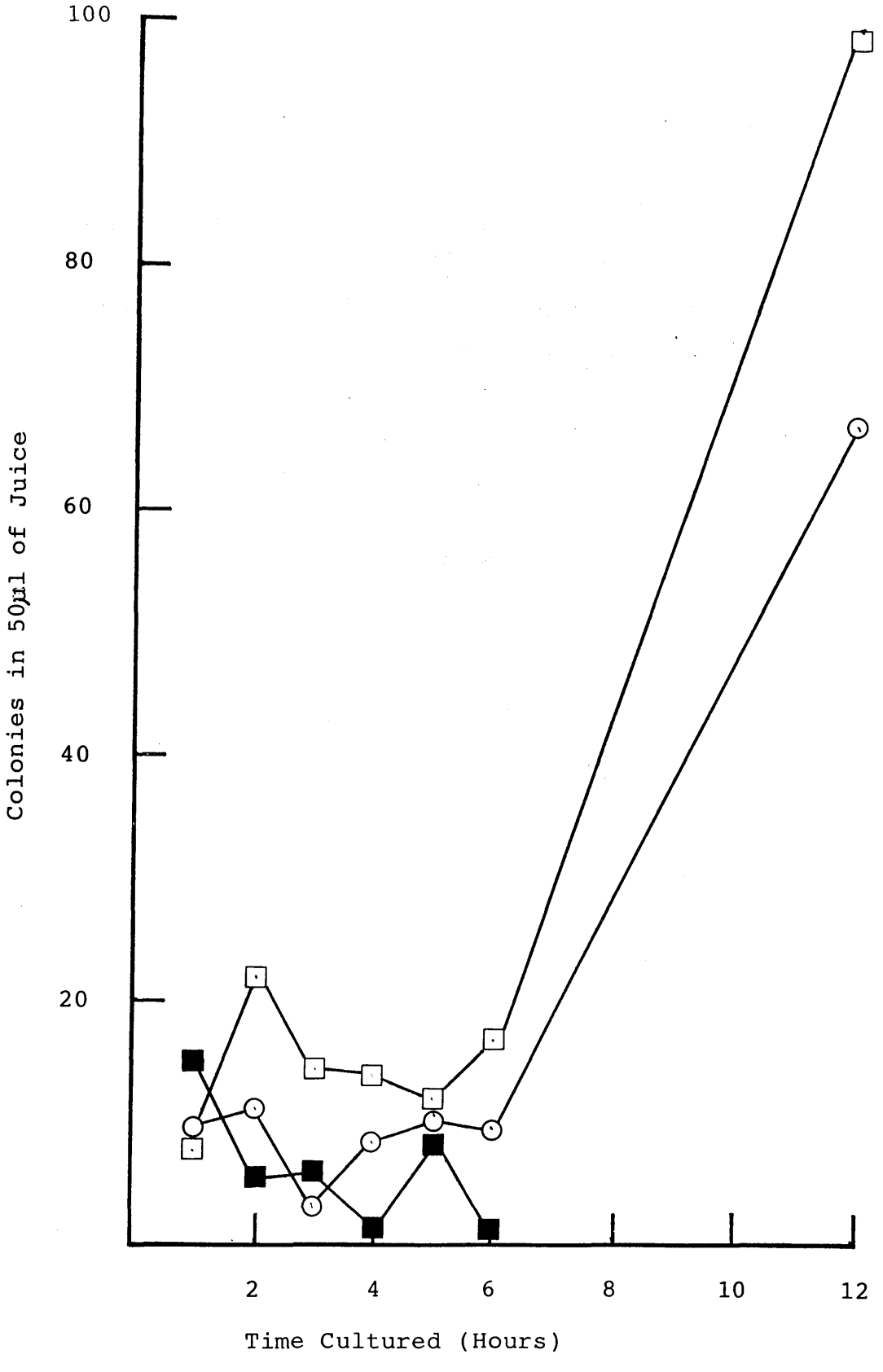


Figure 51 Survival curves of C.albicans in new (■), old (□) and steamed (○) lemon juice.

the old lemon juice than the lemon juice which had been steamed for one hour. *C. albicans* could not survive in the new lemon juice for longer than five hours.

The length of time the new lemon juice was steamed had some effect on the number of viable yeast present. The longer the juice was steamed the less able it became to support the growth of *C. albicans* (Figure 52).

(ii) Content of Sulphur Dioxide In Lemon Juice

The SO_2 content of seven samples of lemon juice was determined by a distillation method and the results, expressed as parts per million (ppm), are given in Table 49.

Table 49: Levels of SO_2 in samples of lemon juice.

Best By Date	Condition	SO_2 Content
Before Date	2 weeks old	92.1
Before Date	2 weeks old	83.4
New	Steamed 5 minutes	53.1
New	Steamed 15 minutes	26.9
New	Steamed 30 minutes	0
Beyond Date	5 Months Past Date	0
Beyond Date	7 Months Past Date	0

The permitted level of preservative for lemon juice is 350 ppm. However, the actual level in all of the lemons tested was far below this. The level of SO_2 in heated juice was considerably less than the new juice and after 30 minutes steaming SO_2 could not be detected. The level

of the SO_2 was too low for detection in the two lemons which were beyond their "best by date".

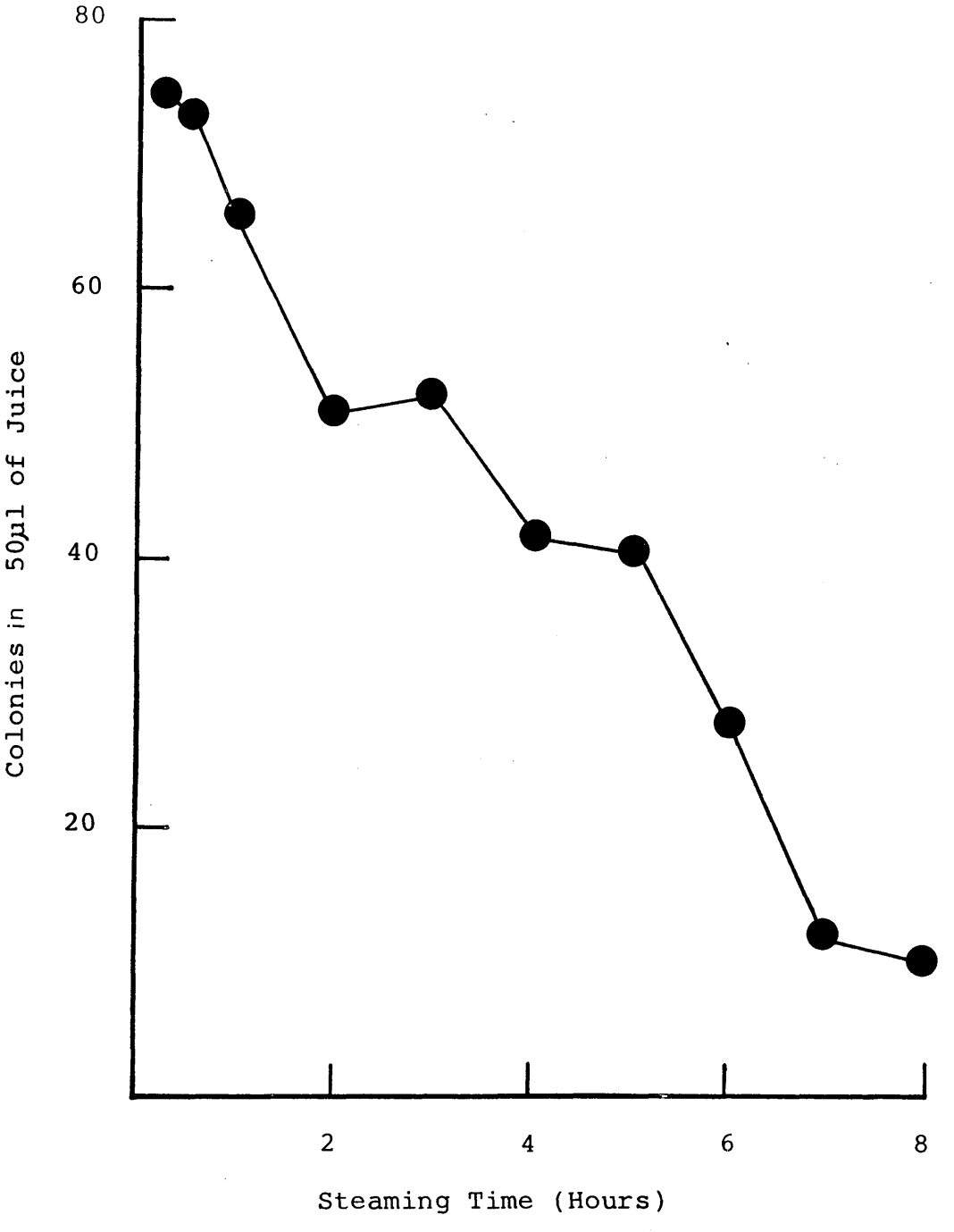


Figure 52 Number of viable C.albicans present after 12 hours culture in juice which had been steamed for an increasing amount of time before inoculation.

5. DISCUSSION

The advent of immunosuppressive therapy for the treatment of cancers and prevention of rejection in transplant patients, in conjunction with longterm antibiotic therapy has resulted in an increase in opportunistic fungal diseases in man. Diagnosis of these infections by serological or antigenic detection methods is not always satisfactory. In many cases the definitive diagnosis is only made at autopsy when there may be no unfixed material available for culture and the identification of the aetiologic agent can only be made by histological examination of tissue sections.

The conventional staining techniques of Periodic acid Schiff's and methenamine silver are adequate for detecting the presence of fungi in tissue. However, these methods seldom distinguish one fungus from another. The various fungi may closely resemble each other in tissue and the ideal staining technique would be one which was able to stain the infecting fungus in such away that it allowed the fungus to be identified accurately.

This study examined a peroxidase anti-peroxidase (PAP) immunological staining method as a means of identifying fungi in tissue sections. The PAP technique was chosen as it is one of the more sensitive of the immunoperoxidase methods. DAB was employed as the substrate because it allows the staining to be viewed using a light microscope and since the reaction product is permanent the slides may be kept indefinitely.

The selectivity and the sensitivity of the antisera employed in the technique had to be ascertained by screening for cross-reactions using several different methods. These included a novel agar block technique developed to test the reagents in the staining method without interference from tissue binding or endogenous peroxidase activity. The agar block staining method successfully simulated three dimensional fungal growth through tissue and enabled cross-reactivity testing with fungi for which positive control material was not available.

The PAP staining method was also applied in the investigation of an animal model of endogenous *C. albicans* endophthalmitis. The model was evolved to investigate the pathology of the disease after an outbreak of the infection in heroin addicts in Glasgow.

Development of an Immunohistochemical Staining Method for the Recognition of Fungal Elements in Tissues

The accuracy of any detection system involving the use of antibodies, is only as reliable as the specificity and sensitivity of the antibodies employed.

The successful application of the immunohistochemical staining method described in this work was dependent on the strength and specificity of laboratory produced and commercial rabbit antisera raised against fungal antigens.

The strength and specificity of both the commercial and the laboratory produced antisera were determined by several methods. These included: counter-immunoelectrophoresis, agar gel double diffusion, ELISA for the detection of antibody, ELISA for the detection of antigen and a PAP immunostaining method on sections from agar blocks.

When tested by CIE all antigens of the *Aspergillus* species reacted with their homologous antisera and also with some of the heterologous sera. The *A. niger* antigen was the least cross-reactive, and demonstrated a reaction only against the homologous serum.

By agar gel double diffusion none of the *A. fumigatus* antigens cross-reacted with antisera raised to *C. albicans* or *R. oryzae*.

In an ELISA system for the detection of antibody, the ability of the antigens to cross-react with the antisera from other species or genera was dependent on the concentration of the solid phase antigen. For *A. flavus* and *A. niger*, if the concentration of the solid phase coating antigen was increased from 3 mg/ml to 30 mg/ml then there was increased cross-reactivity. This may have been due to an increase in the number of sites to which the heterologous antiserum could bind resulting in a detectable colourmetric reaction. The antigens of aspergilli, other than the *A. fumigatus* antigen, could not

be detected by *A. fumigatus* antisera using an ELISA for antigen detection.

The staining results from the *Aspergillus* blocks presented a complex picture as cross-reactions within the genus were evident with the PAP staining method. All the sections from blocks inoculated with *Aspergillus* species were stained by their homologous antisera and, apart from two instances, by all the heterologous *Aspergillus* antisera. No staining was seen when either *C. albicans* or *R. oryzae* antisera were used as the primary antibody. This technique indicated a lack of cross-reactivity of the non-*Aspergillus* antisera with all of the aspergilli tested. Moreover, the method demonstrated that an antiserum raised against one *Aspergillus* species would cross-react with other aspergilli and lead to positive staining.

The *A. fumigatus* antigens consistently reacted against the *A. fumigatus*, *A. flavus*, and *A. niger* antisera regardless of which test method was employed. These same antisera also cross-reacted with *A. fumigatus* in the agar block staining method. *A. fumigatus* antigens cross-reacting with *A. flavus* antisera has been recorded before (Kim and Chaparas, 1979).

The *A. flavus* antigen varied in its pattern of cross-reactions depending on which method was employed although in all cases it reacted with its homologous antisera and with the *A. niger* antisera. *A. nidulans* antigen showed no reactions with the non aspergilli antisera and little

cross-reaction with the other aspergilli by antibody-ELISA and CIE. However, the *A. nidulans* sections from the agar blocks were stained by all the *Aspergillus* antisera with the exception of *A. fumigatus*. The *A. niger* antigens only produced precipitins against the homologous sera when tested by the CIE system. The antigen however did show considerable cross-reactivity with heterologous *Aspergillus* antisera in the antibody-ELISA system and when employed as the antigen in the agar block staining. This is in contrast to other investigators (Kim and Chaparas, 1979) who found *A. flavus* and *A. niger* antigens to have a high degree of specificity. However these workers were using partially purified antigens and they attributed the majority of the cross-reactions to the polysaccharide portion of the mycelial extract.

A. terreus appeared to be the antigen which elicited most response from the heterologous antisera. Although it was not so reactive in the CIE system, giving precipitins only against *A. terreus* and *A. fumigatus*. In the antibody-ELISA and agar block staining method it cross-reacted with all the *Aspergillus* antisera and, in the antibody-ELISA system, even the *C. albicans* antisera.

That there was such a great deal of cross-reactivity is not suprising since the extracts were all water soluble components prepared from whole mycelium. There is some evidence to suggest that wall located antigens are more specific when tested against rabbit antisera. However,

when these cell wall derived antigens were tested against patient sera, cross-reactivity was evident, indicating shared antigenic determinants (Hearn, Proctor and M$^{a c}$kenzie, 1980).

When tested by CIE, the laboratory raised *A. fumigatus* antisera did not cross-react with antigens of other genera, although they gave precipitin bands when tested against some of the aspergilli. The commercial *Aspergillus* antisera demonstrated variable cross-reactivity. The *A. terreus* antisera only gave a precipitin line against its homologous antigen but the *A. niger* antibody showed cross-reactivity against antigens of all the aspergilli apart from *A. terreus*. The *A. fumigatus* antisera did not cross-react with the antigens of the other genera when tested by double diffusion.

There was a great deal of cross-reactivity of the antisera demonstrated by the antibody-ELISA technique. All of the *Aspergillus* antisera showed some cross-reactivity with heterologous antigens within the genus. The commercial antisera even demonstrated some cross-reactions with *C. albicans* antigens. ELISA for antigen detection did not reveal any cross-reaction of the rabbit anti-*A. fumigatus* antiserum with any of the antigens from the other aspergilli. However, this may be attributable to the sensitivity of the test.

The laboratory raised antisera proved to be more specific than the commercial antisera in all of the tests.

The laboratory produced *A. fumigatus* sera was specific and stained only the *A. fumigatus* containing sections. The commercial *Aspergillus* antisera showed a great deal of cross-reactivity. This cross-reactivity was also demonstrated by the other techniques employed in this study and is probably due, in part, to a vast amount of antigenic determinants ubiquitous to the genus *Aspergillus*. Based on the findings of this and other work this cross-reactivity may be unavoidable if the antigens used to raise the antisera are crude preparations. Only if antigens which are peculiar to each species can be found will there be any improvement in the antisera raised to the aspergilli. Moreover, the antigenic factors must be present in all the strains of that species if the antisera is to be of any value in the diagnosis of clinical disease. If the antisera were only active against some strains of the *Aspergillus* species then lack of immunohistochemical staining of the fungi could result and an incorrect diagnosis could be made.

The commercial *Aspergillus* antisera showed more cross-reactivity than the laboratory raised antisera. This may be due to the hyperimmunity of the commercial rabbit antisera or the possibility that the sera were pooled from a number of rabbits, which would increase the possibility of introducing antibodies to the other aspergilli. The rabbits may have natural antibodies to the aspergilli the spores of which are frequently found in hay used as a

supplement to rabbit diets. The cross-reactions with *Candida* antigens may be due to some intrinsic antigenic similarities between the genera *Candida* and *Aspergillus* as has been suggested by Magaldi and M^c^kenzie (1984).

All the *C. albicans* antigens were detected by the *C. albicans* antisera by CIE. No cross-reactivity occurred with *R. oryzae* or *A. fumigatus* antisera. The *C. parapsilosis* antigen cross-reacted with the *C. albicans* antisera but not the *A. fumigatus* antisera. The *C. albicans* antigen only reacted with its homologous antisera when tested by agar gel double diffusion.

The *C. albicans* antibodies all reacted with the homologous antigens using ELISA for antibody detection but not with the *R. orzyae* or *A. fumigatus* antisera. However, there was evidence of cross-reactivity with three of the commercial antibodies. This phenomenon again may be due to hyperimmunity of the sera or common antigenic features between the aspergilli and *Candida*. It is significant that this occurred only with the commercially produced antisera.

Sections from agar blocks inoculated with *C. albicans* stained only with the homologous antisera. The sections from the *C. parapsilosis* inoculated block were not stained by any of the antisera tested, including the *C. albicans* antisera. This was in contrast to the results obtained by CIE and the antibody-ELISA which demonstrated that the *C. albicans* antisera could bind to some *C. parapsilosis*

antigens. The antigenic sites expressed by the *C. parapsilosis* in the block were somehow different from those expressed by the solutions of lyophilysed antigen prepared from this yeast. It is possible that the antigen solutions exposed a greater number of sites for binding antibody than did the relatively small amount of yeast present in the sections. The antigenic determinants of *Candida* appeared to be specific for that genus when PAP staining was employed. Little cross-reactivity was demonstrated with members of this genus and heterologous antibodies.

The *C. albicans* antigens demonstrated no cross-reactivity by CIE or double diffusion. However, the antibody ELISA demonstrated cross-reactions with three of the four commercial *Aspergillus* antibodies. This cross-reactivity may be due to the sensitivity of the ELISA technique but since the *Candida* antigens were not tested against the commercial antisera by CIE or double diffusion the results could not be compared. The *C. parapsilosis* antigen did form a precipitate with the *C. albicans* sera in CIE. These results suggest the presence of shared antigenic factors between *C. albicans* and *C. parapsilosis*. Although only one common antigenic factor is reported by Miyakawa *et al.*, (1986) these workers could not demonstrate agglutination of *C. parapsilosis* and *C. krusei* using monoclonal antibodies to *C. albicans*.

The *C. albicans* antisera did not cross-react with either the *R. orzyae* or *A. fumigatus* antigens in the CIE test system. The *C. albicans* antisera showed no evidence of cross-reactivity with the heterologous sera but gave good precipitins with the *C. albicans* antigen by agar gel double diffusion.

The *C. albicans* antisera reacted well with the *C. albicans* antigens in the ELISA antibody detection system. The antisera also reacted with the *C. parapsilosis* antigen. There was no cross-reactivity with the *A. fumigatus* or *R. orzyae* antigens. However, there were cross-reactions with the *A. flavus* and *A. niger* antigens at the higher concentrations tested.

The laboratory produced *C. albicans* antisera proved to be the most specific in the PAP staining method. The *C. albicans* antisera stained the *C. albicans* sections but they did not stain any of the sections with heterologous fungi.

The *C. albicans* antibody demonstrated strong reactions to its homologus antigens. Cross-reactivity with the *C. parapsilosis* antigen was seen in the CIE and antibody-ELISA systems. Monoclonal antibodies to exoantigens of *C. albicans* have been used for the serological analysis of yeast isolates by the western blot method. The work demonstrated cross-reactions of *C. albicans* monoclonal antibodies with *C. parapsilosis* antigens even though the

C. parapsilosis antigen had a different molecular weight (Polonelli and Morace, 1986).

The *C. albicans* antisera cross-reacted with the higher concentrations of the *A. niger* and *A. flavus* antigens when tested in the antibody-ELISA system. These results may be another indication that *Candida* and *Aspergillus* may express antigens common to both genera.

The *R. oryzae* antigen only reacted with its homologous antserum in the CIE system. Double diffusion showed no evidence of cross-reactivity of the *Rhizopus* antigen when tested against heterologous antisera but it did form a precipitin line with the homologous antiserum.

The ELISA system for antibody detection showed that the *R. oryzae* antigen reacted well with its homologous antiserum. The only heterologous antiserum with which it demonstrated cross-reactivity was *A. flavus*.

The mucoraceous fungi varied in their staining response. *Absidia glauca* hyphae which stained well by PAS did not stain with any of the antisera tested in the PAP system therefore no cross-reactivity was demonstrated. The sections containing hyphae of *M. hiemalis* and *R. oryzae* stained with the *R. oryzae* antiserum and when some of the commercial *Aspergillus* antisera were employed.

There was only one *Rhizopus* antigen investigated and it appeared to be specific. The only evident cross-reaction was with a commercial antiserum and it may have been the result of a natural antibody present in the rabbits.

CIE did not demonstrate any cross-reactivity of the *R. oryzae* antiserum with heterologous antigens. The antiserum only produced precipitin bands with the *R. oryzae* antigen when tested by double diffusion. The *R. oryzae* antiserum did not cross-react with any of the heterologous antigens it was tested against by this method but it did demonstrate strong reactivity with the homologous antigen, demonstrating the specificity of the antisera.

The *R. oryzae* antiserum appeared to be the most specific of all the antisera tested as it did not demonstrate any cross-reactions. However, the antibody was not tested against any of the mucoraceous fungi by CIE, double diffusion or ELISA but in the PAP staining of agar sections it did stain sections with *M. hiemalis*.

The quality of the antisera used in immunoassays is dependent on several factors. The strain of fungus from which the antigen is produced will determine the antigenic properties of the extract used for antiserum production. Different strains of the same fungus may have different antigenic properties some of which may be similar to those produced by other genera. Whether the antigen was prepared from a culture filtrate, the entire mycelium or mycelium components will have a bearing on the final antiserum. The method of extraction and preparation of these components, eg. water soluble fractions or detergent extracted fractions will alter the nature of the antigen. The antigens used in this study were polyvalent preparations

utilising all of the mycelium, thus they were composed of many different antigenic components.

Individual animals may recognise different components of the antigens and may exhibit slightly different immunological reactions to the same antigen. The commercial sera may have been the product of several rabbits while the laboratory raised antisera were from single rabbits and were never pooled.

The different assay systems in this study, employed different reagents and this accounts for the lack of consistency of results obtained by the various tests. The number of steps required to perform the tests may also contribute to variation of the results. The double diffusion and CIE systems were the least sensitive at detecting cross-reactions, which is not surprising since there are no stages in the procedures which intensify the reaction.

The heterogenicity of fungal antigens and antibodies employed in different studies may be one explanation of the discrepancies in results found in this and other laboratories eg. Magaldi and M$^{a c}$kenzie (1984) did not find the *A. flavus* antigen reactive with the *A. niger* antisera in the antibody-ELISA test.

The cross-reactivity found in various tests during this investigation indicate the possible complications that may be encountered when applying the PAP immunoperoxidase technique to the diagnosis of fungal disease.

The laboratory produced sera all proved to be selective for their homologous genus. Unfortunately a great deal of inter-specific cross-reactions were demonstrated within genera. However, the commercial antisera were less specific and inter-genus cross-reactions were revealed. There was little evidence that false positive staining would occur when the laboratory produced antisera were employed. This indicates the suitability of these antisera for use in a diagnostic PAP staining system. The commercial *Aspergillus* antisera would be less suitable for use in such a system since an unacceptably high rate of false positive staining may occur.

Peroxidase Antiperoxidase Staining of Tissues

Having established the suitability of the various antisera for use in an immunohistochemical staining method, the peroxidase anti-peroxidase staining technique was evaluated for several clinical manifestations of opportunistic fungal diseases. The fungi were clearly stained and the technique performed well. There were several tissue types examined for each fungal infection and there were no apparent differences in background staining or the performance of the method with differing tissues. Regardless of which fungus or which organ was involved, staining was evident with the homologous primary antibody.

C. albicans antisera was not absolutely specific since staining was evident when the antiserum was used as the primary antibody to stain the contents of an aspergilloma. This was in contrast to the findings with the agar block method, although the *C. albicans* antisera did show some cross-reactivity with *A. flavus* and *A. niger* in the antibody-ELISA test. The positive staining of the aspergilloma may have been due to the large amount of dense hyphae present which made a great number of common antigen sites available for binding. The *A. fumigatus* antisera did not stain tissues from diseases attributed to other fungi but clearly stained hyphal elements of *Aspergillus* in sections from patients with invasive aspergillosis, paranasal aspergillosis and aspergilloma. The *R. oryzae* antiserum was reasonably specific as it did not stain any of the sections from patients with aspergillosis or candidosis. However, all the sections from cases of suspected mucormycosis were stained although the aetiologic agent had not been determined in every case and possibly was not *R. oryzae* but some other mucoraceous fungus.

In summary, the PAP staining method appeared to be sensitive and able to demonstrate and identify fungi present in tissue even if present only in small amounts. There was a problem with background staining which was stronger than desirable on some occasions despite the blocking of endogenous peroxidase activity at the start of

the staining procedure. The dilution of the second stage antibody in human serum in addition to tris buffer helped to reduce this.

The technique is suitable for use as a diagnostic tool to aid the identification of the aetiologic agent of fungal infection. The method can distinguish to which family a fungus belongs and in many cases the particular genus. The PAP method produced consistant, reproducible results. It is extremely sensitive, although this sensitivity increases the chances of demonstrating cross-reactions with other fungi. The species of fungus can not be determined since there was evidence of cross-reactivity among species of the same genus.

Greater specificity may be achieved eventually by the use of monospecific or monoclonal antibodies but such antibodies would have to detect an antigen which is not only specific for the species but is ubiquitous to that species. The antisera would have to recognise a specific determinant common to every member of that species *in vivo*. Absorption of the antisera with antigens of other opportunistic fungi may help to eliminate some of the cross-reactivity but large quantities of antibody would be required.

The PAP method is a sensitive immunoperoxidase staining method and with the exception of the fungal antisera, the reagents employed are relatively inexpensive, readily

obtainable, have a long shelf life and are interchangeable for detection of various antigens.

A drawback is perhaps the time required for processing the material because of the numerous antibody layers required. However, the need for more and better aids in the diagnosis of these serious and and ever more common infections ensures immunoperoxidase staining a place in the mycological laboratory in the future.

Epidemiology of *Candida albicans* Endophthalmitis in Glaswegian Heroin Addicts

An outbreak of endophthalmitis in heroin addicts in Glasgow was studied to determine the aetiologic agent, the source of the outbreak, the method of spread and, if possible, a method of containing the infection. Clinically the patients presented with optic symptoms suggestive of *C. albicans* endophthalmitis. This diagnosis was confirmed by culture of vitrectomy specimens and supported by *Candida* serology results. The possible sources of the infecting organism appeared to be the street drug, adulterants of the drug, the drug diluent, the injection paraphernalia or the addicts endogenous flora.

Some of the patients suspected a "pusher" may have been supplying them with heroin cut with bakers' yeast and initially *S. cerevisiae* was thought to be the aetiologic agent of this outbreak of endophthalmitis. Culture of dealers' and "pushers'" drug and their packets failed to yield any yeasts and indeed, whenever street heroin has been investigated by culture neither *C. albicans* nor *S. cerevisiae* have ever been grown (Mellinger *et al.*, 1982; Moustoukas *et al.*, 1983). This has been attributed to many factors the most common being the small size and number of samples available but even when one hundred samples were cultured no evidence of yeast contamination was found (Tuazon *et al.*, 1974).

An explanation of the lack of cultural evidence for contamination of street heroin by yeasts, may be that diamorphine hydrochloride inhibits the growth of yeast. The results of this investigation indicate that the neat drug in the powdered form is not only fungistatic but fungicidal for yeasts. However, when the drug was diluted in water and placed in wells in agar seeded with yeast no inhibition was detected. The passive diffusion of the drug through the agar may have been prevented by the drug binding to agar constituents. Some inhibition was evident when the diamorphine hydrochloride was incorporated in the agar. Broth dilution experiments showed that the concentration of the drug required to inhibit yeast growth was 20-50 mg/ml, which is substantially higher than the spectrum of activity of antifungal drugs.

The exposure time required to kill the strains of yeast, tested in a broth dilution system varied. The *C. albicans* isolates were more resistant than those of *S. cerevisiae*, taking a minimum of 4 hours to be killed at 100 mg/ml.

IC_{50} determinations were used to assess the effect of diamorphine hydrochloride. The measurement of MICs for drugs with an antifungal effect by agar incorporation and broth dilution methods can sometimes give misleading results. These methods can be dependant on inoculum size, temperature and duration of incubation. The end point of MIC assays can be subjective if there is partial

inhibition over a range of concentrations (Johnson *et al.*, 1984). The IC_{50} method was beneficial as there was a definite end point. Moreover, the amount of drug required is small and a large number of isolates can be screened at once. This made it an ideal system for testing all the isolates received from patients and injection apparatus. All of the yeast isolates cultured from the patients and their injection paraphernalia were killed by diamorphine hydrochloride in the range 20-78 mg/ml.

In an attempt to mimic the situation on the street, diamorphine hydrochloride was mixed with freeze-dried cultures of *C. albicans* and *S. cerevisiae*. Even at proportions of eight parts diamorphine to one part yeast the yeasts remained viable and were recovered on glucose peptone agar medium. This is in contrast to the findings of Dupont and Drouhet (1985) who found that a paste of *C. albicans* and drug was able to inhibit the growth of the fungus after being stored at room temperature for one week. It may be significant that these workers chose to moisten the mixture and rehydration of the diamorphine hydrochloride may be required before its antifungal activity is expressed. However, in the present study, when a solution of diamorphine hydrochloride was stored for 24 hours before it was tested no antifungal activity remained.

If there are any yeasts present in street heroin they may not be revealed by culture because they may be killed

by the diamorphine hydrochloride, which, according to the results in this study has fungicidal activity. When the situation on the street was simulated despite employing pure diamorphine hydrochloride, a drug of greater quality and higher concentration than that purchased by addicts, the yeast was able to survive and remain viable in culture.

If the street drug was not the source of infection then the yeast may have originated from the addicts own flora of micro-organisms. Culture of body fluids and stool specimens from the addicts and culture of items of their injection utensils yielded several isolates of *C. albicans*. The biotyping system of Odds and Abbott (1980; 1983) was employed to determine common features among the isolates. Biotyping fingerprints isolates of *C. albicans* by analysing their growth patterns on a range of different media. A biotype has no formal status and refers to a strain which has been differentiated experimentally beyond the level of species. The system was employed to try to discover if any common or similar strains of *C. albicans* had been isolated from the various samples cultured during the study.

Apart from addict number 22 who had two different biotypes the isolates from each of the individual addicts were similar and biotype number 153 was found from five different addicts. Addict number 23 harboured biotype 455, a different biotype from the other addicts. Unfortunately

no one addict was available to donate a complete set of samples. The overall picture demonstrated a preponderance of biotype 153. This biotype has also been implicated in infections of heroin addicts in Madrid where it has been isolated from addicts skin lesions (F.C. Odds, personal communication). Every isolate tested was positive for citric acid perhaps indicating their ability to survive and grow in lemon juice.

The practice of using "Jif" lemon juice to dissolve their heroin was peculiar to the addicts of the Castlemilk area of the city. Other addicts in different areas were not afflicted with endophthalmitis and used water or vinegar as a solvent. Fresh lemon juice has been implicated in this disease in heroin addicts before in Australia (Newton-John, 1984). The ability of *C. albicans* to survive in lemon juice from plastic "JIf" lemons, the preferred solvent of the Castlemilk heroin addicts, was examined. This investigation found that *C. albicans* could be grown in old or steamed lemon juice but after five hours in contact with new "Jif" lemon juice it was non-viable. Measurement of the levels of SO_2 present in samples of "Jif" lemon juice indicated that even a short period of steaming drove off significant amounts of the preservative. The samples of old juice tested had no detectable levels of preservative. The number of yeasts remaining viable in old lemon juice and steamed lemon juice with low levels of SO_2 increased dramatically over a

24 hour period, whereas the number of viable yeasts in new juice dropped to zero after 24 hours illustrating the antifungal effect of the SO_2.

These results indicate the possibility of *C. albicans* surviving and multiplying in old "Jif" lemons near or past their "best by" date because the level of SO_2 preservative has been depleted to a level below which it could not be detected. "Jif" lemons past their "best by" date could be purchased in local shops in the area where the heroin abusers lived and indeed one of these lemons yielded a growth of *A. niger*. The addicts did not store their lemons under the ideal cool conditions but kept them with the rest of their paraphernalia at room temperature. This would enhance escape of the gaseous preservative through the plastic container and promote the growth of any micro-organisms that were present. *C. albicans* was isolated as a confluent growth from Castlemilk addicts plastic lemons. Dupont and Drouhet (1985) believe that contamination from the diluent is unlikely to cause infection as the addicts heat the drug mixture before injection and they speculate that this will have a sterilising effect. This has yet to be substantiated by experiment.

If a heavy suspension of *C. albicans*, from a contaminated "Jif" lemon, was introduced into the blood stream of a heroin addict, it would be a larger inoculum than normally encountered in candidal infection. In addition the immune status of heroin abusers might be

impared. McDonough *et al.*, (1980) reported a significant decrease in the total number of T lymphocytes *in vivo* and a concomitant increase in the number of null lymphocytes. Leukocytes may be a prime binding site for exogenous opiates in the peripheral circulating system since it has been determined thatphagocytic leukocytes have receptor sites for opiates. It has been demonstrated that the intracellular killing of *C. albicans* by polymorphonuclear leukocytes was reduced in one heroin abuser with pulmonary candidosis but in symptom-free addicts only minor abnormalities were noted (Lazzarin *et al.*, 1985).

The culture results demonstrated that *C. albicans* was isolated from the needles, syringes and lemons of the addicts in addition to the addicts themselves whether as commensal colonisers in the mouth or gastrointestinal tract or as opportunistic pathogens isolated from vitreous or scalp lesions. The lemon juice may have become contaminated by *C. albicans* which possibly originated as part of the commensal flora of an addict. The yeast could grow in the lemon juice which was probably shared among several addicts, although the addicts involved in the outbreak claimed not to know each other and were adamant that they never shared syringes.

The similarity of the biotypes of the isloates is an indication that the yeasts may have had a common source or that a particularly viulent strain was involved. The isolates from skin of a group of heroin abusers from

Madrid have a preponderance of the same biotype. It is interesting to note that all the *C. albicans* tested were able to grow on the citric acid medium used as one of the tests in the biotyping procedure.

In conclusion, endophthalmitis in heroin abusers has been reported previously and in most reports it appears in clusters indicating a common source of infection. The most likely source is the drug itself but *C. albicans* has never been found. This work has indicated that the heroin diluent may be one reservoir of infection. A cocktail of lemon juice and *C. albicans* is perhaps being injected into the addict's blood stream along with other impurities present in the drug. The flakes of lemon present in the juice may cause obstructions that block the fine capillaries of the eye contributing to the start of the infection. The *C. albicans* may adhere to the endothelium of the capillaries more easily if the blood flow is slowed by a blockage. The yeast can reach and locate in the eye very quickly, as was demonstrated by the animal model and from there it may grow to form an infection focus.

A Mouse Model For Haematogenous *Candida* Endophthalmitis

This study has shown that the intravenous injection of mice with 1 X 10^6 cells of *C. albicans* into their tail veins was able to produce an experimental model of *Candida* endophthalmitis. This concentration of *C. albicans* consistently produced eye infection in Balb/c mice and, as illustrated by the mortality curve, allowed the mice to survive for two to eight days which is sufficient time for ocular lesions to develop or for the infection to clear. In other experiments carried out under the same conditions the mice survived for up to 21 days. This may have been because some of the mice did not receive their full inoculum as the injection technique is difficult to perform. Alternatively, there may have been a greater range in the weights of the mice inoculated in these experiments with the larger mice receiving a proportionally smaller challenge for their body weight.

Fundus examination of selected animals revealed the start of ocular changes in the mice at three days post infection which is the same time interval after injection that Edwards *et al.* (1975) detected lesions appearing in the eyes of infected rabbits. The mice showed detachment of the retina, which was visible as an increase in parallax between the retina and choroidal vessels. There was segmentation of the blood vessels. This may be a consequence of retinal vasculitis (an infiltration of

inflammatory cells around the blood vessels) and may be an indication of a focus of infection. Vasculitis is also seen in cases of *Candida* endophthalmitis in man (Edwards, 1974). Figure 37 illustrates inflammatory cells in the retina adjacent to a blood vessel. There was tortuosity of parts of the blood column which may have been caused by inflammation elevating and distorting the vessels. Examination by an indirect ophthalmoscope did not reveal the fluffy, snowball like lesions characteristic of human disease (Michelson *et al.*, 1971). This may have been due to the small size of the lesions, perhaps insufficiently developed, or to the small sample of mice examined by this technique not accurately demonstrating the formation of lesions by the rest of the population.

The model demonstrated that the yeast reached the eye within a few minutes. This is not a surprising finding given that 0.2 ml of a blastospore suspension was injected into the mouse's blood system which has a total volume of about 3 ml.

The speed at which *C. albicans* is cleared from the blood of mice has been investigated by radiolabelling *C. albicans* with ^{14}C and ^{144}Ce (Trnovec *et al.*, 1978). It has been demonstrated that more than 85% of the radioactive cells were cleared from the blood within 30 minutes and that by 24 hours less than 2% of the original radioactivity remained in the blood (Mardon and Robinette, 1978). The earliest events in haematogenous *Candida*

endophthalmitis must be adherence and penetration. In this particular study it was found that after one hour yeast was still present in the retina. This would be unusual if the fungus was being cleared from the blood stream unless some yeast adherence mechanism was taking place. Klotz *et al.* (1983) reported that *C. albicans* and *C. tropicalis* which adhered to vascular endothelium *in vitro* were capable of transversing the endothelial surface before the production of germ tubes. These workers found adherence to be complete within 15 minutes and that as early as 15 minutes after attachment disruption of the endothelial cell surface was evident. Rotrosen *et al.* (1985) studied the attachment of *C. albicans* to endothelial cells and found this was inhibited by rabbit antiserum to *Candida* which blocked the attachment by binding serum constituents to the organism. These workers implicated mannan associated cell wall constituents as the *Candida* adhesin in this system.

An alternative explanation is that the sheer size of the yeast relative to the small size of the blood vessels may have caused mechanical blockage. By 24 hours pseudomycelium and germ tubes developed in the retina and the number of visible yeasts increased, indicating some movement from the capillaries or perhaps as a result of bursting of these vessels.

C. albicans was able to divide and by 36 hours there were clumps of yeast cells seen throughout the retina. The

yeast cells developed into focal lesions of infection which started to show disturbances of the nucleated cells of the retina. From these lesions the yeast could be seen spreading into the nerve fibre layer, the ganglion layer and the inner plexiform layers. This resulted in a bump being formed by the retina protruding into the vitreous. The protrusions started and grew at different rates in different mice. The raised lesion contained yeasts, pseudomycelium and inflammatory cells which were mainly mononuclear. Inflammatory cells were also seen in other layers of the retina. The results of this inflammatory process were the formation of discrete lesions surrounded by inflammatory cells which, in some cases, presumably resulted in the resolution of infection. The mice sacrificed later showed no evidence of infection. This may have been due to the mice overcoming the infection and clearing the *Candida* from their tissues. In the human manifestation of *Candida* endophthalmitis, spontaneous resolution is sometimes seen (Dellon *et al.*, 1975).

The animal model showed a marked localisation of the lesions and there was no generalised inflammatory infiltration throughout the retina or choroid. The fungus appeared to be restricted to the areas associated with inflammatory cells. The damage of the adjacent retinal cells may have been caused by the *Candida* when it invaded the retina initially or been caused by the inflammatory cells releasing hydrolytic enzymes as has been suggested

by Cohen *et al.*(1977). Further evience that*C. albicans* can cause tissue damage is provided by Danley and Polakoff, (1986). In their study *C. albicans* was described as having a cytotoxic effect. *C. albicans* was described as exhibiting a cytotoxic phenomenon 15 to 30 minutes after phagocytosis and this may represent an immediate reaction of blastospores when they are engulfed by phagocytes.

Quantitation of the fungal lesions by histological examination of the retinal sections indicated that the number of yeasts present in the eye immediately following inoculation was large. However, the number of yeasts visible decreased after one hour, illustrating the efficacy of the mouse system at clearing the fungus. The number of lesions increased at 36 hours as a consequence of multiplication of the viable yeasts which remained in the eye. The number of lesions reached a maximum at three days, after which time the number decreased. The decrease in the number of lesions may have been due to the mouse immune system arresting the development of the infection, resulting in survival of the mouse.

When the quantitative results of the lesions were compared with those of the average colony counts from eyes of mice sacrificed at the same times, a similar overall pattern emerged. There was an increase in the number of yeasts cultured, which reached a maximum at three days, decreasing at five days and dropping to zero at 22 days. However, the colony numbers in the first 24

hours of the experiment did not reflect the number of lesions seen on histology. Demant and Easterbrook (1977) attempted to correlate culture results with clinical appearance in a rabbit model of the disease. They found the results could not be related consistently to the number and size of the lesions but the eye with the highest count was the only one with a vitreous haze. The authors attributed the lack of consistency to "inadequate homogenisation or poor dilution before plating". The discrepancy in the results of the present study may be due to the large range in the colony numbers for different mice sacrificed at the same time post challenge and the relatively small number of eyes investigated at each time.

The mice which were selected for early sacrifice were the ones not expected to survive the next 24 hours. Therefore mice which survived longest were either the ones most resistant to the infection or those mice which did not receive a full inoculum. Edwards *et al.* (1975) noted flat chorioretinal scars in the eyes of rabbits caused by spontaneously healing lesions 52 days after infection.

In the present study the presence and severity of ocular lesions correlated with the presence of lesions in other organs. Histological examination of other organs showed development of lesions in the animals up to six days post challenge. The brain was heavily involved but the quantity of the brain lesions did not correlate with the other organs. This may have been a factor attributable

to the small sample of mice subjected to detailed histological examination.

C. albicans endophthalmitis in the heroin addicts appeared to have an asymmetric distribution with the left eye being more frequently involved than the right. In the present mouse model there was no predominant involvement of one eye. However, some asymmetrical distribution of the fungus between the eyes was noted. Three groups of mice had their eyes removed after sacrifice, the eyes were cultured and the colony counts per eye were ascertained. Each of the three groups of animals analysed showed overall significant differences in the distribution of the yeast between the two eyes at the 5% level. In the third group, the mice sacrificed at five minutes, one hour and six hours showed a significant difference in the number of yeasts between the eyes of the mice. However, when group two was examined for asymmetrical distribution of fungus between the eyes at each time interval, there was no significant difference in the number of yeasts per eye in the mice sacrificed at three and five days post challenge.

When the results of the three groups were collated there was a significant difference for the first six hours post challenge. From 24 hours onwards there was no significant difference in the number of yeasts cultured from each eye of the mice. When the results were analysed overall there was a significant difference in the number of yeasts cultured from each of the two eyes of the mice,

indicating an asymmetric distribution of the *Candida*. This peculiar phenomenon is difficult to interpret since there was a large range in the number of yeasts cultured from both eyes of different mice sacrificed at the same time post infection. The change in the significance of the yeast distribution, may be a reflection of the course of the disease. There may be an uneven distribution of the yeasts in the eyes or there may be an uneven clearing of the yeasts from the eyes. The original focus of infection may be unilateral and may act as a reservoir of the fungus which could then spread to the other eye.

The infection appears to take 24 hours to become established and for the yeasts to multiply. After it becomes established and forms a focus of infection the lesion may shed yeasts into the blood stream and these yeasts, if not destroyed by the mouse's phagocytes, may initiate a futher focus of infection elsewhere including the other eye. However, the infected lesions in the eyes appear to be discrete and contained by inflammatory cells. Moreover, if re-infection occurs, spontaneous resolution would be more difficult to explain, unless the yeast disseminates within the first 24 hours and before the host defences have succeeded in containing the infection. After 24 hours the mice may be able to contain the fungus by surrounding the original focus with inflammatory cells thus preventing reinoculation of the blood stream. The immune system may then overcome the infection.

The theory that yeasts are present in the blood and eyes of the animals is borne out by the culture results of the eyes, regardless of how long post challenge minimal growth of *C. albicans* was found. Blood cultures in animals with haematogenous *C. albicans* endophthalmitis have been consistently negative (Edwards, 1985).

The presence of *Candida* antigenaemia in the mouse model was investigated to study the kinetics of antigen release following systemic challenge with *C. albicans*. Two kits were compared in the tests but the Immy kit produced 60% false positive results. The Mercia Diagnostics kit showed that no mannan was detectable until 24 hours post challenge. The fact that the kit did not detect the presence of mannan at 5 minutes post infection when there was a large number of yeasts in the mouse's body may be explained by the theory that the yeast cells may have to be phagocytosed before the mannan is released (Taschdjian *et al.*, 1971) and that this method of detection is most useful in diagnosing acute infection. If the patient has no immune function and the yeasts are not damaged sufficiently to release mannan then the kit will not detect the presence of the yeast. Detectable mannan persisted in the mice until the end of the experimental period of 72 hours. This indicated a pocket of infection releasing mannan into the blood stream. Mannan has been shown to be cleared from the blood rapidly, the plasma

half life of *Candida* mannan is two hours (Kappe and Muller, personal communication).

There requires to be a level of 25 μg /ml of mannan before it is detectable by the Mercia Diagnostics kit as used in this laboratory.

For future work it may be informative to look for the presence of mannan in the murine eyes and relate this to the presence of mannan in the sera. The volume of the vitreous of a mouse is too small to extract and test. However, the eye could be homogenised in a small volume of distilled water before testing. Preliminary results have demonstrated that after five minutes in water, viable *C. albicans* at a concentration of 1 x 10^3/ml, releases mannan, at levels which could be detected by the Mercia Diagnostics kit.

The protocol used in this study produced a reliable model for *Candida* endophthalmitis. Ocular changes were seen by fundus examination and study of the histological sections revealed the development of lesions containing yeasts and inflammatory cells within the retina. The number and severity of eye lesions correlated with lesions seen in other organs of the mice and reflected the progress of the infection as determined by culture of mouse eyes. This mouse model for *Candida* endophthalmitis has great potential for the study of the treatment of the disease by antifungal drugs and the assessment of their retinal toxicity.

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