DISSEMINATED CANDIDA INFECTION IN MAN,

WITH PARTICULAR REFERENCE TO THE DISEASE AS A COMPLICATION OF STEROID AND IMMUNOSUPPRESSIVE THERAPY

by

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INTRODUCTION

The investigations described in this thesis were undertaken in an attempt to throw some light on to the problem of "opportunistic" infection in man; that is, infection caused by organisms which are normally not pathogenic, but which cause disease and even death when the normal defence mechanisms are undermined by other disease processes. Since many of the organisms which cause such infections are ubiquitous in nature and man is frequently exposed to them, and presumably has been so for much of his evolutionary development, and since infection of otherwise healthy persons is exceptionally rare, it follows that these normal defence mechanisms must be highly developed and extremely efficient. Conversely, disease processes which predispose to such infections, assumedly do so by virtue of profound impairment of the normal resistance.

Fungal infections are of special importance in this context. Candida infection was studied in this thesis for several reasons. It is probably the commonest cause of opportunistic fungal infection in man. In the patient with undermined immunity it may produce severe disease and the result may be fatal. It was felt that principles applying to Candida infection were probably applicable to various other fungal infections as well. The organism is easily identified, and it is easily handled in the laboratory. It produces characteristic disease in the laboratory mouse, and the natural history of the experimental infection in mice fairly closely resembles that of the infection in man. Koch's postulates are easily applicable.

In this study the history of Candida infection is reviewed and it is shown how it has been recognized for a long time that Candida infection may be a "disease of the diseased". It is shown from a study of the literature that disseminated Candida infection has become increasingly common since the introduction of broad spectrum antibiotic therapy,

immunosuppressive and cytotoxic drug regimes, and treatment with adrenal cortical hormones. In latter years it has become a very real hazard of transplantation surgery.

Little is known of the ways in which these different pharmacological agents predispose to such infection, but with accelerated interest in immunology and with advances in immunological techniques in recent years, an investigation along these lines suggested itself. Both cellular and humoral aspects of normal immunity to Candida were studied and the effects of various steroid and immunosuppressive drug regimes on the normal immune mechanisms were observed. Although classification of immunity to infectious organisms into "cellular" and "humoral" is somewhat rigid and possibly a little artificial, in that it is likely that both are closely interlinked and interdependent in their protection of the host, this classification represents the limits of knowledge at the present time and it offers convenient immunological parameters for study in respect of this problem (Soothill, 1968).

Cellular, or cell-mediated, immunity was studied by examining the phenomenon of lymphocyte transformation in response to antigenic stimulation by Candida. From a comparison of a fairly large series of normal persons with patients receiving variously long-term steroid therapy, combined prednisone and azathioprine therapy, and cyclophosphamide therapy, it was shown that there is significant impairment of cellular immunity to Candida with each of these drug regimes. On the other hand humoral immunity, as reflected by both agglutinating antibodies and by quantitative fluorescent antibody estimations, did not appear to be so impaired.

An attempt was made to follow up recent work which has suggested that the normal serum exerts anti-candidal or candidacidal activity. Although the experimental systems described by the previous workers could

be reproduced and their results confirmed, it was felt that the assay systems were largely artificial and that inference from these results was necessarily strictly limited.

Experimental Candida infection was produced in mice, and it was shown that immunosuppressive and steroid drug regimes significantly modified the natural history of the infection by virtue of the anti-inflammatory properties of these drugs.

Finally, the relevance of these findings in the context of the clinical situation, and the possible wider application of these principles to several medical and biological problems are considered.

All the experimental work reported in this thesis, with the exception of the preparation of the Candida antigens, was carried out personally by the author.

The adaptation and standardisation of the technique of lymphocyte transformation testing for the investigation of lymphocyte responses to stimulation by Candida antigens, the study of the lymphocyte response of the normal population to Candida antigens, the investigation of the effect of steroid and immunosuppressive drug therapy on the in vitro phenomenon of lymphocyte transformation, and the effect of these drug regimes on levels of antibodies to Candida in the blood, and on experimental candidiasis in mice are put forward as original contributions to medical knowledge.

CONTENTS

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CHAPTER 1 : REVIEW OF THE LITERATURE

"He hath a fever, a thrush and a hickup, all three together, which are it seems very bad symptoms."

Pepys's diary. 17th June, 1665.

The history of Candida infection

Palaeopathologists consider that the first fauna were free from infection, but from the time of the Devonian period (350 to 400 million years ago) there is evidence of bacteriological and fungal infection of prehistoric fish, amphibia and insects. Infectious disease seems to have increased progressively in subsequent eras. Pathological reactions to disease, on the other hand, are thought to have remained unchanged since the time the vertebrates appeared on Earth (Jannsens, 1970).

Following the invention of the light microscope in 1590, Robert Hooke published his book "Micrographia" in 1677, in which is to be found the first description of the microscopic fungi. Yeasts were first observed microscopically by Leeuwenhoek in 1680. Linnaeus in 1752 collected all that was known about the fungi, and at about the same time John Hill (1751) used the name of Monilia to describe a genus of fungi isolated from vegetable sources. This organism bears no resemblance to the yeast-like fungi, nor to the causative organism of thrush (Benham, 1931). In 1839 Langenbeck demonstrated the fungus responsible for the lesion of thrush, and from that time advances in knowledge of medical mycology largely went hand in hand with the development of concepts of medical microbiology in general.

The use of the word "thrush" seems to date from the 17th Century. Its derivation is obscure, but it is probably of Anglo-Saxon or Scandinavian origin (Swedish "torsk"; Danish "troske"). (A New English Dictionary on Historical Principles, 1919).

The disease, however, was already a well known malady. For centuries it had been recognised that white patches and ulcers might appear in the mouth in the course of severe and debilitating diseases. Hippocrates (Epidemics) described two such cases, one occurring in the course of fatal erysipelas and the other in what appears to have been an epidemic of severe enteric fever. A chapter on the aphthae or thrush appeared in a Swedish textbook of paediatrics in 1771 (Rosen von Rosenstein, 1771) which makes it seem certain that the condition was already well recognised. Von Rosenstein, who was First Physician to His Swedish Majesty and Knight of the Polar Star, described oral thrush thus:

> "When a child gets pimples or bladders in its mouth which soon grow ulcerated or with crusts on them, it has the disease we call thrush."

Even at this time it appears that it was recognised that oral lesions might be followed by involvement of the intestines and the bronchi and lungs, and that the infection was potentially lethal. It was at this stage in the knowledge of the condition that Langenbeck made his discovery of the causative organism in 1839.

Only gradually was it realised that thrush infection was most likely to produce disease in a patient whose resistance was already undermined by another disease process. In 1844 Bennett in Edinburgh found a yeast-like fungus growing in the sputum and subsequently, at post mortem examination, in the lungs of a man who had died from tuberculosis and a pneumothorax. It is quite possible that this was the causative organism of thrush. His illustrations were of a dimorphic fungus (Fig. 1). His conclusions regarding these organisms were remarkably modern; that such vegetations always arose in living animals previously diseased; that their presence was indicative of great depression of the vital powers and impairment of the nutritive functions of the economy; and that the

cachexia favourable to their growth was the tubercular or scrofular (Bennett, 1844). In 1853 Charles Robin had written:

> "du reste les nombreuses observations des muguets developpent chez enfants bien portants mais ordinairement mal nouris; sur des adultes à la dernière période de maladie de longue durée."

So striking was this relationship that Parrot, writing in 1877 on the illnesses of the newly born, stated categorically:

> "le muguet est toujours la conséquence d'un état morbide antérieure et ne constitue pas une maladie."

Trousseau (1869) had largely expressed the same opinion. In the first report of systemic spread of thrush (Zenker, 1862), in which both buccal mucosal lesions and cerebral infection were described, it was emphasised that host disturbance, in this case "debility", was characteristic of those who suffer from thrush.

Subsequent reports up until the early 1940's served to draw attention to the occasional dissemination of the organism in man, and its frequent company in association with other infections such as typhoid fever, tuberculosis and streptococcal septicaemia. Before 1940 there were no authenticated reports of systemic yeast infection occurring in otherwise healthy persons (Winner and Hurley, 1964).

Disseminated Candida infection: Modern concepts

The "medical monilias" were given the name of "Candida" by Berkhout in 1923, and the generic name Candida was officially adopted by the Eighth Botanical Congress at Paris in 1954. This designation has been accepted as correct medical terminology (Medical Research Council Memorandum, 1958).

Biological aspects of the Candida organism, such as its morphology, cytology, variation, physical characteristics, metabolism, cultural

characteristics, ecology and habitat have been thoroughly described by Winner and Hurley (1964). The organism has been found occurring both as commensal and as pathogen in human beings all over the world. It has also been found to be the cause of spontaneous disease in several animal species. Candida has been isolated from the soil, and from vegetable sources by a few workers, but the usual cycle of infection appears to be from animal to animal, or from man to man. There does not seem to be any evidence that Candida normally multiplies and maintains itself in a non-animal habitat.

The criteria for the diagnosis of disseminated candidiasis are: either the clinical picture of septicaemia, combined with the culture of a member of the genus Candida from the blood stream during life, or, culture or identification from post mortem specimens combined with morbid anatomical evidence of mycotic disease (Winner and Hurley, 1964).

The clinical and pathological features of disseminated Candida infection have been reviewed by several authors in recent years (Braude and Rock, 1959; Andriole, Kravetz, Roberts and Utz, 1962; Louria, Stiff and Bennett, 1962; Hurley, 1964; Symmers, 1964a; Winner and Hurley, 1964; Felten, Klintworth and Hendry, 1967; Louria, 1967).

The causative organism is generally Candida albicans (C. albicans), but occasionally C. parapsilosis, C. guilliermondii, C. krusei and C. tropicalis may be responsible (Andriole et al., 1962; Winner and Hurley, 1964). All of the Candida species produce similar clinical and pathological features.

The characteristic clinical features are fever (almost invariably the initial symptom), oral Candida infection, constitutional disturbance with malaise and anorexia, diminished response to external stimuli and a depressed sensorium. Other features depend on the site of the metastasis of the fungus, and on the primary pathology. Braude and Rock

(1959) stressed the feature of intestinal bleeding, but this has not been confirmed in subsequent reports. Splenomegaly, petechiae, embolic phenomena, tachycardia, tachypnoea and lethargy are common physical findings (Louria et al., 1962). The patient is usually anaemic. The white cell count is variable (Andriole et al., 1962). Males and females are equally affected.

The portal of entry of the organism may be from the mouth (occasionally histological examination of oral Candida lesions has shown penetration of sub-mucosal blood vessels), from ulcerative lesions of the intestine, particularly the large intestine, or from intravenous introduction of the organism (Symmers, 1964a). In a review of twenty-nine cases of disseminated candidiasis, twenty-three had had indwelling venous catheters or constant intravenous infusions (Louria et al., 1962). Less commonly, the organism may be introduced directly at cardiac surgery, or by direct intravenous administration by narcotic addicts (Louria et al., 1962). Lehner (1964) described four cases of fatal systemic candidosis following dental extraction.

The pathological picture of septicaemic candidiasis is of an acute, severe, widespread mycotic infection. The kidney is the organ most frequently involved (75% of cases); the heart is affected in about 50% of cases, and the gastrointestinal tract and brain are found to be affected in about 33% of cases. Less frequently other organs, e.g. liver, lungs, blood vessels, spleen and thyroid gland are diseased. Occasionally at post mortem there is evidence of infection of organs such as bone marrow, retina, muscles and bladder (Hurley, 1964).

In human infection and in infection of laboratory animals the kidney appears to be the target organ in disseminated candidiasis. The organisms appear to be trapped in the renal capillaries, and to cross the

blood vessel wall (the mechanism of this remains unexplained) to reach the interstitial tissues of the renal cortex, and to a lesser extent of the medulla, where they elicit an interstitial inflammatory response. The organisms penetrate to the renal tubular lumen in both cortex and medulla, whence they may either re-rupture into the interstitial tissue, or move down to the collecting ducts where, often in their hyphal forms, they may produce obstruction, hydronephrosis and secondary infection. Knowledge of this sequence is obtained from serial histological analysis in laboratory animals. Proximal and distal ureteral obstruction, and obstruction of the bladder by hyphal masses have been observed. The overall effect of these morbid anatomical changes is of progressive loss of renal function (Louria, 1967). (Fig. 2.)

Andriole et al. (1962) and Louria (1967) have described the features of Candida endocarditis. In general, it mimics subacute bacterial endocarditis, but with the difference that the vegetations are frequently a large size and may be several centimetres in diameter. These vegetations are friable, so that emboli to the major arteries of the extremities, the brain, the kidneys and the skin are common. Histologically both yeast and hyphal forms may be seen and often there is pathological evidence of focal or diffuse myocardial involvement. Candida pericarditis has been described in association with the endocardial and myocardial lesion. More than 50% of patients give a history of previous rheumatic heart disease. Fever is invariably present, and a significant cardiac murmur is usually heard. Splenomegaly and anaemia are generally associated.

Haematogenous dissemination to the brain and meninges may be characterised by signs of meningitis, with cerebrospinal fluid changes, by cerebral abscess or granuloma formation, or by invasion of the walls of blood vessels with thrombus formation (Felten et al., 1967).

Morbid anatomical features of intestinal infection are generally

less striking. The organism is often present as a commensal, and mycotic ulcers of the gut may be small and they may be overlooked at post mortem examination unless carefully sought (Winner and Hurley, 1964).

The prognosis of systemic infection in the adult is poor, and with the rare exception the disease is lethal. There were very few reported cases of recovery before the introduction of Amphotericin B. The outlook in childhood infection is somewhat better, and recovery may even take place in the absence of specific anti-fungal treatment (Winner and Hurley, 1964).

Candida infection - a "disease of the diseased"

Metchnikoff (1893), who was both pathologist and zoologist, and who was embued with the recently developed evolutionary concepts of Darwin and the microbiological concepts of Pasteur, studied the comparative pathology of infectious disease and inflammation. He showed that all organisms, however simple, have infectious diseases produced by different classes of parasite. He recognised that the most characteristic features of the organisation of an animal or of a plant are the organs of attack and defence. In a classical study, he produced experimental infection of an amoeba by the organism, Microsphaera. Under normal environmental conditions the amoeba was perfectly able to resist infection, so that no normal function of the amoeba was adversely affected, and there was no evidence of a diseased state. However, by altering the environment or the physiological conditions of the amoeba unfavourably, e.g. temperature, electrolyte, glucose or pH change of the surrounding medium, the Microsphaera was able to undergo continued division within the amoeba, which would become less and less active, "showing that it is not in a healthy condition". This experimental system possibly demonstrates the most primitive example of infection of one organism by another organism. The

infecting agent, which to all appearances is insignificant, had yet the power of resisting the digestive influence of the amoeba and, when circumstances became unfavourable to the larger organism, of bringing about its death (Fig. 3).

Metchnikoff's conclusions were that "we are justified in assuming generally that the relations between the protozoa and the microorganisms which infect them are to be regarded in the light of a struggle between two living species. These phenomena do not come under the heading of the struggle for existence in the strictly Darwinian sense (i.e. competition for the survival of the fittest among individuals of the same species), yet they are all more or less directly connected with the struggle for survival that is always going on between the representatives of the different orders of living beings."

Macfarlane Burnet (Burnet, 1962) has expanded further on those aspects of biological science that deal with the interaction of organisms with other organisms, and with the modification of these relationships by environmental factors. In the ecology of a species he sees a constant balance between the "population pressure" of the species, i.e. the constant production of more young than can hope to survive, and limiting factors set by nature, which are chiefly the available food supply and the activity of enemies (predators or parasites). It follows that the reproductive potentialities of most species are so great that it needs only a surprisingly short time for a vast increase in numbers to occur following some change in the environment unusually favourable to the species concerned. In the case of parasitic infestation there is thus a "climax state", i.e. the development of an approximately balanced condition between contending species which, in a constant environment, would tend to result in a virtual equilibrium in which both species would survive indefinitely. Man, however, lives in an environment which is

invariably being changed by his own activities, and which is liable to be changed by disease processes and by pharmacological agents, so that few of his infections have attained such an equilibrium.

It is that characteristic of organisms, established in a "climax state" with man, whereby they adapt themselves to make profitable use of the circumstances of the moment and consequently cause morbidity of the host, that has earned the description of "opportunism". The word was adopted from Italian politics, and probably brought into the English language by Lord Granville, who defined its meaning as "adapting yourself to those circumstances which are most fitted to getting you into power and to maintaining you there" (Granville, 1882).

There is very good evidence from the medical literature that man in many instances lives in such a "climax state" in association with organisms of the genus Candida. Candida has been isolated from human beings in all parts of the world, and it has been found to occur in birds, domestic fowls, dogs, cats, pigs, sheep, monkeys and many other animals (Winner and Hurley, 1964). It has been isolated from the soil by several workers, from fresh water lakes and from purely vegetable sources (Winner and Hurley, 1964). The organism establishes a parasitic relationship with man without producing any evidence of clinical disease in 15 to 40 per cent of the adult population, according to various observers. Barlow and Chattaway (1969) showed in a study of 155 persons that 35% of them were carriers of Candida, i.e. harbouring the organism without suffering any adverse effects whatsoever; there was no sex difference in the carriers; the sites of isolation of the organism were the mouth, the anus, the vagina and occasionally the skin. The majority of the carriers harboured the organism at one site only, but a small number harboured it at two or three sites.

Thus it appears that Candida organisms are fairly ubiquitous in nature, that they are vigorous organisms with a propensity for establishing

a parasitic relationship with man and various animals as host, and that although the hosts may become re-infected from vegetable sources, there can be little doubt that the more usual cycle of infection is from animal to animal, or from man to man.

This carriage of Candida by man is essentially local, and spontaneous dissemination of the organism in an otherwise normal person is exceptionally rare (Zimmerman, 1955; Chick, 1962; Winner and Hurley, 1964). The inference is that Candida, with its modest requirements for nutrients, is perfectly able to remain viable within the lumen of the gastrointestinal tract of man, and that man has become absolutely resistant under normal conditions to penetration and dissemination of the organism. It is conceivable that man has been exposed to Candida for much of his evolutionary scale, and that he has developed an impenetrable defence to the organism.

Dubos (1955) comments: "A physiological disturbance of sufficient magnitude in the host can result in the destruction of the equilibrium between host and parasite, converting latent infection into overt disease." Candidiasis is a disease which ranges from a mild superficial infection to a malignant fulminating deep-seated mycosis with an extremely poor prognosis. The transition from the carrier to the disease state in man follows with almost unerring constancy a change in the clinical state from the normal. While oral and cutaneous thrush are common conditions, deep mycoses caused by Candida are rare. It appears probable that a physiological disturbance not only destroys the equilibrium between the host and the parasite of thrush, but also that the magnitude of the disturbance largely determines the type of candidiasis which will ensue. Fatal candidiasis is a complication of serious diseases. It does not occur in those who are subject to minor ailments, although they may suffer from local thrush.

It is virtually impossible to reproduce local thrush in the mouths of the healthy, with the possible exception of the newborn. If the buccal mucous membrane is damaged, the disease becomes reproducible (Cawson, 1969). Similarly, skin candidiasis can only be reproduced experimentally if the skin is macerated before the introduction of the fungus (Maibach and Kligman, 1962). Local damage is clearly a predisposing factor in the causation of local disease. However, it is not the only predisposing factor, as is apparent from the large number of reported cases of candidiasis, both local and general, which have occurred in diverse morbid conditions. The only feature common to the pathology underlying and preceding the appearances of thrush which can be discerned is a deviation from the normal, a state of disease and derangement of physiology.

Winner and Hurley (1964) have categorised those abnormal states in which the likelihood of thrush as a complication is well recognised, as follows:

1. Physiological: pregnancy, infancy.
2. Local trauma, maceration, allergy of the skin.
3. Disorders of the endocrine system: diabetes mellitus, hypoparathyroidism, Addison's disease, pancreatitis, hypothyroidism.
4. Malnutrition.
5. Malabsorption syndrome.
6. Antibiotic and steroid therapy.
7. Blood dyscrasias, in particular acute leukaemia, agranulocytosis and aplastic anaemia.
8. Post-operative states.
9. Malignant disease.

The conditions that determine the critical transformation of a localised mucosal candidiasis into progressive septicaemia and dissemination

of the organism are still largely unknown (Symmers, 1966). Zimmerman (1955) has outlined the factors which predispose to disseminated Candida infection:

a) a general state of increased susceptibility, e.g. lymphoma, leukaemia, leukopenia, debilitating disease;

b) a local lesion which serves as a portal of entry, e.g. in the respiratory or gastrointestinal tracts. This may be a mucosal lesion induced by X-rays, avitaminosis, etc., or a pre-existing disease such as tuberculosis, bronchiectasis or peptic ulceration; and

c) an ecological disturbance brought about by various combinations of antibiotics, hormones, etc. Zimmerman indicated that in the majority of cases all three factors are operative.

Parenteral entry of the organism to the body as a result of prolonged intravenous therapy, indwelling venous catheterisation, cardiac surgery, by-pass surgery, direct intravenous administration by narcotic addicts, severe local trauma to the skin by a burn or gunshot wound and surgical procedures such as tooth extraction, abdominal and genitourinary operations has come to be recognised in recent years as being of importance (Louria, Stiff and Bennett, 1962; Andriole et al., 1962; Dennis, Peterson and Fletcher, 1968; Lehner, 1964; Winner and Hurley, 1964).

The role of pharmacological agents in the pathogenesis of Candida infection

The weight of epidemiological evidence is considerable that the morbidity and mortality of local and disseminated Candida infection has increased significantly over the last twenty-five years, and that this increase has been associated with antibiotic, steroid and cytotoxic drug

therapy. Although greater awareness of the problems, and improved mycological laboratory techniques account to some extent for this increased incidence of Candida infections, and drug therapy has modified the natural history of various diseases so that survival is longer and the primary disease process is attenuated, thus rendering the patient more susceptible to fungal infection which in the past might not have had time to evolve, these factors cannot wholly account for this increased incidence, and a more direct predisposing effect of the various drug regimes is implicated (Symmers, 1964a). The incidence of primary fungal infections, e.g. histoplasmosis, actinomycosis, etc., on the other hand, has not changed since 1947 (Keye and Magee, 1956). There appears to be heightened danger with drug combinations, and a greater risk with higher dosage and more prolonged treatment (Symmers, 1964). Hutter and Collins (1962), in an analysis of the occurrence of opportunistic fungus infections in a cancer hospital over a twelve year period, showed that over the years 1948-1951, when antifolic agents, steroids and antibiotics such as penicillin and streptomycin were available for the treatment of malignant disease, there were seven cases of "opportunistic" fungal infection, whereas over the period 1952-1960, with a broader spectrum of antibiotic, antimitotic and hormonal agents, there were 194 such cases. There had been no significant change in the numbers of hospital patients in the two periods studied. An important and peculiar problem of the "miracle drug era" had begun to show itself. In another study, in a search for evidence of mycotic infection at 1,000 unselected autopsies, sixty-six instances were found. Ninety-four per cent of these cases had received treatment with antibiotics and corticosteroids, together or separately. Fifty-four of these sixty-six cases were due to Candida (González-Mendoza and Aguirre-Garcia, 1967).

The asymptomatic carrier rate, and a quantitative increase in the

number of Candida organisms in the mouth and gastrointestinal tract, without the production of clinical disease, has been shown to follow antibiotic and steroid therapy, even over a short period of time (McGovern, Parrott, Emmons, Ross, Burke and Rice, 1953; Sharp, 1954; Barlow and Chattaway, 1969). Cawson (1969) has shown that acute Candida stomatitis may develop within forty-eight hours of the commencement of oral or parenteral therapy with broad spectrum antibiotics. The use of antibiotics and of steroids has been shown to be associated with a higher incidence of Candida isolations from the rectum (Minkin and Smith, 1969). Vaginal candidiasis may complicate broad spectrum antibiotic therapy (Pappenfort and Schnell, 1951). Louria, Stiff and Bennett (1962) and Winner and Hurley (1964) have stressed the remarkable frequency with which patients with systemic Candida infection have received antecedent multiple antibiotic and/or steroid therapy. Of the thirty cases of systemic infection reviewed by Winner and Hurley, twenty-nine had received multiple antibiotics.

A recent problem, and one of increasing importance, is that of Candida infection developing in patients who have undergone renal transplantation. Utz (1970) has pointed out some of the possible combinations of factors which seem to be associated with such infection, viz. uraemia, azathioprine therapy, leukopenia, prednisone, drug-induced diabetes mellitus, anti-lymphocyte serum therapy, hypogammaglobulinaemia, x-irradiation, repeated surgical procedures, indwelling urinary catheter, Scribner shunt, haemodialysis, and antibiotic regimes. Starzl (1964) has stated that of thirty-five patients who developed severe infections following renal transplantation (thirty-three of these thirty-five patients died), the infection developed in thirty-two of the cases at the time of intensification of azathioprine, prednisone and actinomycin therapy for threatened rejection crisis. Rifkind, Marchioro, Schreck

and Hill (1967) analysed their findings in the follow-up of 111 patients following renal transplantation over a five year period. Fifty-five of these patients (50%) had died. Of these, fifty-one post mortem studies were made, and twelve were found to have disseminated Candida infection. Although it is difficult in the transplantation situation to assess the role of any particular factor in the pathogenesis of infection, these authors concluded that there can be little doubt about the association of immunosuppressive therapy and systemic fungal infection.

The effect of drugs on experimental Candida infection in laboratory animals has been studied by several workers. The natural history of local and systemic lesions, and survival times have been variously investigated. O'Grady, Cotton and Thompson (1964) produced a local Candida lesion in the mouse thigh, and this model was used by Thompson (1966) to demonstrate that cortisone therapy depressed markedly the normal local inflammatory response to the organism. Louria, Fallon and Browne (1960) showed a marked increase in mortality in cortisone-treated mice infected intravenously with Candida. This mortality, which was dose-dependent, was attributed to progression of infection which was largely confined to the kidneys, liver and spleen. Aureomycin and Terramycin have an enhancing effect on mouse-peritoneum candidiasis (Sheldon and Bauer, 1962). A similar effect has been noted with roentgen radiation (Roth, Friedman and Syverton, 1957), and the effect of combined cortisone therapy and roentgen radiation was greater than additive. Both Louria et al. (1960) and O'Grady (1966) considered that the natural history of the Candida experimental lesion in mice closely resembled that of the infection in man, and these experimental findings may contribute to an understanding of some cases of disseminated candidiasis in man.

The mechanism whereby drugs increase the incidence, pathogenicity and morbidity of Candida infections

Substantial clinical evidence has been accumulated to show that the large majority of Candida infections consequent on drug therapy are of endogenous origin (Kligman, 1952; McGovern et al., 1953; Zimmerman, 1955; Rifkind, Marchioro, Waddell and Starzl, 1964; Starzl, 1964).

As Symmers (1965) has stated, for an infection to develop there has to be contact with the organism, a portal of entry, a sufficient number of organisms or sufficient proliferation, and the organism must be able to overcome the patient's resistance. Hence, drugs which serve to change the "climax state of equilibrium" between man and Candida generally do so either by enabling the organism to proliferate, or by lowering the patient's capacity to resist the establishment of infection.

The mechanisms by which antibiotics increase the incidence and severity of candidiasis have been reviewed by Seelig (1966). Broad spectrum antibiotic therapy nearly doubles the carrier rate of Candida (Barlow and Chattaway, 1969), even when given for a short period of time, and the number of Candida organisms which can be isolated from the intestinal tract is quantitatively increased (Tomaszewski, 1951; Johnston, Chick, Johnston and Jarvis, 1967). Aureomycin, Neomycin and chlortetracycline have been shown to have a direct stimulatory effect on the growth of Candida in vitro (Huppert and Cozin, 1955), but it is not thought that superinfection is due to a direct stimulatory effect by antibiotics. pH changes in the gut lumen induced by antibiotics have been incriminated, but this theory is not widely accepted because of the considerable range of pH at which Candida can readily grow. By the elimination of competitive organisms and the creation of a "bacterial vacuum", it is argued, antibiotics make more nutrition available to the

fungi, themselves resistant to the antibiotic. It is unlikely that this factor is of major importance as the change in gut flora is often qualitative rather than quantitative. In addition, it is quite clear from in vitro observations that Candida requires only a minimum of growth factors for survival and even proliferation. It is possible that with the death of a large number of gram-negative bacilli there is release of endotoxin which enhances growth of Candida, or alternatively there is inhibition of a normal direct antifungal effect of E. coli (Isenburg, Pisano, Carito and Berkman, 1960).

It is fairly well established that the mycelial form of the organism is required for dissemination and penetration (Seelig, 1966). Penicillin, streptomycin and chloramphenicol have been shown in vitro to induce hyphal formation, proliferation and penetration of the wall of the bowel (Beemer, Pryce and Riddell, 1954). The possibility that with the elimination of the normal gut flora there is decreased vitamin production and as a result a loss of the integrity of the mucosal wall has not been substantiated, as invasive candidiasis has not been prevented by supplemental vitamin therapy (Cawson, 1969).

Although antibiotics have been shown to modify immunological responses to Candida such as the production of agglutinating antibodies in response to infection (Winner, 1955), alpha- and gammaglobulin production in goats infected with Candida (Seelig, 1966), and the phagocytic rates of mononuclear cells (Donomae and Kawamori, 1955), it is generally considered that the role of antibiotics in predisposing to both local and systemic Candida infection is largely at the level of the gastrointestinal tract.

Steroid therapy, cytotoxic therapy and immunosuppressive drug regimes on the other hand appear to predispose to Candida infections mainly through their inhibitory effects on immune mechanisms. The infection-

enhancing quality of corticosteroids to fungal disease generally parallels the anti-inflammatory and glycogenic activities of the compounds (Frenkel, 1962). Although adrenocortical hormones affect adversely both humoral and cellular aspects of immunity to infections in general (Beisel and Rapoport, 1969), it is thought that it is the effect of steroids on lymphoid and reticulo-endothelial components of immunity ("cellular immunity") that is of critical importance (Frenkel, 1962).

The mechanisms whereby cytotoxic and immunosuppressive agents predispose to fungal infections are but vaguely documented. Attention has been drawn to this hiatus in knowledge by several authors (Chick, 1962; Frenkel, 1962; New England Journal of Medicine, 1964; Starzl, 1964; Symmers, 1964a; Tripathy and Mackaness, 1969; Pepys, 1970; Utz, 1970).

The immunological aspects of candidiasis

Tran van Ky, Biguet and Andrieu (1963) showed by immunoelectrophoretic investigations that no fewer than nine antigens can be isolated from Candida albicans. Faux (1968) showed that immunological responses can be elicited in man to antigens from three discrete parts of the organism, i.e. cell wall antigens, somatic antigens and culture filtrate antigens.

The cell wall of Candida albicans is thought to be about 150 mμ in thickness, and to consist of a relatively electron-dense outer layer, about 40 mμ wide, and containing complexes of both mannan and protein and glucomannan and protein. The inner layer of the cell wall is less electron-dense but greater in diameter, and it is thought to consist mainly of a glucan-protein complex (Dobias, 1964). The electron-microscopic features of the cell wall of Candida albicans are shown in Fig. 4. The specific circulating antibodies to Candida are thought to be directed mainly against the cell wall.

The somatic antigens and the culture filtrate antigens are protein,

but their exact chemical nature is not known. Antibodies are thought to be produced in vivo to the somatic antigens only in the event of deep-seated or disseminated infection, presumably due to lysis of the yeast cell in such conditions by host defence mechanisms, with release of antigen in sufficient amount to stimulate antibody formation. (Faux, 1968).

Lehner (1970) has shown that serum antibodies produced in response to Candida infection are of all three of the main immunoglobulin classes, viz. Ig G, Ig M and Ig A.

Various immunological techniques have been used in order to demonstrate serum antibodies to Candida albicans, of which agglutination (Pepys and Longbottom, 1967), the indirect fluorescent antibody technique (Lehner, 1966) and precipitation techniques (Stallybrass, 1964) are the most widely used and the best understood.

The relationship of serum agglutinins to Candida infection was largely elucidated by Comaish, Gibson and Green (1963). In their investigation of eighty-one patients they considered that there was a clear relationship between the level of agglutinins in the serum and clinical infection. Using a quantitating technique, their conclusions were that a titre of 1:8 or higher indicates the presence of Candida albicans somewhere, and a level of 1:16 or higher indicates active Candida infection. Eighteen patients, studied by these workers, with titres of 1:16 or higher, all had evidence of Candida infection. However, the converse was not true and there were several cases of clear-cut Candida infection and an agglutination titre less than 1:8. Thus, their opinion was that a high titre seems to indicate Candida infection but a low titre does not necessarily exclude it. Lehner (1970) has shown by Cellulose Ion Exchange chromatography fractionation that agglutinating antibodies to Candida consist predominantly of Ig M, less of Ig G and least of Ig A.

Quantitative estimation of serum antibodies to the cell wall of both yeast and hyphal forms of Candida albicans, made by the indirect fluorescent antibody technique, has shown similar results. Lehner (1966) showed that a titre up to 1:8 was commonly present in control subjects; Candida carriers frequently had a titre up to 1:16 and a titre of more than 1:16 was commonly present in clinical candidiasis, although some patients with infection showed a much lower titre. Lehner (1970) has shown that Ig M, Ig G and Ig A are all capable of producing this immunofluorescent phenomenon. There appears to be some overlap between this antibody system and the agglutinating system, but with the development of clinical infection due to Candida there may be discrepant rises in the two types of antibody (Lehner, 1970). This fact strongly suggests that there are differences in the nature of the two sets of antibodies (Murray, 1970; Lehner, 1970).

A large majority of the healthy population has circulating antibodies to Candida which can be demonstrated by one or other, or both, of these techniques. Drake (1945) showed in a study of 1,150 healthy persons that 89% of them had agglutinating antibodies in their sera. Norris and Rawson (1947) found 64% of 496 normal persons to have serum agglutinins. The possible reasons for this high incidence of agglutinating antibodies in the population are that clinical and subclinical infection with Candida is common, or alternatively in some instances, that infection in the past may have occurred with antigenically related organisms.

Lostia (1960) considered that precipitating antibodies in the serum would be of value in the diagnosis of candidiasis, and this prediction was largely confirmed by Stallybrass (1964), who showed that precipitins were only found in the disseminated form of the disease. Lehner (1970) has shown that precipitating antibodies are only present in the Ig G fraction. Presumably these antibodies are directed against somatic

antigens of the Candida organism. Murray and Buckley (1966) considered that the titre of the antibody probably reflected the degree of the infection.

Whereas humoral antibodies seem to be produced in significant amounts in response to both local and disseminated Candida infection in many instances, there is good circumstantial evidence that it is those aspects of immunity intrinsic to lymphoid cells, i.e. cell-mediated immunity, which are primarily responsible for natural immunity against Candida infection. The evidence for this is well reviewed by Brody and Finch (1960) and by Hermans, Ulrich and Markowitz (1969). The latter authors pointed out how candidiasis tends to be associated with those immune-deficiency states characterised by abnormality of the thymus-dependent modality of the immunological system, and by inability to react to antigenic stimulation with delayed hypersensitivity,[1] and not with states of humoral antibody deficiency with intact cellular immunity.[2] It is quite conceivable that defects in cellular immunity to Candida may differ quantitatively between individuals. However, the precise role of cellular defence mechanisms against Candida in healthy individuals is poorly understood.

In recent years several models have been developed for the analysis of the performance of lymphoid cells when they are confronted with antigen. Skin testing was most frequently used in the past. This depended in essence on the local production of inflammation, i.e. vascular permeability and cellular infiltration consequent on the interaction in the dermis of

1 e.g. dysplasia of the thymus with agammaglobulinaemia (Swiss type of agammaglobulinaemia); dysplasia of the thymus without agammaglobulinaemia (Nezelof-Allibone syndrome); third and fourth branchial pouch syndrome (Di George).

2 e.g. hypogammaglobulinaemia of the Bruton type, and of the acquired type.

antigen with sensitized lymphocytes and macrophages. Quite recently this antigen-sensitized lymphocyte interaction has come to be assessed *in vitro*, either by virtue of its ability to stimulate enlargement, DNA production and mitosis of lymphocytes (the lymphocyte transformation technique), or by virtue of the ability of the stimulated sensitized lymphocytes to produce a soluble factor capable of inhibiting macrophage migration (the macrophage migration inhibition factor).

Skin testing with Candida antigens has been fully discussed by Salvin (1968). His conclusions were that a positive reaction was suggestive of either past or present exposure to the fungus, and that a negative reaction indicated either insufficient contact with the fungus in the past to initiate hypersensitivity, or an excess of the antigen in the tissues so that anergy results, or finally, an inability, through disease, of the host tissues to respond to specific antigenic stimulation. Longbottom, Murray and Pepys (1968), reported that between 80 and 94% of normal adults develop a positive skin reaction to Candida antigen.

In vitro lymphocyte transformation response of the normal population in response to antigenic stimulation with Candida has not been studied. There are, however, several recent reports suggesting that in chronic mucocutaneous candidiasis lymphocyte transformation is not different to that of paired normal controls (Chilgren, Meuwissen, Quie, Good and Hong, 1969; Canales, Middlemas, Louro and South, 1969; Hermans, Ulrich and Markowitz, 1969; Valdimarsson, Riches, Holt and Hobbs, 1970). Valdimarsson et al. (1970) demonstrated in one patient suffering from chronic mucocutaneous candidiasis that *in vitro* challenge of her lymphocytes showed a normal blastoid response to Candida but no detectable production of macrophage migration inhibition factor (M.I.F.). Lehner (1970), on the other hand, noted defective lymphocyte response to Candida in four out of six cases of chronic mucocutaneous candidiasis.

Finally, mention should be made of what has been referred to as the "candidacidal" activity of normal human blood. Anti-candidal serum activity was first described by Roth and Goldstein (1961) and subsequently, using a different experimental system, by Louria and Brayton (1964). Both groups of workers concluded that the serum of most normal adults was capable of specifically inhibiting Candida albicans, and both concluded that the serum factor responsible did not conform to the classical conception of an antibody. Louria and Brayton considered that this activity was located in the alpha and beta globulin fractions of the blood, mainly the alpha globulin fraction. From its physicochemical properties it appeared to be either a small protein or a polypeptide.

This normal anti-candidal activity was considered to be reduced in cirrhosis, diabetes with uraemia, uraemia due to non-diabetic chronic renal disease, acute blood dyscrasias, Hodgkin's disease and multiple myeloma. It was thought likely by these authors that deficiency of this anti-candida factor was critical in the development of local and systemic candidiasis in these disorders.

In a later study, Louria, Shannon, Johnson, Caroline, Okas and Taschdjian (1967) suggested that children with hypofunction of the parathyroid glands and/or adrenal glands, who are known to be particularly susceptible to mucocutaneous candidiasis, lack serum candidacidal activity.

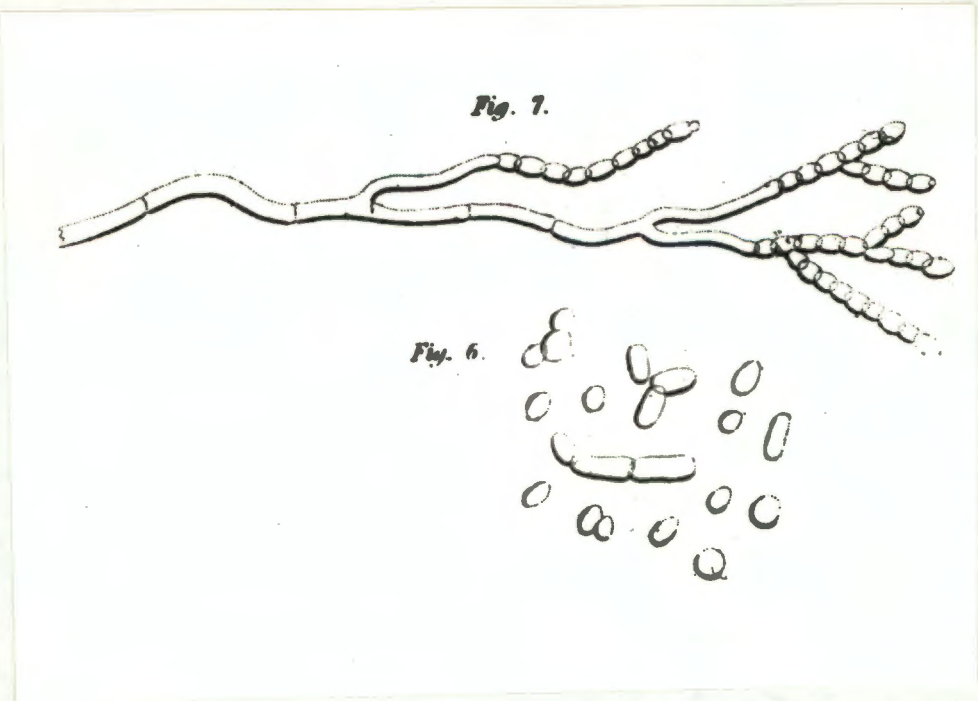

Figure 1: Illustration of dimorphic fungus. (Bennett, 1844.)

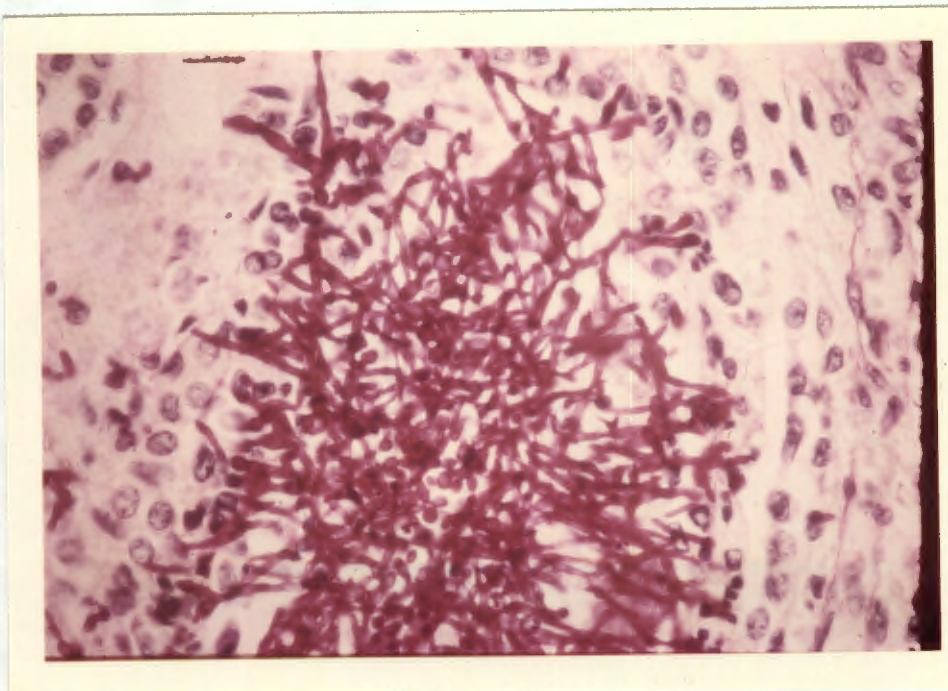

Figure 2: Disseminated candidiasis - renal lesion; in this case complicating Candida endocarditis.
[Histology of kidney of a patient who died at Guy's Hospital, 1969.]

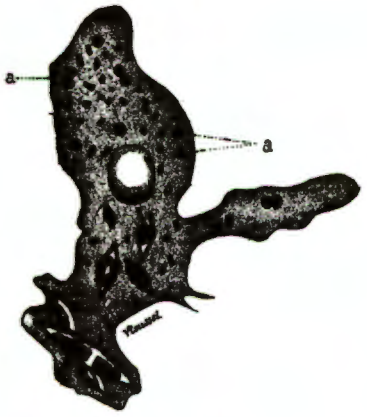

Fig. 6.—Amœba infected by the *Microsphæra*.
a. Early stage.

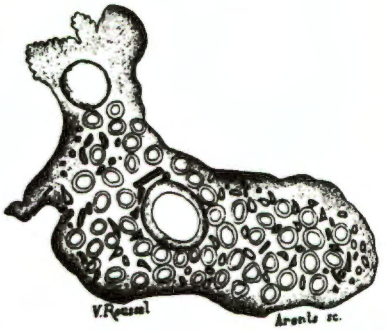

Fig 7.—A dying Amœba, full of parasitic Microsphæræ.

Figure 3: Infection of Amoeba by Microsphoera. (Metchnikoff, 1893.)

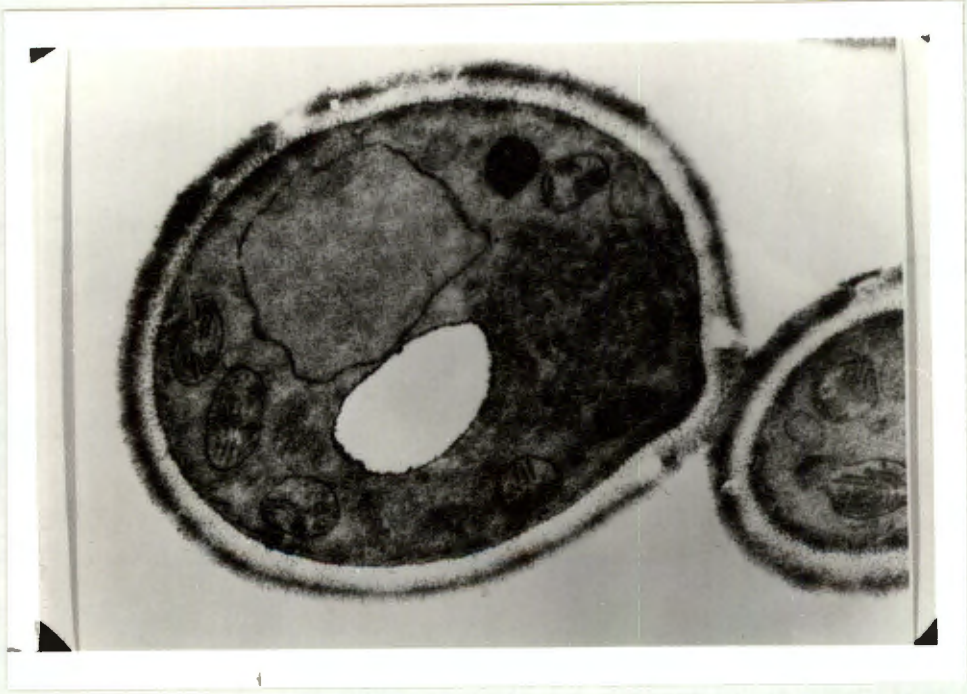

Figure 4: The electron-microscopic features of the cell-wall of C. albicans. (Reproduced by kind permission of Dr. B. Partridge.)

CHAPTER 2 : MATERIALS AND METHODS

A) LYMPHOCYTE STIMULATION IN RESPONSE TO CANDIDA ANTIGENS

The method described here was used with the objective of comparing the response of lymphocyte preparations obtained from normal persons and from patients treated with various immunosuppressive and steroid drug regimes when these cell preparations were subjected under tissue culture conditions to antigenic stimulation by Phytohaemagglutinin (P.H.A.) and by Candida antigens.

The method was adapted and developed from that described by Coulson (1966) who described a technique for measuring lymphocyte stimulation by tuberculin antigens.

This investigation will be described under the following headings.

1. The preparation of mononuclear cell suspensions.
2. The preparation of purified Candida albicans antigen.
3. Tissue culture technique.
4. Quantification of the results;
   a) by direct microscopy
   b) by liquid scintillation counting of tritiated thymidine uptake; method, calibration of a liquid scintillation counting method, autoradiography.

1. The preparation of mononuclear cell suspensions

Introduction

The objective of this procedure was to obtain a fairly pure lymphocyte culture from a sample of peripheral blood. Fibrin, together with a large number of platelets, polymorphonuclear cells and monocytes, was removed

by a simple process of defibrination. The red blood cells were sedimented due to rouleaux formation induced by the addition either of methyl cellulose or of a gelatine preparation, and most of the polymorphonuclear cells remaining were removed by virtue of their phagocytic effect on finely divided iron which was added, thus increasing their specific gravity.

In later experiments, less effort was made to produce a pure lymphocyte preparation so that there was "contamination" with other mononuclear cells. This method was more convenient for routine use and it was felt to be equally or even more effective since it has been clearly shown that phagocytic cells are necessary for optimal lymphocyte response (Wilson, 1966; Hersch & Harris, 1968).

Materials

Universal containers: 25 ml capacity (Gallenkamp, London).

Carbonyl iron powder: type SF of average particle diameter 3.0 μ was obtained from G.A.F. (Great Britain), Manchester. Quantities between 75 and 150 mg were placed in Universal containers and sterilised in a hot air oven.

Methyl Cellulose: Methocel Grade USP, type MC, premium, viscosity 15 c.p.s., manufactured by Dow Chemical Company, Michigan, supplied by R. W. Greeff and Company Ltd., London. A 1% solution was prepared in 0.9% phosphate-buffered sodium chloride pH 7.2 and sterilised by autoclaving at 15 lbs for twenty minutes to ensure complete dissolution of the methyl cellulose in saline. The mixture was then placed in the refrigerator for 48 hours.

Plasmagel: Gelatine 3 g., sodium chloride 0.7 g., and crystalline anhydrous calcium chloride 0.2 g., made up to 100 ml with distilled water, supplied by Laboratoire Reger Bellon, Neuilly, Houts-de-Seine.

White blood cell counting fluid: 1% aqueous gentian violet 1 ml.,

glacial acetic acid 1 ml., de-ionised water 98 ml.

Glassware: Universal bottles were fitted with silicone liners and all glassware was cleaned in chromic acid, washed in de-ionised water and sterilised before use.

Method

Venous blood (10 - 20 ml) was obtained by venepuncture. The blood was transferred immediately to a universal container and defibrinated slowly using three sterile cherry sticks (15 cm by 2 mm in diameter), or a sterile glass rod. Care was taken to prevent partial clotting of blood which gave substantial reduction of the yield of lymphocytes.

The defibrinated blood was placed in an incubator at 37°C for a period not exceeding two hours before processing. The defibrinated blood was poured into a pre-warmed universal bottle containing 75 mg of carbonyl iron powder for every 10 ml of blood added, and 0.3 ml of methyl cellulose per ml of blood was added. The container was rotated on a suspension mixer for thirty minutes at 37°C.

While holding a magnet to the side of the container to retain most of the iron, the blood was poured carefully and slowly into a clean, sterile, universal container, and allowed to sediment at 37°C for thirty minutes. Most of the red cells and all the phagocytic cells containing iron settled to the bottom of the container during this period, and the supernatant fluid containing non-phagocytic mononuclear cells was then removed under sterile conditions and centrifuged at 1,200 r.p.m. for twenty minutes.

In later experiments high molecular weight gelatin (Plasmagel) was used to obtain a preparation of lymphocytes according to the technique of Coulson and Chalmers (1964). One volume of Plasmagel previously warmed to 37°C was added to three volumes of blood, i.e. 5 ml Plasmagel was added to 15 ml samples of defibrinated blood. This preparation was

then allowed to stand in an incubator at 37°C. for thirty minutes. The supernatant fluid was then removed and centrifuged precisely as before (Figure 5).

After centrifugation, the fluid layer was removed and the sedimented mononuclear cells were washed once with warm Eagle's Minimal Essential Medium (M.E.M.), and centrifuged again at 1,200 r.p.m. for twenty minutes.

On each occasion, prior to centrifugation, a 0.1 ml. aliquot was taken up in a white cell counting pipette, to which white blood cell counting fluid was added and a total white cell count was performed in Neubauer haemocytometer.

The lymphocytes were finally resuspended in 10% autologous serum in Eagle's M.E.M. in a volume sufficient to give a final concentration of nucleated cells of between one and two million per ml. If the initial lymphocyte population was too low the sample was discarded.

2. The preparation of Candida albicans antigen

The method of production of the purified Candida albicans antigen preparation was that described by Maibach & Kligman (1962). This material was prepared specifically for the purposes of this study by the Bencard Allergy Unit, Beecham Research Laboratories, Worthing, Sussex, England. Candida albicans was grown on Sabouraud glucose agar in Roux bottles and subsequently washed five times with water and then four times with acetone. The cells were then dried for a week in a desiccator. The dry cells were ground for six hours in a stainless steel ball mill. After this period of time the vast majority of the cells (about 80 - 98%) was disintegrated. The cell debris was then suspended in distilled water (five times the weight of the cells), and shaken for ninety minutes on a vibrator. The suspension was kept in a refrigerator

overnight. The following day it was shaken again for about one hour. Then, it was centrifuged at 14,500 r.p.m. for four hours. The supernatant fluid was filtered through an ultra-fine ground glass filter.

The filtrate, which was clear, yellowish fluid was lyophilised, stored in a refrigerator, and reconstituted with a small amount of normal saline just before use. (About 100 - 300 mgm. of dry powder in one ml. of saline.)

3. Tissue culture technique

Introduction

After the preparation of a mononuclear cell suspension it was necessary that the cells be maintained in a tissue culture system which would support their life without inducing spontaneous cell transformation. Eagle's Medium (Eagle, 1959) was used, it being one of the most popular and simple culture media available for lymphocyte studies (Ling, 1968). Homologous serum was used to supplement the tissue culture medium. The reason for the addition of serum was to provide macromolecules, which appear to have a protective function, or to promote lymphocyte growth in some ill-defined manner (Ling, 1968), and to provide small molecules, such as nucleosides, vitamins, hormones and co-enzymes which are essential trace nutrients not present in the medium. It was considered that by using homologous serum the *in vivo* situation was more closely simulated. There were positive reasons for not using heterologous serum or foetal calf serum. The former may result in serious experimental errors in *in vitro* culture systems, and the latter has been found to be both toxic (Parker, 1969) and stimulatory (Johnson & Russell, 1965) to lymphocytes, and it may contain numerous potential antigens. (Ling, 1968.)

The lymphocyte preparations were cultured with phythohaemagglutinin (PHA) and with Candida antigens.

It became very clear that the variables in tissue culture studies of lymphocytes are numerous and significant, and in an attempt to obtain meaningful results, the whole procedure was standardised for all patients at all times.

Materials

Tissue culture medium: Eagle's Minimal Essential Medium (M.E.M.)

10 × strength (Wellcome Reagents, Ltd., Beckenham, Kent):	10 ml.
De-ionised sterile water	90 ml.
Benzyl penicillin	200 units
Streptomycin sulphate B.P. (Glaxo Laboratories, Ltd.)	100 µg/ml.

When required for use, the pH was adjusted to 7.2 - 7.4 with one molar tris (hydroxymethyl)methylamine (i.e. 2-amino-2(hydroxymethyl) propane - 1:3 - diol).

Tris-hydrochloride buffer: This was made up as follows: 50 ml. of 2 molar tris-hydrochloride (242 g/litre) with 43 ml. of 2 molar hydrochloric acid, made up to 100 ml. in distilled water. The final pH was approximately 7.5.

Phytohaemagglutinin: The Wellcome freeze dried product was used. The powder (50 mg.) was stored at 4°C. and reconstituted when required with 5 ml. of sterile distilled water. The solution was discarded after one month's storage.

Candida antigen: Bencard, Sussex.

Bijoux bottles: (C.X. - 200, Gallenkamp, London.) 7 ml. capacity. The liners were made of non-toxic siliconed rubber. (Esko Rubber Company, London.) The tissue-culture glassware was prepared in the following way: it was rinsed in hot water, boiled up with tap water and detergent and rinsed again in hot tap water. It was immersed overnight in chromic acid, then it was rinsed in numerous changes of de-ionised water to remove the chromic acid, and finally, dried and sterilised in a hot air oven at 120°C.

Method

The lymphocyte preparations were transferred to bijoux bottles in sterile, graduated pipettes. The cell suspensions were dispensed in 2 ml. aliquots (six cultures per patient). Phytohaemagglutinin (0.21 ml.) and Candida antigen (4 units) were added to different bottles. There was always a minimum of 2 control bottles without added antigen and two bottles for each antigen studied. The containers were lightly screw-capped. All the cultures were maintained at 37°C. and the temperature of the incubator was checked regularly. The culture bottles were stood upright and they were kept stationary throughout the culture period. The total duration of the culture was 120 hours. The reason for this period of culture is given in Chapter 3.

4. Quantification of the results

Introduction

At the termination of the lymphocyte cultures, two methods were used to assess the degree of lymphocyte transformation; in the early stages of the investigation, counting was by direct microscopy of the proportions of transformed cells (Coulson & Chalmers, 1967), and latterly, when satisfactory correlation with the direct counting method had been achieved, the assessment was by liquid scintillation counting (Sell and Gell, 1965). In the direct method, the number of transformed cells and mitoses found was expressed as a percentage of the total number of lymphoid cells counted and this was compared with the percentage of transformation obtained in control cultures. The second method of assessment was by the incorporation of a radio-active DNA precursor followed by liquid scintillation counting of the extracted cellular deposit. Tritiated thymidine was used in this case. The incorporation of isotope in stimulated cultures is compared with the incorporation into

non-stimulated, control cultures. This method had the great advantage of objectivity.

Four assumptions are made when these quantitating techniques are used. The first assumption is that transformed cells develop from small lymphocytes and not from other peripheral blood leucocytes. There is good experimental evidence that monocytes and large lymphocytes do not in fact make any numerically significant contribution in vitro to the transformed cell compartment (Coulson, 1966). The second assumption, which is as yet unproved, is that the background transformation seen in control cultures is also present in test cultures and that all the stimulation over and above the background is due to the added stimulus in test cultures. In other words, it is assumed that the transformation induced by the added stimulus in test cultures does not enhance or depress the background transformation. The third assumption is that transformation stimulated by antigen in the culture is related to an immunological binding of antigen and cellular receptor site at a molecular level. If this assumption were correct, then transformation would be related to the ratio of antigen to cell in culture, and to the immunological status of the lymphocytes. The fourth assumption is that small lymphocytes do not transform into other cell types in significant numbers in the culture systems employed in the present work. Thus the transformed cell is regarded as the only positive cellular expression of small lymphocyte stimulation seen in vitro. The evidence for the validity of this rests largely on the negative observation that in pure, small lymphocyte cultures, cell types other than lymphocytes, other lymphoid cells and transformed cells are not seen. Plasma cells, fibroblasts and giant cells are never seen (Coulson, 1966).

At least three separate events occurring in the cell suspension have a direct bearing on the final percentage of transformed cells. Firstly, part of the original small lymphocyte population may die and disintegrate,

either as a result of manipulatory procedures, or by the lethal effect of antigen on sensitised cells. This phenomenon of antigen-induced cytotoxicity has been reviewed by Heilman (1963). The second factor governing the percentage transformation is that the number of transformed cells increase with time in the culture. This increase in the number with time is due to continued introduction of small lymphocytes into the transformed cell compartment and to proliferation of transformed cells. A third factor which affects the percentage is that transformed cells may die in culture. These factors were taken into account in the planning of the experimental system (Chapter 3).

a) Quantification of the results by direct microscopy

Materials

Leishman's stain: (Eosin methylene blue compound.) British Drug Houses, Poole, Dorset.

Preparation of the smear:

The cells, in bijoux bottles, were resuspended by swirling, and then they were poured into centrifuge tubes. These tubes were centrifuged at sufficient speed to sediment the cells (1,200 r.p.m.) and the supernatant fluid was removed with a fine-tipped Pasteur pipette. The cells were then lightly sucked up into the tip of a clean, fine, Pasteur pipette. A small volume was transferred to several clean, dry slides, smears were made and they were air dried and fixed in Methanol.

Staining of the smears:

Conventional staining of the smears with Leishman's stain and a buffer of distilled water, pH 6.8, gave very good results.

Counting of the cells:

The characteristics of a transformed cell were taken to be those

described by Coulson and Chalmers (1967). A typical transformed cell has an oval shape and an average diameter of about 20 μ. The nucleus-cytoplasm ratio is reduced compared with small and large lymphocytes because of the greatly increased amount of cytoplasm. The nucleus often appears eccentric and with Leishman's staining, the chromatin appears to be a combination of a stippled and fine reticular pattern. The nucleus contains either one large nucleolus or as many as three discrete nucleoli. The cytoplasm is deeply basophilic and it often contains small, well-defined vacuoles and azurophilic granules.

Cells intermediate in morphology between large lymphocytes and transformed lymphocytes, and macrophages, were not included in the count. A total of at least 1,000 nucleated cells were counted on each slide.

Calculation of the transformation ratio

The transformation ratio was simply calculated from the formula:

$$\text{Transformation ratio} = \frac{\text{Number of transformed cells}}{\text{Total number of lymphoid cells}} \times 100$$

(Levene, 1970.)

b) Liquid scintillation counting of tritiated thymidine uptake

This method was used in latter experiments when the tests had become routine and when good correlation had been obtained with this method and with the results of direct microscopical examination

The basis of the test is that the transformed lymphocytes are able to synthesise DNA, and that by addition of a radioactive-labelled DNA precursor to the culture medium, a measure of the degree of DNA synthesis and therefore of transformation of lymphocytes, can be obtained. Tritiated thymidine (^{3}H-Tdr) was used as it was reasonably inexpensive and a suitable scintillation counter for ^{3}H-Tdr was available.

It was undoubtedly of value to have a parameter by which stimulated

cell activity could be objectively measured. On the other hand, this technique is "blind", compared with direct microscopy and autoradiography, and it has been justly criticised on these grounds. However, there is no doubt that small, meaningful differences between cultures can be detected by this technique, which would otherwise be overlooked (Ling, 1968).

Liquid scintillation counting should detect the amount of label incorporated into the DNA of the transformed cells en masse. Two possible sources of error should be mentioned in this regard. Firstly, there is a need to show a constancy of relationship between transformed in the S (synthetic) compartment (that phase of the cell cycle during which DNA is synthesised, and DNA precursors are taken up), and the total transformed cell population. Secondly, Pelc (1963) has shown that in many tissues DNA is not perfectly stable and that the labelled precursors of DNA can be incorporated into non-dividing cells. It is possible that the conditions prevailing in tissue culture may induce or exacerbate instability in the DNA of those lymphocytes which are not responding by transformation. The subsequent incorporation of labelled precursors into the total DNA of the culture would thus not be correlated directly with DNA synthesis in the transformed cells. This would interfere with accurate quantitation.

Autoradiography was used on samples of tissue culture cell populations concurrently with liquid scintillation counting in a few instances in an attempt to demonstrate that the large proportion of ^{3}H-Tdr taken up was incorporated into DNA material in the nuclei of transformed cells.

Materials

Desoxyribonucleic acid precursor: 2.0 microcuries of ^{3}H-thymidine (specific activity 5 C/m.mole, Radiochemical Centre, Amersham) in 0.1 ml.

of 0.9% sodium chloride was added to each culture.

Filter Holders: Millipore No. Xx 10 025 03, Millipore (U.K.) Ltd., London.

Glass Fibre Discs: GF/A 2.1 cm. Whatman discs, obtained from Gallenkamp, Ltd., London.

Trichloroacetic acid (TCA): "Analar" trichloroacetic acid 100 g. De-ionised water to make 1,000 ml. Stored at 4°C.

Normal Saline: "Analar" sodium chloride 9 g. De-ionised water to make 1,000 ml. Stored at 4°C.

Methyl alcohol: Methanol A.R. (Fisons Scientific Apparatus, Ltd., Loughborough, Leics.), stored at 4°C.

Liquid scintillation vials: Beckman low potassium glass (borosilicate glass) vials, with plastic caps (Beckman U.K.).

Polythene closures: Robert Moss Ltd., Kidlington, Oxford.

Liquid scintillant: 0.6% Butyl P.B.D. (2 - (4'-T-Butyl-phenyl) 5 - (4"-biphenyl) - 1,3,4 - oxadiazole (fluorescence maximum 366)). Ciba (A.R.L.) Ltd., Cambridge, in "analar" toluene (British Drug Houses, Dorset).

Liquid scintillation counter: (Beckman LS - 100 U.S.A.)

Method

To each 2 ml. culture 1.0 microCi of ^{3}H-thymidine was added and the suspensions were incubated at 37°C. for a further 24 hours, exactly. At the end of this period the cultures were placed in an ice bath to terminate the thymidine incorporation. The cells were transferred quantitatively with cold saline at 4°C. to cold centrifuge tubes and centrifuged at 1,200 r.p.m. for ten minutes.

The cells were resuspended in cold saline and transferred to glass fibre discs held in chilled Millipore filter holders (Fig. 6). Using

suction (50 mm. mercury) the cells were washed with 2 × 10 ml. volumes of saline, followed by 2 × 10 ml. of 10% TCA to precipitate the nucleic acid, and finally with 2 × 20 ml. of methanol to remove all traces of TCA and haemoglobin.

The glass-fibre discs were removed from the filter holders, dried in a drying oven at 60°C. for one hour so that they were absolutely dry, and then placed in liquid scintillator vials, to each of which 5 ml. of scintillant was added. Each sample was counted for ten minutes in a Beckman liquid scintillation counting system.

Calibration of the liquid scintillation counting method

The efficiency of the counting method was determined by the preparation of a quench correction curve using ^{3}H - hexadecane as the internal standard.

Materials

Radioactive isotopes:

(i) ^{3}H-thymidine (specific activity greater than 5,000 µCi/mM.)

(ii) n-Hexadecane - 1,2-T (specific activity 2.32 µCi/G)

Both supplied by the Radiochemical Centre, Amersham.

Liquid scintillants:

Scintillant A:	Butyl BPD	6 g.
	Analar toluene to make	1,000 ml.
Scintillant B:	Butyl BPD	6 g.
	Absolute ethyl alcohol	200 ml.
	Analar toluene to make	1,000 ml.

Method

Tritiated hexadecane (0.2318 g.) was dissolved in scintillant B in a 20 ml. volumetric flask and the activity per ml. calculated as follows:

$$\text{Activity of 1 g.} = 2.32\ \mu Ci$$

$$\therefore \text{Activity of 0.2318 g.} = \frac{0.2318}{1.0000} \times 2.32$$

$$= 26.88 \times 10^3\ \mu Ci/ml.$$

This is equivalent to 59,693.14 disintegrations per minute (d.p.m.)/ml. (i.e. activity × 3.7×10^{10} Ci × 60).

A sample of 50 µl. of ^{3}H-thymidine (which had been previously diluted to 0.5 µCi/ml. in order to contain approximately 20,000 c.p.m./ 50 µl) was transferred with a 500µl Hamilton syringe to each of five counting vials; 10 ml. of scintillant B. was added and the samples were counted. To the same vials, 50 µl. of the diluted ^{3}H-hexadecane was added and the samples were recounted.

The same volume (50 µl.) of ^{3}H-thymidine were then placed on each of five glass-fibre discs, dried and then counted in scintillant A.

Expression of results:

The rate of synthesis of DNA was expressed as counts per million lymphocytes. As there was no significant quenching of the samples (Chapter 3) it was not necessary to convert the results to disintegrations per minute (d.p.m.).

The index of response was calculated from the formula:

$$\text{Index of response} = \frac{\text{c.p.m. of test preparation}}{\text{c.p.m. of control preparation}}$$

(Levene, 1970.)

Autoradiography

The technique of Moorhead, Nowell, Mellman, Battips and Hungerford (1960) as described by Parker (1969) was used. The objective of the technique was to demonstrate that the tritiated-thymidine taken up and

counted by the scintillation counting method was indeed taken up by transformed cells only.

Materials

Film: Kodak AR-10 stripping film. (Kodak Ltd., Middlesex.)

Emulsion: Ilford nuclear research emulsion in gel form (type K.5) was diluted 1 in 1 with de-ionised water. (Ilford Ltd., Essex.)

Developer: Kodak D-19b (Kodak Ltd., Middlesex).

Fixer: Kodak Acid Fixing Bath (Kodak Ltd., Middlesex).

Leishman stain: eosin-methylene blue compound (British Drug Houses, Poole, Dorset).

Method

After the lymphocytes had been incubated with tritiated-thymidine (1 micro-Ci/2 ml.) for twenty-four hours, they were washed twice with saline and fixed for thirty minutes at room temperature in a mixture of methyl alcohol and glacial acetic acid (3 : 1). The cells were concentrated by centrifugation before being dropped on to clean, methanol-washed, cold, dry slides. The slides were then washed for thirty minutes in cold running water and allowed to dry.

A box of Kodak AR-10 stripping film was opened, using an indirect safe-light, containing a fifteen-watt bulb and fitted with a Wratten 2 (dark red) filter. A plate coated with emulsion was held, emulsion uppermost, and scored with a scalpel, three lines parallel to the short edge and two parallel to the long edge. Squares of film were lifted with a scalpel and transferred face downwards on to a bowl of water at 26°C. When the film was saturated with water (a few minutes, indicated by a change from shiny to dull) a slide was dipped into the water, smear uppermost, and lifted so that the film was squarely on it and wrapped itself closely around the slide. The slides were dried in a rack in

the dark in an air-stream from a rubber-blade fan. The slides were then transferred to slide boxes which were placed inside another cardboard box containing desiccant (silicone gel) and stored at 4°C. for ten days.

The slides were than put in a metal rack and placed in a pot containing Kodak D-19b developer at 19°C. for six minutes. After a brief rinse in tap water, they were transferred to acid hard fixative at 19°C. for twelve minutes. They were then washed in running water (below 19°C.) for thirty minutes and dried.

The cells were stained through the emulsion with Leishman stain. The cell types labelled were those actively engaged in DNA synthesis.

B) QUANTITATIVE INDIRECT FLUORESCENT ANTIBODY INVESTIGATION

Introduction

The introduction of immunofluorescence techniques followed earlier observations of the use of coloured markers to demonstrate interaction between an antigen and its homologous antibody.

The "indirect fluorescent antibody technique" was developed by Weller and Coons (1954). The authors treated tissue culture preparations of varicella and herpes virus, first with human antisera and subsequently with fluorescene labelled anti-human globulin; the resulting fluorescent staining of the virus-infected tissue culture cells indicated the presence of human antibody globulin surrounding viral antigen. In this two-stage system, therefore, the human globulin played the dual role of antibody in the first stage and antigen in the second. This method has been applied to the serological diagnosis of the principal causes of all microbial infection.

Vogel and Padula (1958) applied the fluorescence method to the detection of human serum antibodies against Candida albicans, Histoplasma capsulatum, Blastomyces dermatatidis and Cryptococcus neoformans. Serum from patients with mycotic infections were found to be reactive with the corresponding fungal cells at dilutions comparable with the agglutination titre in the case of candidiasis.

The application of the method to the detection of serum antibody to Candida albicans was studied by Lehner (1966). The serum fluorescent antibody titre against this organism was found to be significantly higher in patients with clinical candidiasis, than in uninfected patients, and the method was judged to be more sensitive than other available techniques for detection of antibodies to Candida.

The basic principle of the technique is as follows: a dried film

of known Candida organisms (the antigen) is first exposed to the serum under investigation; after washing to remove excess serum, the film is next exposed to fluorescene-labelled anti-human globulin, washed again and it is then examined under the fluorescence microscope.

Homologous antibody if present, is bound to the antigen in the first stage of the test, and is detected by the fluorescent antiglobulin used in the second stage. Green fluorescence of the wall of the organisms, in response to the appropriate light stimulus, therefore indicates the presence of homologous antibody in the test serum. Quantitation of this antibody can be performed by serial dilution of the serum.

The objective of the investigation described below was to establish whether or not the titre of the antibody responsible for the indirect immunofluorescent antibody phenomenon against Candida albicans was influenced by steroid drug regimes and by immunosuppressive drug therapy in man.

Materials

Microscope slides: These were carefully cleaned by washing with soap and water, rinsing in hot water, then in ion-free water and finally in methanol. They were then thoroughly dried.

Candida: Candida albicans group A was used. It was obtained from patients with clinical candidiasis. It gave characteristic sugar reactions and formed germ tubes and chlamydospores. The strain was maintained by monthly subculture on Sabouraud agar slopes.

Preparation of antigen: a suspension of the micro-organisms was prepared by harvesting the culture in 0.9% sodium chloride and standardising it to a density of approximately 4 million yeast cells/ml. The cells were inactivated by heating at 60°C. for one hour. The antigen was kept at 4°C for up to three months.

Phosphate-buffered saline: Physiological saline buffered at pH 7.1 by 0.01 M. phosphate was prepared.

Ingredients:	di-sodium hydrogen orthophosphate (anhydrous)	1.07 g.
	sodium di-hydrogen phosphate	0.39 g.
	sodium chloride	8.5 g.
	distilled water to	1,000 ml.

The solution was used as a general medium for diluting test sera and reagents, and for washing films of Candida albicans following exposure to these agents.

Fluorescene-conjugated sheep anti-human globulin: prepared by Burroughs Wellcome, Beckenham, Kent. Diluted one in eight in phosphate-buffered normal saline.

The Mountant: a mixture of nine parts of glycerol with one part of buffered saline was used for mounting films prior to examination under the fluorescence microscope.

The Optical System: the preparations were all viewed with a fluorescence microscope using a 250 watt M.E./D mercury discharge lamp with an ultra violet interference filter (365 m.μ) and Chance-Watson heat absorbing filter ON22, three primary filters OX1, OX7 and OX1, and a secondary absorbing filter OY12. An Abbey bright field condenser and an X50 water-immersion objective were used.

Method

The smears were prepared by placing a loopful of the antigen suspension on a slide which was then heated gently at 60°C. for one hour to fix. Up to ten slides had to be prepared for each test serum, the number depending upon the antibody titre of the serum. Serial dilutions of 1 in 2, 1 in 4, 1 in 8, etc. were prepared from the serum using buffered saline.

The smear was covered with the serum for thirty minutes in a humid

chamber. Then the slide was washed in two changes of phosphate-buffered saline for fifteen minutes. Fluorescene-conjugated anti-human globulin was applied for thirty minutes and finally the slide was washed in two changes of phosphate-buffered saline for one hour. The smears were mounted with buffered glycerol saline.

The preparations were all viewed on the same day using the optical system described above. All observations were made in a darkened room.

The highest dilution of serum giving a complete ring of green fluorescence around the cell wall and "track lines" along the hyphae was taken as the titre of the serum. Observation of the gradual fading of the fluorescence with increasing dilution of the serum was also of assistance in arriving at the final reading, and in cases of doubt the titration was repeated.

A negative control was run for each serum tested, in which phosphate-buffered saline was applied to Candida, in place of the test serum.

C) QUANTITATIVE AGGLUTINATING ANTIBODY INVESTIGATION

Introduction

Serological investigations of human sera by several workers have shown that between 22.5 and 64 per cent of normal persons possess agglutinating antibodies to Candida albicans.

Winner (1955) stated that although it is possible that agglutinins are normal serum constituents whose ability to react with Candida albicans is fortuitous, the evidence suggested that they arise as a result of clinical or subclinical infection either with Candida albicans or with antigenically related organisms. This was largely confirmed by Comaish et al. (1963) who showed in a thorough clinico-pathological investigation that there is a clear relationship between Candida infection and the level of serum agglutinins.

The technique described here is a simple agglutination method, testing for the capacity of serial dilutions of test sera to agglutinate inactivated Candida organisms. This method was described by Winner (1955).

Materials

The antigen: A strain of Candida albicans was used which had been maintained on a Sabouraud agar slope. The same strain was used for all the tests performed. The organism was shown to be pathogenic to mice. Stock suspensions were prepared by washing down fresh overnight subcultures with 0.9% sodium chloride, and standardising them to a strength of forty million organisms per ml. by direct spectrophotometry. This strength of organisms was found to give good visible agglutination, and the prozone phenomenon was less marked than with weaker suspensions. The organisms were then inactivated by heating in a water bath at 60°C. for one hour.

Method

0.2 ml. volumes of the serum under test in dilutions of 1 : 4, 1 : 8, etc. in normal saline, were prepared in "welled" trays specially designed for serial dilution techniques. 0.4 ml. of the antigen suspension was added to these, and to a control tube of 0.4 ml. of normal saline. The tray was then covered, and shaken well. It was left in an incubator at 37°C. for two hours, and then refrigerated at 4°C. overnight. The results were read the following morning.

Prior to reading the results the tray was shaken once. The tray was placed over a darkened "viewing box" with indirect light. The results were checked by microscopical examination.

D) THE ANTI-CANDIDAL ACTIVITY OF NORMAL HUMAN SERUM

Introduction

The method used in this investigation was based on that described by Louria and Brayton (1964) and by Louria et al. (1967). The claim made by these workers was that the majority of normal adults had a serum factor capable of inhibiting the growth of Candida albicans. The substance was thought to be a protein in the alpha or beta globulin fraction. Deficiency of this factor was thought to be associated with susceptibility to candidiasis.

The technique is in essence an assay method in which a known number of viable Candida organisms is added to a standard volume of serum. A viable count of Candida organisms is performed on a carefully measured aliquot, both at the time when the organisms are originally added and at given intervals subsequently (e.g. six and twenty-four hours). A log. 1 fall of Candida census was taken to indicate significant anti-candidal activity of the serum.

Materials

Normal sera: These were obtained from normal donors to the Blood Transfusion Service at Guy's Hospital.

Bijoux bottles: 7 ml. capacity (Gallenkamp, London).

Sabouraud maltose agar: (Oxoid, London).

Candida albicans: Freshly isolated Candida albicans was obtained from patients with clinical disease. Identification was by cultural characteristics, germ tube formation in serum, and characteristic sugar reactions. Quantification was by spectrophotometry. Details of this are given in the following section.

Method

To 1 ml. of serum in sterile bijoux bottles 10^6 washed Candida

albicans cells from an overnight culture were added, and the serum-Candida suspension was shaken continuously in a water bath at 37°C. At 0 and 6 hours small aliquots were removed after vigorous pipetting, and 0.03 ml. was dropped on to Sabouraud agar (Miles and Misra, 1938). The plates were incubated for 48 hours at 37°C. and then read. The total number of colonies which had grown from the original 0.03 ml. was counted.

E) THE CANDIDA MOUSE-THIGH LESION

Introduction

In the experiments to be described in this section, standard numbers of viable, freshly isolated Candida albicans were injected into the thigh of mice with the objective of producing a localised inflammatory response which could be measured, and examined histologically. The natural history of the experimentally produced infection was observed over a period of time. The modifications produced by steroid therapy and by several immunosuppressive drug regimes on such aspects of the infection as mortality rate, degree of local swelling, histological changes and dissemination could thus be studied in a controlled experimental situation.

Selbie and O'Grady (1954) first used the mouse thigh to study infection (tuberculosis) under laboratory conditions. The advantage of this experimental lesion was that the infection remained localised under normal conditions and the general condition of the mouse remained good. Subsequently, this technique was used to study Candida infection (O'Grady and Thompson, 1958).

The modifying effects of cortisone on the Candida mouse-thigh lesion have been described by O'Grady, Cotton and Thompson (1964). Cortisone had a profound suppressant effect on the size of the lesions and on the inflammatory reaction.

Materials

Candida albicans: Strains of freshly isolated Candida albicans obtained from patients with oral or vaginal candidiasis were used. These organisms gave characteristic sugar reactions for Candida, and they produced germ tubes in serum and chlamydospores on corn meal agar.

Mice: Adult male mice, strain S.A.S.-I.C.I. supplied by Scientific

Animal Service (Elstree, Herts.) were used. All the mice were approximately three months old and they weighed between 35 and 50 g. They were fed on a fully balanced mouse diet (F.F.G., Dixon; Ware, Herts.) and water *ad libitum*. The mice were kept in polypropylene boxes, and conditions of husbandry, ventilation, humidity and temperature were satisfactory.

Sabouraud liquid medium: (Oxoid, London).

Sabouraud maltose agar: (Oxoid, London).

Micrometer calipers: A micrometer "Schnell taster" caliper, accurate to 0.01 mm. was used (Fig. 7).

Cortisone: 1 mg. of cortisone acetate suspended in 0.5 ml. of 0.9% sodium chloride was administered subcutaneously each day.

Azathioprine: Daily intraperitoneal injections of azathioprine in solution in water for injection were administered in the dose of 250 mg. per kg. of body weight.

Horse anti-mouse anti-lymphocyte serum: (Burroughs-Wellcome, Beckenham, Kent).

Spectrophotometer: S.P. 600. (Unicam Instruments, Ltd., Cambridge).

Method

The Candida organisms were quantitated by spectrophotometry. By a technique of viable counting (Miles and Misra, 1938) and correlation with light absorbance it was demonstrated that during the log-phase of growth of Candida there was a direct relationship between the degree of light absorbance and the viable count (Fig. 8). Subsequent estimations of Candida counts were performed by spectrophotometric estimation of light absorbance only. From time to time check viable counts were performed to confirm correlation with the spectrophotometric findings.

An overnight growth of Candida albicans in Sabouraud liquid medium was obtained. The cells were washed twice in physiological saline, and

a suspension of the organisms was prepared in physiological saline. Eight million organisms in 0.1 ml. of physiological saline were injected through a fine needle into the quadriceps group of muscles of the right thigh of the test mice. Care was taken to avoid the production of a haematoma (Fig. 9).

Before injection, and on each day subsequent to injection for eight days, the thigh was measured across its largest diameter with a micrometer caliper. Readings were taken to the nearest one-tenth of a millimetre. All measurements were taken with the same instrument and by the same observer.

Mice injected with 0.1 ml. of physiological saline served as negative controls.

Cortisone was administered subcutaneously; the combination of azathioprine and cortisone was introduced into the peritoneal cavity as a mixed solution in water for injection, B.P.; and azathioprine dissolved in water for injection B.P. was administered by intraperitoneal injection.

Horse anti-mouse anti-lymphocyte serum was injected subcutaneously in 0.2 ml. doses daily.

The weight of the mice was recorded daily.

A series of four mice from each treatment group was killed on alternate days; a full blood count, blood culture and a complete post mortem examination was performed on each. Histological examination of the thigh, heart valves and myocardium, kidney, liver and spleen was performed. The histological sections were stained with haematoxylin and with periodic acid Schiff.

F) THE METHOD OF STATISTICAL ANALYSIS BY THE RANK SUM TEST

In comparing the lymphocyte stimulation responses of normals and of the various treatment groups studied, the Rank Sum test (also known as the Wilcoxon or Mann-Whitney test) was used. This test was used because the distributions of response of the various groups appeared to deviate considerably, and the samples were of moderate size only. The Rank Sum test assumes only that the basic variable has a continuous frequency function.

Each test compared n_1 sample results, n_1, n_2 ..... n_{n1} from one group, with n_2 sample results, y_1, y_2 ..... y_{n2} from a second group. Given that the samples of n and y values were from populations with continuous frequency functions f1(n) and f2(n) respectively, the hypothesis being tested was Ho: $f1(n) = f2(n)$.

To perform the Rank Sum test, the results from the two groups were pooled, and arranged in increasing order of magnitude, or "array". The ranks (i.e. order in the array) of the n values, were summed, giving a value T .

Now if $f1(n) = f2(n)$ and the two groups being compared are in fact members of the same population, then the results can be considered as one sample, of size $(n_1 + n_2)$. Since the sampling is random, the observations from the two groups should be thoroughly mixed, with no tendency for either the n or y values to cluster at either end of the array. In this case the statistic T, (given the moderate sample sizes used in this case) will follow an approximately normal distribution with mean and variance given by the formulae

$$\text{Mean, } E(T) = \frac{n_1 (n_1 + n_2 + 1)}{2}$$

$$\text{Variance, } \sigma^2(T) = \frac{n_1 n_2 (n_1 + n_2 + 1)}{12}$$

The statistic, $T = \frac{T - E(T)}{\sigma_T}$ is thus an approximate standard normal variable, and is used in the normal way to test whether T is significantly different from E (T) and hence whether f1(n) = f2(n) .

For the purposes of analysis of the results reported in this investigation a one-tailed test was used, at a significance level of 0.01 or 1%.

The Rank Sum test is powerful, having a minimum A.R.E. (Asymptotic relative efficiency) of 0.864 compared to "Student's" t (Kendall and Stuart, 1967).

Figure 5: Defibrinated blood with plasmagel added; the sediment contains red blood cells and large numbers of polymorphonuclear white blood cells.

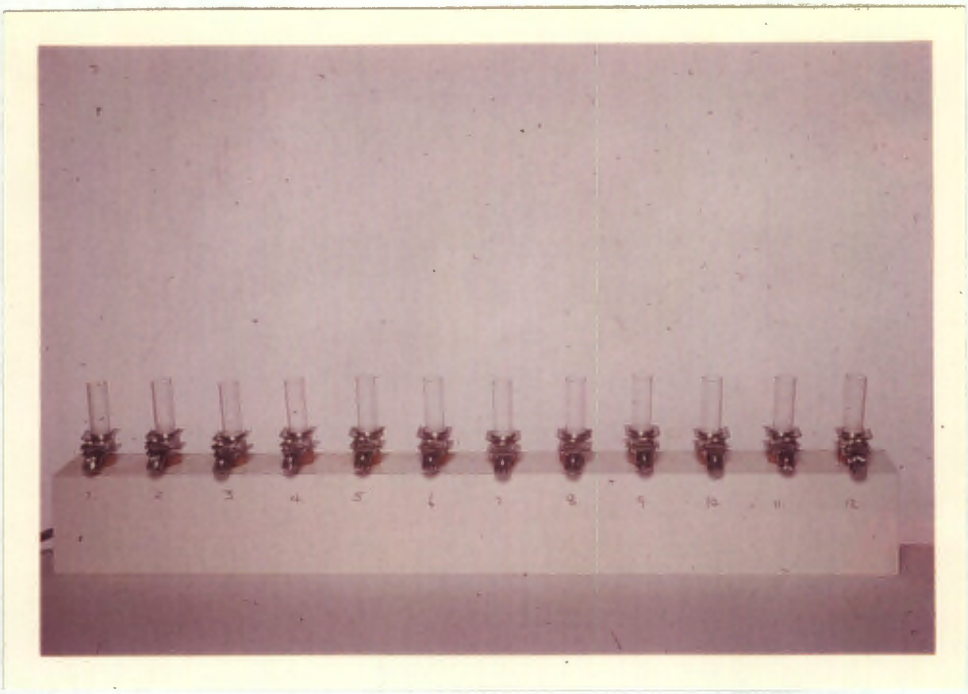

Figure 6: Millipore filter system.

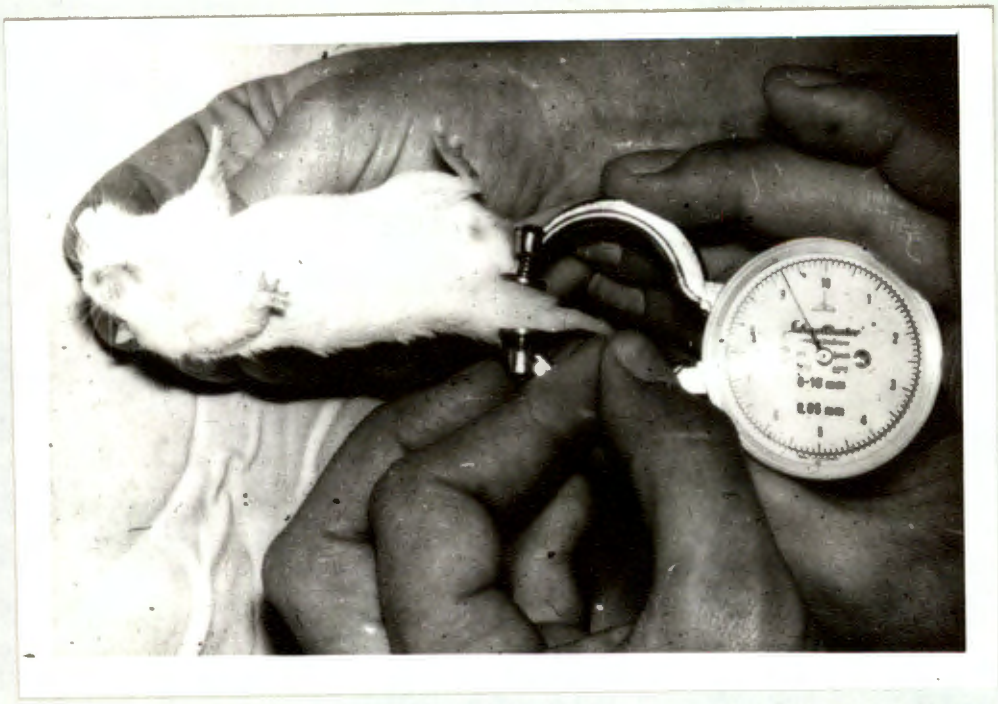

Figure 7: Micrometer caliper, used for measurement of the mouse-thigh.

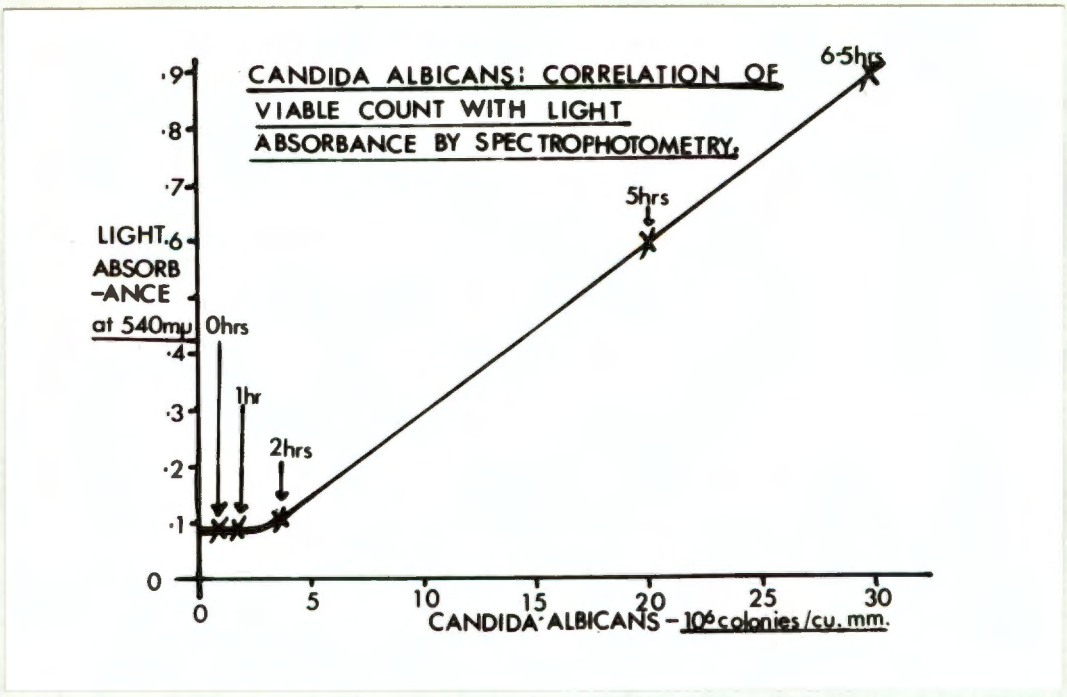

Figure 8: Quantitation of Candida organisms.

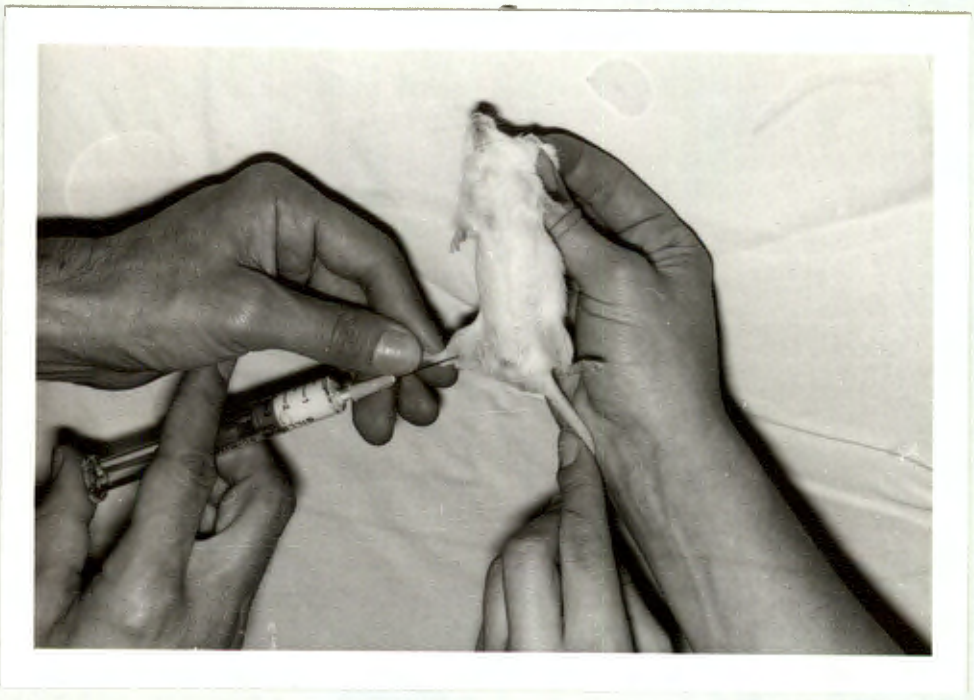

Figure 9: Administration of Candida into the mouse-thigh.

CHAPTER 3 : RESULTS

A) RESULTS OF LYMPHOCYTE TRANSFORMATION STUDIES

Introduction

The objectives, basic principles and methodology of the series of experiments described in this chapter were detailed in Chapter 2.

There is considerable evidence now available that the ability of lymphocytes to undergo morphological transformation in the presence of P.H.A. or specific antigens is a measure of the immunological competence of these cells. Levene has stated recently, in an assessment of the clinical significance of lymphocyte transformation *in vitro*, that it can be said with certainty at the present time that lymphocyte transformation reflects the immunological activity of the donor of the lymphocytes to the particular antigen (Levene, 1970). Humphrey (1967), Turk (1969) and Marshall, Cope, Soothill and Dudgeon (1970) have equated the *in vitro* phenomenon of lymphocyte recognition of and transformation in response to antigen with *in vivo* cell-mediated immunity. Hall (1969) considers that the function *in vivo* of sensitised lymphocytes is to provide very rapid defence of tissues exposed to antigen - notably the gut and the mucous surfaces. It seems that lymphocytes provide the very first line of defence. The evidence that lymphocyte transformation in response to antigenic stimulation is an index of cell-mediated immunity, may be summarised as follows:

1. There is good correlation with the immunological status of healthy individuals; e.g. B.C.G., tetanus toxoid, T.A.B. and pertussis immunization (Ling, 1968).

2. Impaired ability of lymphocytes to transform in response to P.H.A. is characteristic of those diseases in which deficiency of

cellular immunity is a feature, e.g. Hodgkin's disease, chronic lymphatic leukaemia, sarcoidosis, miliary tuberculosis, lepromatous leprosy, measles, thymic agenesis and ataxia telangiectasia (Pentycross, 1970; Marshall et al., 1970; Ling, 1968). In sex-linked hypogammaglobulinaemia, a condition in which the only immunological deficiency is thought to be a depletion of circulating antibodies, there does not appear to be any impairment of lymphocyte response to P.H.A. (Marshall et al., 1970; Elves, 1966).

3. In laboratory studies, animals antigenically challenged have developed lymphocyte response to the antigen to which they have been previously sensitised (Elves, Roath and Israels, 1963). Zweiman, Besdine and Hildreth (1966) sensitised guinea pigs with mycobacterial antigens, following which the animals developed positive lymphocyte transformation to tuberculin in cell culture.

4. Finally, in transplantation surgery, the responsiveness of lymphocytes to blastogenic agents in vitro, has been noted roughly to parallel the capacity of children and adults to exhibit homograft immunity (Lischner, Punnett and DiGeorge, 1967).

In this Chapter the experimental results are detailed and analysed of the investigation into the response of lymphocytes of the normal population to stimulation with Candida antigen, and the modification of this response by various drug regimes.

Experiment I : The optimum duration of incubation.

This experiment was planned with the objective of establishing the optimum duration of incubation of the lymphocyte preparations with Candida antigen. The ideal time for termination was taken to be, as defined by Coulson and Chalmers (1967), as "that time at which all the

cells capable of doing so have transformed, the minimum number of transformed cells has divided, and the decline in the total cell population in adequate cultural populations has not progressed to a significant degree".

Method

The blood of two normal adults was examined:

1. E.F. Male 44 years
2. P.I.F. Male 31 years

A lymphocyte culture was prepared as described in Chapter 2. A sufficiently large volume of blood was taken originally so that the final lymphocyte preparation could be divided into 27 2ml. aliquots. One-tenth of a ml. of reconstituted Candida antigen was added to each 2 ml. sample. At 0 hours and at 20-hour intervals for a total of 140 hours, three samples from each subject were examined for the total mononuclear cell count per ml. of culture fluid and the differential mononuclear cell count. In the differential count one thousand cells were counted in each estimation. The mean of the three results for each reading at each time interval was calculated.

Results

The total mononuclear cell count, and the differential mononuclear cell count of E.F. and P.I.F. are tabulated in figures 10 and 11 respectively.

Graphical representation of the degree of transformation of the lymphocytes of the two subjects, with the progress of time over the 140-hour period is shown in figure 12. In this figure, the time of the first appearance of mitotic cells in the cell preparation is indicated.

It should be mentioned at this stage that it was quite apparent from direct microscopical examination that lymphocytes which had transformed

in response to stimulation by Candida antigens did not differ morphologically in any apparent way from lymphocytes stimulated by P.H.A. or by other antigens.

Four basic cell types were identified in cultures examined microscopically:

a) Small lymphocytes, 6 - 9 μ in diameter.

b) Large lymphocytes, 8 - 12 μ in diameter with one to two azurophilic granules in the cytoplasm of some.

c) Intermediate lymphocytes up to 12 μ in diameter. The nuclear material of these cells was less condensed and there was more cytoplasm. There was no increase of basophilia and no increase of RNA in the cytoplasm.

d) Transformed lymphocytes (figures 13, 14, 15 and 16). The features of these cells included the following: diameter 10 - 25 μ ; nucleus eccentric, with chromatin in columns and 1-3 nucleoli in each nucleus; cytoplasm basophilic with varying numbers of azurophilic granules; streaming of the cytoplasm of some of the cells; mitotic figures. The intermediate lymphocytes were considered to represent the result of morphological change induced by the culture conditions rather than an early stage of transformation. They were not included in the count of transformed cells.

Comment

From an assessment of figures 10, 11 and 12, in the light of the statement of Coulson and Chalmers referred to above, it was felt that a culture time of 120 hours would be optimal for the assessment of the stimulatory effects of Candida antigen on lymphocytes of normal persons.

Experiment II : Dose-response curve.

It was necessary at this stage to determine a dose-response curve

for Candida antigen for lymphocyte stimulation.

It is thought that at very low concentrations of antigen there is insufficient to activate all the cells capable of response, and that the response increases linearly with dose until all sensitised cells have combined with antigen. This principle is thought to apply to all antigens capable of activating lymphocytes (Coulson, 1966). On the other hand, very large doses of P.H.A. were found by Wilson (1966) to be inhibitory and this possibility applies to specific antigens as well. It was, therefore, important to establish an optimum dose of Candida antigen.

Method

Lymphocyte preparations were obtained from four normal medical students:

1. C.R.M. Male 20 years.
2. R.A.B. Male 18 years.
3. J.C. Male 18 years.
4. P.L. Male 18 years.

Six, increasing doses of Candida antigen, in the same volume of diluent, (0.1 ml.), were added to cultures from each subject. Two cultures were tested for each dose of Candida antigen added, and the mean of the readings was calculated. The duration of each culture was 120 hours.

Results

The results of this investigation are tabulated in figure 17, and represented graphically in figure 18. It was reasonable to assume from this data that for future experiments three units of Candida antigen, diluted in 0.1 ml. of diluent, was suitable.

It can be seen from figure 18 that large doses of Candida antigen exerted an inhibitory effect on the normal level of lymphocyte activity seen in control cultures.

Experiment III : Autoradiography.

When reproducible results had been obtained with the direct counting method, it was felt to be expedient to convert from this method of quantitation to that of the more objective method of liquid scintillation counting of tritiated-thymidine uptake.

The correlation between this method of quantitation and direct microscopy is good, as has been discussed by Schellekens and Eijsvoogel, (1968).

This experiment was undertaken in order to demonstrate that tritiated-thymidine counted by the liquid scintillation counting system, under the experimental conditions as described, was indeed thymidine taken up into newly synthesised DNA by transformed lymphocytes alone, and that the reading obtained was not due to uptake by unstimulated cells or due to background radiation.

Method

A lymphocyte preparation from six normal donors was subjected to culture with Candida antigen over a 120-hour period. One μCi of tritiated-thymidine was added 24 hours prior to termination of the culture. The technique of autoradiography used was described in Chapter 2.

Results

The results obtained were photographed; all results were similar and a representative result is shown in figure 19.

Tritiated-thymidine was shown to be taken up into the DNA of large, transformed cells only, i.e. those cells actively engaged in the synthetic phase of the cell cycle. Background radioactivity was negligible.

Experiment IV : Calibration of the liquid scintillation counting system.

The underlying principle of this experiment was to compare the

efficiency of the system for counting tritiated-thymidine with its efficiency in counting a known amount of tritiated-hexadecane with an established number of disintegrations per minute (d.p.m.).

Method

The method used was detailed in the preceding chapter.

Results

The efficiency of counting tritiated-hexadecane in scintillant B (0.6% butyl P.B.D. in toluene containing 20% absolute alcohol) was calculated to be 39.8% and the disintegrations per minute (d.p.m.) of 50 ml. of tritiated-thymidine counted with this efficiency was 43,197.7.

The efficiency of counting tritiated-thymidine on glass fibre discs in scintillant A (0.06% butyl P.B.D. in toluene) was found to be 29.3%. The external standard ratio at this efficiency was 4.0 to 5.0, and no loss in counting efficiency was observed in samples with external standard ratios between 3.6 and 5.0. All the samples counted in this investigation had ratios within this range and therefore it was not necessary to correct the results for quenching.

Experiment V : The lymphocyte response to Candida antigens of the normal population.

Before any studies could be made on the effects of drug therapy, a study of the lymphocyte response to Candida antigen of the normal population had to be undertaken.

Method

Fifty-five normal adult subjects between the ages of eighteen and fifty-one years, and with an approximately equal male-female distribution, were studied. Each subject was questioned and examined personally, in order to ensure that he or she was in perfect health, not receiving any

drug therapy, and not suffering from any form of Candida infection. In each case, mouth washings were cultured for Candida, and any positives (Candida carriers) were discarded from the series. (Rectal and vaginal examinations were not performed.)

Lymphocyte transformation testing was performed as detailed in Chapter 2.

Results

The results of this investigation are tabulated in figure 20; the frequency distributions of the responses are tabulated in figure 21, and represented graphically in figure 22.

Taking an index of response of 2.0 or greater as being of significance, it can be said that in this study 65% of the subjects had a significant response to Candida antigens.

Experiment VI : The lymphocyte response to Candida antigens of patients on intermittent haemodialysis.

It was necessary to ensure that chronic renal disease with uraemia, treated by intermittent haemodialysis, was not inhibitory to the lymphocyte transformation response to Candida. This disease group was selected because the patients were not receiving any form of drug therapy, and because they represented a control group suffering from the same clinical problem as a large majority of the test patients described later.

Method

Eight patients were studied. All had the following in common: chronic renal disease with uraemia; all were on a chronic intermittent haemodialysis programme; none of the patients was being treated with any form of drug therapy. The clinical details of these patients were:

1.	D.S.	Female	38 years	Chronic proliferative glomerulonephritis.
2.	D.H.	Male	37 years	"End-stage renal disease" - Membrano-proliferative glomerulonephritis.
3.	P.G.	Female	21 years	Chronic pyelonephritis; ureteric reflux.
4.	G.W.	Male	20 years	"End-stage renal disease". ? chronic glomerulonephritis.
5.	J.L.	Female	35 years	Chronic pyelonephritis.
6.	A.B.	Female	45 years	Chronic pyelonephritis.
7.	C.L.	Female	47 years	Chronic glomerulonephritis; hypertension.
8.	H.G.	Male	39 years	Bilateral polycystic kidneys.

The method of lymphocyte transformation testing was performed as before.

Results

The results of the lymphocyte response to Candida antigen in this series of patients are tabulated in figure 23.

Statistical analysis by the rank sum test of the response of this group compared with the response of the normal subjects detailed in Experiment V. showed that:

$$T = -0.48$$

$$P\ (T \leq -0.48) = 0.3156 \text{ or } 31.56\%$$

i.e. the response of this group of patients was not shown to be different from the response of normal subjects.

Comments

The inference from the result of this experiment was that chronic renal disease per se, when managed by intermittent haemodialysis, does not impair the normal lymphocyte response to antigenic stimulation with Candida.

This finding is in accordance with that of Ling (1968) who reported that the lymphocyte responses to P.H.A. of patients with uraemia were normal.

Experiment VII : The lymphocyte response to Candida antigens of patients receiving combined azathioprine and prednisone therapy following renal transplantation.

In 1967, Hersh and Oppenheim (1967) showed that intensive combination therapy with parenteral 6-mercapto purine and methotrexate, with or without prednisolone, abolished lymphocyte transformation induced by P.H.A. and by smallpox vaccine. With intensive therapy with this drug combination the abolition of this response took place within three days of starting treatment; non-toxic therapy which did not induce leucopenia took two to five weeks to cause maximum suppression. Steroid therapy, in doses normally given, does not appear to inhibit lymphocyte responses to P.H.A. (Ling, 1968).

The investigation described in this experiment was of the lymphocyte responses to stimulation by Candida antigens in vitro of twenty-one patients receiving long-term azathioprine and prednisone therapy following renal homotransplantation.

Method

All 21 patients studied had undergone homograft renal transplantation at Guy's Hospital for chronic renal disease. The drug therapy was at maintenance level; all patients had undergone transplantation at least ten weeks prior to the investigation. The maximum period of treatment was three years. All patients were examined personally; none had evidence of Candida infection clinically, and cultures of mouth-washes were negative for Candida. Candida "carriers" were excluded from the series.

The details of the sex, age and drug doses at the time of the investigation are as follows:

	Name	Sex	Age	Daily Azathioprine dosage (mg.)	Daily Prednisone dosage (mg.)
1.	A.F.	Male	14	200	200
2.	W.C.	Female	32	150	60
3.	G.E.	Male	21	200	200
4.	J.C.	Male	49	200	20
5.	W.R.	Female	39	150	50
6.	P.L.	Female	33	150	30
7.	T.Q.	Male	24	150	10
8.	D.S.	Female	31	200	50
9.	D.H.	Male	29	200	50
10.	R.R.	Male	27	150	10
11.	K.M.	Male	35	150	15
12.	D.L.	Male	21	150	60
13.	G.W.	Male	37	150	10
14.	H.A.	Female	33	150	50
15.	C.W.	Female	18	75	15
16.	J.L.	Female	35	200	50
17.	A.W.	Male	39	200	50
18.	P.H.	Male	34	150	50
19.	O.R.	Male	39	150	30
20.	K.M.	Male	40	100	10
21.	D.E.	Male	32	150	30

The experimental methods were precisely as described previously.

Results

The results of the lymphocyte response to Candida antigens in this series of patients are tabulated in figure 24.

Statistical analysis by the rank sum test of the response of this group of patients compared with the response of the normal subjects detailed in Experiment V showed that

$$T = -4.5$$

$$P\ (T \leq -4.5) = < 0.00003 \text{ or } 0.003\%$$

i.e. the response of this group of patients is lower than the response of normal subjects at an extremely high level of significance.

Experiment VIII : The lymphocyte response to Candida antigen of patients receiving long-term steroid therapy.

As indicated in the previous section, steroid therapy, when given in normal doses, does not appear to inhibit lymphocyte responses to P.H.A.

Method

The lymphocyte response to Candida of seventeen patients suffering from various diseases for which they were receiving treatment with either prednisone or prednisolone in doses of 7.5 mg. or more daily for at least two weeks were examined.

The method used was precisely as described in the previous experiments. Candida "carriers" were excluded from the study.

The following are brief clinical details of the patients investigated.

	Name	Sex	Age	Diagnosis	Daily dose of Prednisone or Prednisolone (mg.)
1.	E.H.	Male	64	Chronic obstructive airways disease	20.0
2.	A.D.	Female	58	Asthma	30.0
3.	E.B.	Female	74	Asthma	7.5
4.	J.A.	Female	30	Asthma	40.0
5.	M.D.	Female	14	Nephrotic syndrome	30.0
6.	M.M.	Male	60	Asthma	20.0
7.	M.J.	Male	38	Ulcerative colitis	15.0
8.	C.C.	Female	35	Ulcerative colitis	40.0
9.	F.S.	Female	49	Asthma	20.0
10.	A.D.	Female	66	Asthma	10.0
11.	J.B.	Female	59	Haemolytic anaemia	7.5
12.	B.H.	Female	21	Ulcerative colitis	45.0
13.	J.A.	Female	24	Raynaud's disease	20.0
14.	E.D.	Female	32	Asthma	15.0
15.	J.A.	Male	32	Chronic proliferative glomerulonephritis	15.0
16.	J.O.	Male	69	Chronic obstructive airways disease	15.0
17.	J.D.	Female	30	Asthma	7.5

Results

The results of the lymphocyte responses to Candida antigens of this series of patients are tabulated in figure 25. Statistical analysis by the rank sum test of the response of this group of patients compared with the response of the normal subjects detailed in Experiment V showed that

$$T = -4.315$$

$$P\ (T \leq -4.315) = < 0.00003 \text{ or } 0.003\%$$

i.e. the response to Candida antigens of this group is lower than that of the normal group at an extremely high level of significance.

Experiment IX : The lymphocyte response to Candida antigens of patients receiving cyclophosphamide therapy.

An experiment similar to that described in Experiment VIII was constructed with the objective of testing whether long-term cyclophosphamide therapy significantly depressed the normal lymphocyte response to Candida antigens.

Method

Six patients receiving long-term cyclophosphamide therapy for various diseases were examined. The method was the same as that described previously.

The clinical details of the patients investigated were:

	Name	Sex	Age	Diagnosis	Daily dose of Cyclophosphamide (mg.)
1.	C.R.	Male	55	Nephrotic syndrome	50
2.	A.W.	Male	55	Chronic renal disease	50
3.	B.W.	Male	46	Nephrotic syndrome	25
4.	J.P.	Male	51	Polycythaemia	150
5.	J.R.	Male	58	Chronic proliferative glomerulonephritis	100
6.	K.D.	Female	22	Systemic lupus erythematosus	50

Results

The results of the lymphocyte response to Candida antigens of this series of patients are tabulated in figure 26.

Statistical analysis by the rank sum test of the response of this group of patients compared with the response of normal patients detailed

in Experiment V showed that

$$T = -3.133$$

$$P\ (T \leq -3.133) = 0.00087$$

i.e. the response to Candida antigens of this group is lower than the response of the normal group at a significance level of 0.00087 or 0.087%.

Experiment X : Selective depression of lymphocyte responses to Candida.

An investigation is reported here which appears to be of some interest and possibly of importance from the point of view of lymphocyte function. In addition it may be of direct relevance to Candida infection.

Both Coulson (1970) and Ling (1968) have postulated that sensitised lymphocytes have specific receptor sites on the cell surface, conceivably immunoglobulin in nature, which are capable of combining with and interacting with the specific antigen when it is re-introduced. Biochemical messengers are thought to be released as a result of this interaction which penetrate the cell and induce the RNA and DNA cytoplasmic and nuclear material respectively to turn over at a markedly accelerated rate. It is almost certain that this complex biochemical activating system takes place in several stages (Ling, 1968).

The possibility suggested itself from the observations recorded in this series of investigations that P.H.A. and specific antigen might activate lymphocytes at different sites or by different mechanisms. The corollary is that different drugs might exert an inhibitory effect on the lymphocyte transformation phenomenon at different biochemical stages of this process. The findings recorded in Experiment VIII, showing that steroid therapy significantly depresses the normal lymphocyte response to stimulation with Candida antigen, were at variance with the report of

Ling (1968) that steroid therapy does not inhibit the normal response to P.H.A. The possibility that steroid therapy selectively depressed the response to Candida while not affecting the P.H.A. response, or affecting it to a lesser extent, was explored.

Method

The P.H.A. response of 24 normal subjects was compared with the P.H.A. response of 11 patients being treated with long-term steroid therapy in doses greater than 7.5 mg. daily. Both series of patients were sample populations of those series reported in Experiments V and VIII respectively. The responses to Candida antigens of subjects in these two groups were compared in a similar manner.

The experimental method used was precisely as described previously and statistical comparison was made using the rank sum test and analysis of variances.

Results

The responses to P.H.A. of both the normal and the steroid-treated subjects are tabulated in figure 27. (The responses to Candida antigens of these subjects were given in figures 20 and 25 respectively.)

Statistical analysis showed the following:

1. The P.H.A. response of the steroid-treated subjects was assessed for a difference from the P.H.A. response of the normal subjects. By rank sum testing:

$$T = -1.1$$

$$P\ (T \leq -1.1) = 0.1357 \text{ or } 13.5\%$$

i.e. the response of the steroid-treated subjects was not shown to be different from the P.H.A. response of normal subjects, at conventional levels of significance (0.01 or 0.05). (They are, however, different at a significance level of anything greater than 13.6%.)

2. The lymphocyte response to Candida antigen of the steroid-treated subjects was different to the response of normals at a very high level of significance (0.00003 or 0.003%).

3. By straightforward analysis of variances on ranked observations, the hypothesis that the differences in the responses could be accounted for by treatment alone, and that they were not dependent on the nature of the challenge was overthrown at a significance level of $P < 0.05$.

Comment

It has been shown in this experiment that steroid therapy had a selective depressant effect on lymphocyte transformation in response to antigenic stimulation with Candida, compared with lymphocyte stimulation by P.H.A.

This observation seems to be of some significance in respect to Candida infection, and it possibly gives support to the theory that lymphocytes are activated by a system of biochemical messages. It seems reasonable to conclude that the mechanisms of activation of lymphocytes by Candida antigens and by P.H.A. are different. Drugs, conceivably, inhibit lymphocyte responses at different biochemical sites along this system.

Figure 10: Total and Differential White Cell Count of a Mononuclear Cell Preparation in Tissue Culture Conditions with Candida Antigens (Estimations: 20 hour intervals).

TIME (hours)	0	20	40	60	80	100	120	140
Total number of White Cells/cu.mm.	$3{\cdot}28 \times 10^6$	$2{\cdot}82 \times 10^6$	$2{\cdot}28 \times 10^6$	$2{\cdot}27 \times 10^6$	$1{\cdot}72 \times 10^6$	$1{\cdot}83 \times 10^6$	$1{\cdot}88 \times 10^6$	$1{\cdot}83 \times 10^6$
Lymphocytes	669	682	869	981	977	974	877	824
Monocytes / Macrophages	11	4	1	0	0	0	0	0
Granulocytes	320	314	130	0	0	0	0	0
Transformed Cells	0	0	0	19	23	23	105	168
Mitotic Cells	0	0	0	0	0	3	18	21

NORMAL SUBJECT: E.F. (M.44)

Figure 11: Total and Differential White Cell Count of a Mononuclear Cell Preparation in Tissue Culture Conditions with Candida Antigens (Estimations at 20 hour intervals).

TIME (hours)	0	20	40	60	80	100	120	140
Total number of White Cells/cu.mm.	$2 \cdot 33 \times 10^6$	$1 \cdot 41 \times 10^6$	$1 \cdot 22 \times 10^6$	1×10^6	$1 \cdot 19 \times 10^6$	$1 \cdot 01 \times 10^6$	$\cdot 98 \times 10^6$	$\cdot 92 \times 10^6$
Lymphocytes	934	932	964	946	891	802	748	610
Monocytes / Macrophages	3	0	0	0	0	0	0	0
Granulocytes	63	61	4	0	0	0	0	0
Transformed Cells	0	7	32	54	108	194	240	346
Mitotic Cells	0	0	0	0	1	4	12	44

NORMAL SUBJECT: P.I.F. (M.31)

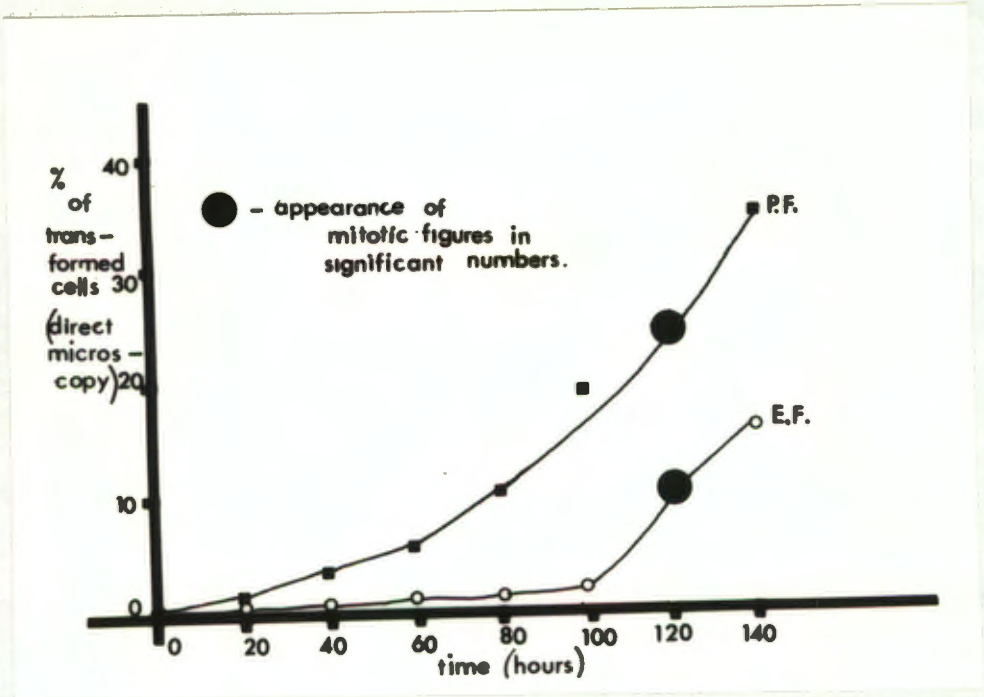

Figure 12: The time of lymphocyte transformation in response to Candida antigens.
[2 normal subjects; P.I.F. and E.F.]

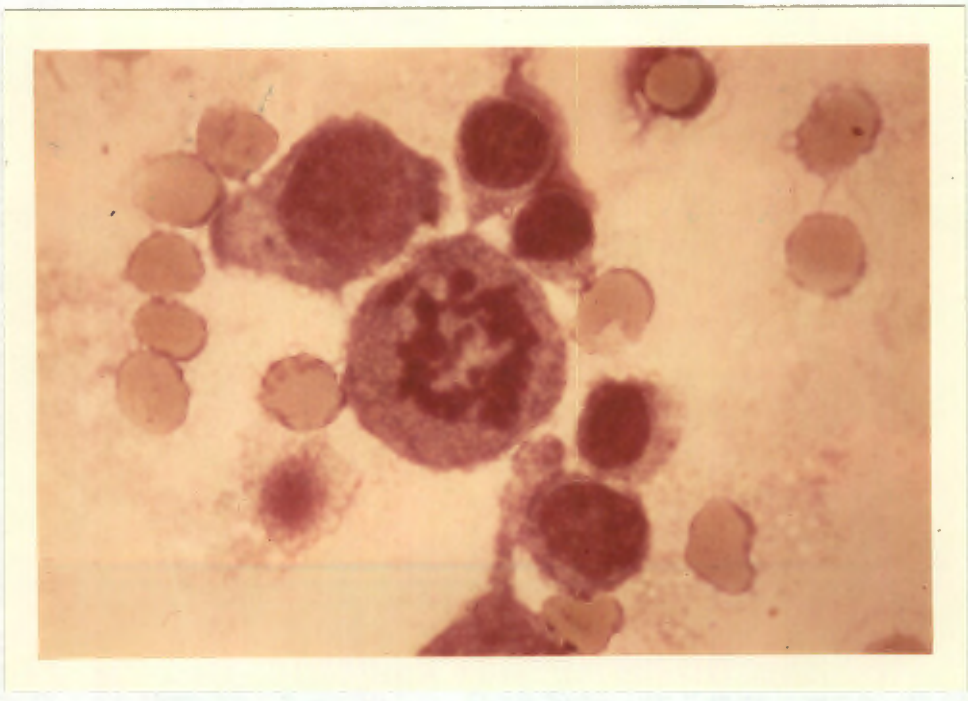

Figure 13: Photomicrograph of two transformed lymphocytes, three unstimulated lymphocytes alongside and an intermediate lymphocyte at bottom right.
[Leishman stain × 950.]

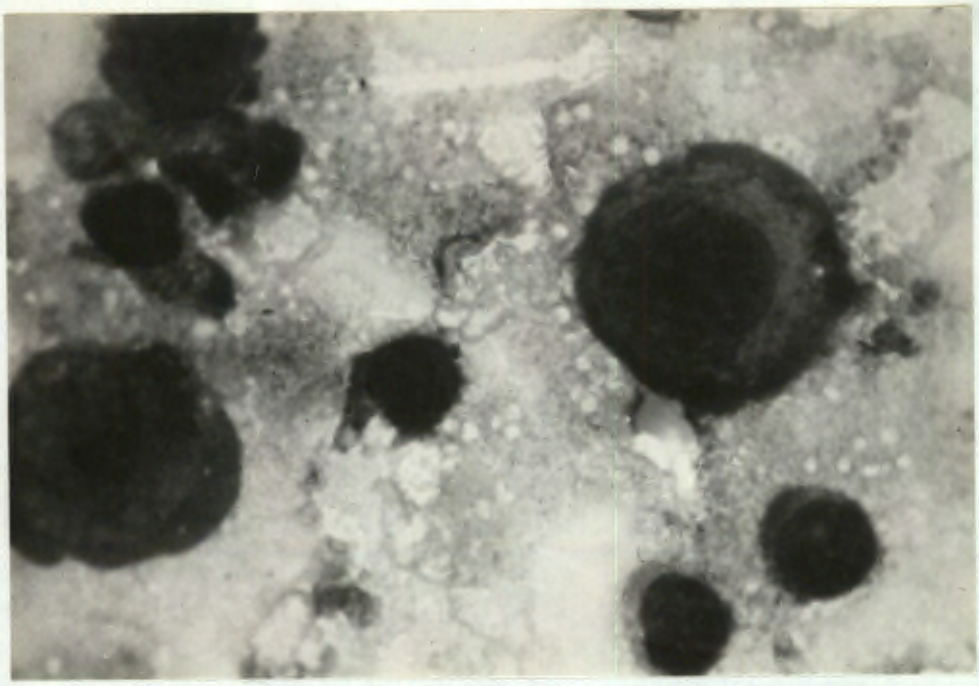

Figure 14: Photomicrograph of transformed lymphocytes - response to stimulation by Candida antigen.
[Leishman stain : × 950.]

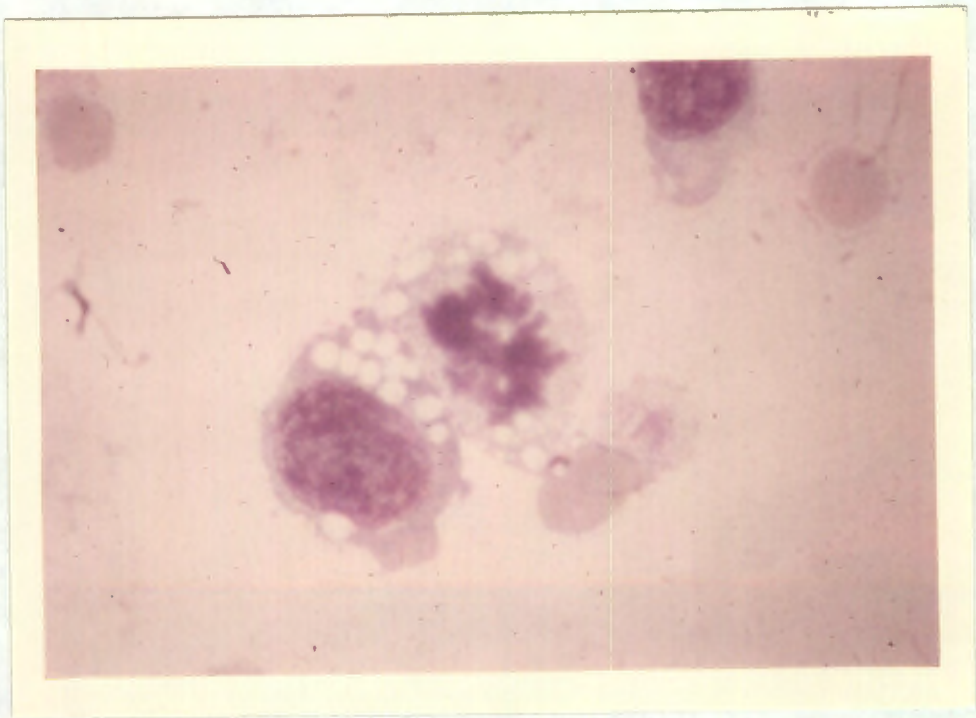

Figure 15: Photomicrograph of a mitotic cell in prophase, and an adjacent transformed lymphocyte showing pronounced vacuolation of cytoplasm.
[Leishman stain : × 950.]

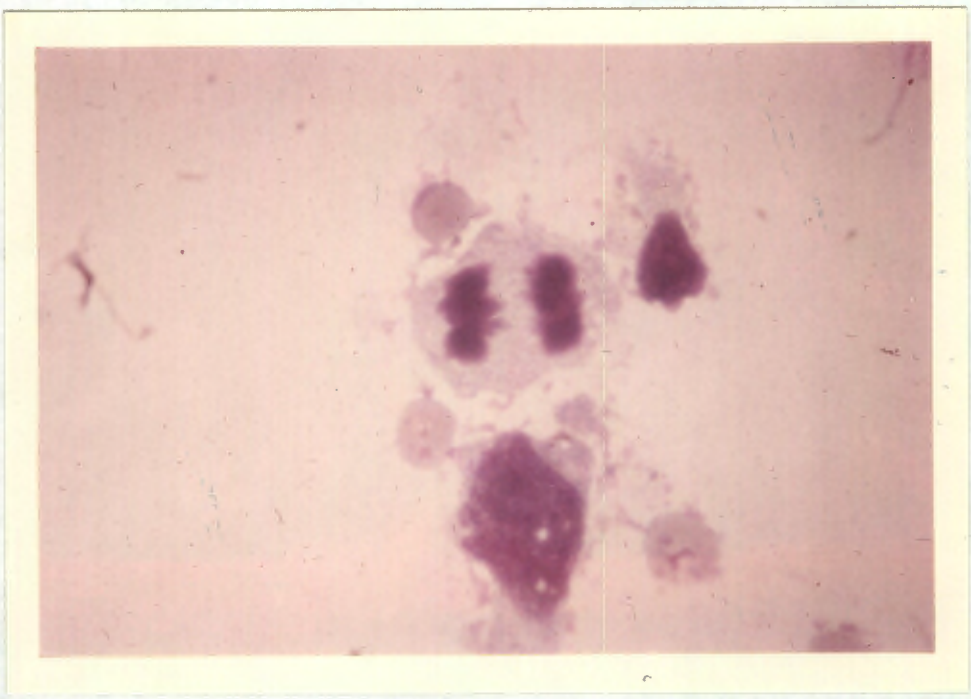

Figure 16: Photomicrograph of a mitotic cell in late anaphase - response to stimulation by Candida antigen. [Leishman stain : × 950.]

Figure 17: Titration of the optimum dose of Candida antigen required to produce maximum lymphocyte stimulation at 120 hours.

Readings: Counts per minute (c.p.m.) of tritiated-thymidine taken up. (The mean of three readings is recorded.)

Results:

SUBJECT	DOSE OF CANDIDA ANTIGEN (units)						
	0	1	2	3	4	5	6
C.R.M.	239	133	400	1,159	445	448	45
R.A.B.	287	451	1,299	3,385	169	71	60
J.C.	68	347	4,288	7,909	3,230	141	68
P.L.	599	6,073	4,814	3,551	818	88	76

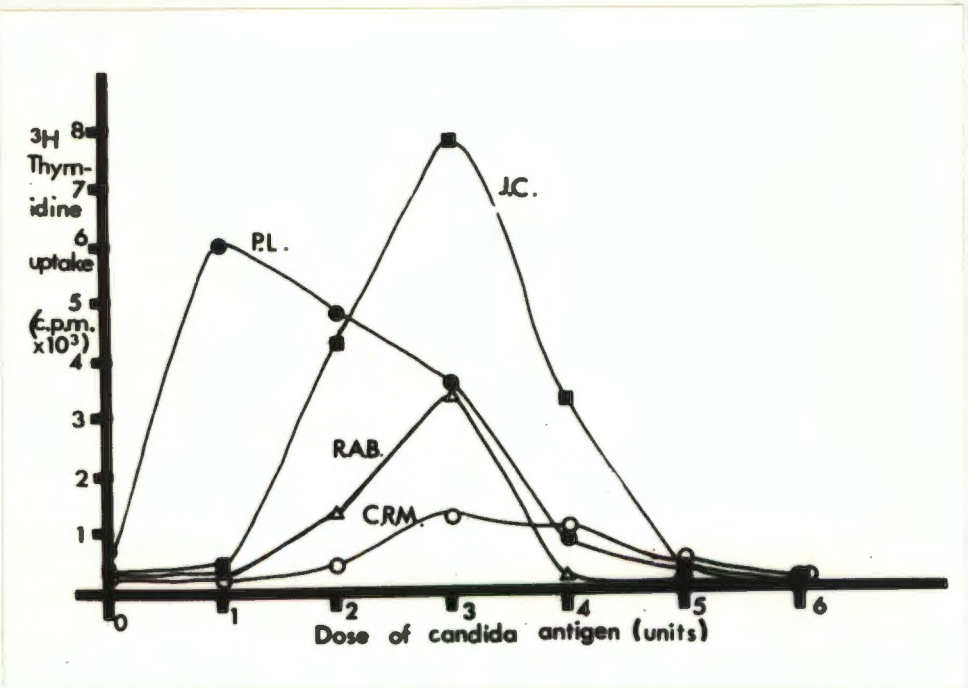

Figure 18: Dose-response curve of lymphocyte stimulation of 4 normal subjects to Candida antigens.

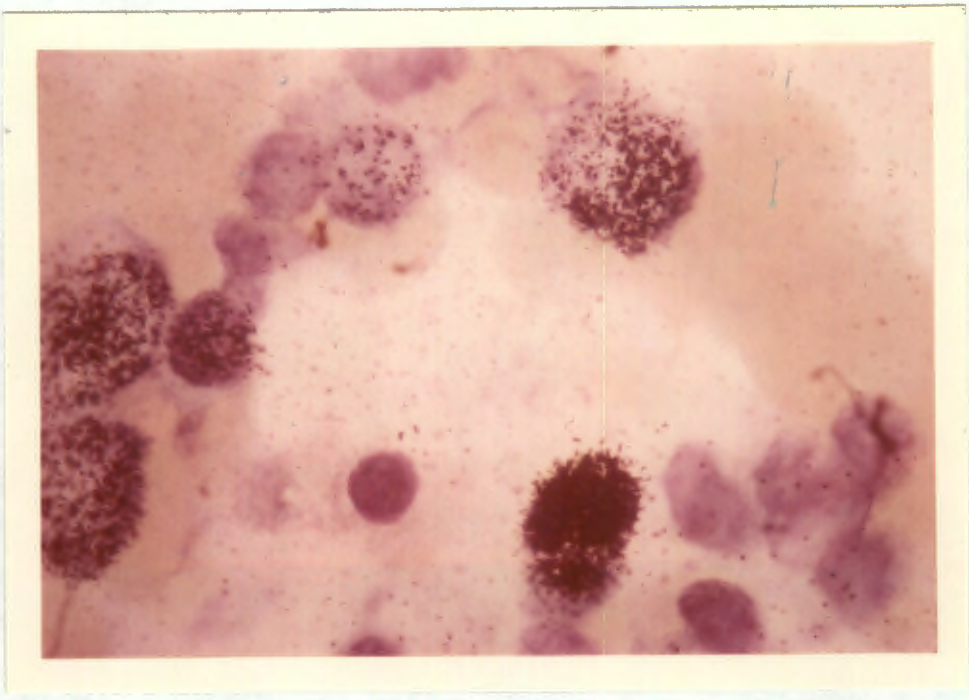

Figure 19: Photomicrograph of autoradiography of lymphocytes stimulated by Candida antigens. ^{3}H-thymidine (black granules) has been taken up into transformed cells only. There is minimal background radioactivity. [Leishman stain : × 950.]

Figure 20: Lymphocyte transformation in response to Candida antigens of 55 normal adult subjects.

Subject	Age and Sex	Mean of Control Readings	Mean Response to Candida	Index of Response
M.T.	M 19	5·4 per cent	25·6 per cent	4·7
G.R.	M 31	11·0 " "	17·8 " "	1·6
A.P.	F 46	2·3 " "	19·2 " "	9·4
A.S.	M 38	1·0 " "	2·6 " "	2·6
R.S.	F 35	1·6 " "	4·3 " "	2·7
S.F.	M 22	1·0 " "	3·7 " "	3·7
E.T.	M 21	1·8 " "	4 " "	2·2
L.H.	F 21	1·0 " "	3·2 " "	3·2
C.K.	F 19	322 c.p.m.	1,114 c.p.m.	3·5
A.D.	F 20	719 "	958 "	1·3
S.H.	F 23	215 "	932 "	4·3
C.M.	M 20	400 "	1,159 "	2·9
R.B.	M 18	290 "	3,385 "	11·7
J.C.	M 18	250 "	7,909 "	31·6
P.L.	M 18	750 "	3,551 "	4·8
A.McI.	F 20	1,998 "	8,753 "	4·4
L.S.	M 19	1,159 "	17,765 "	15·4
F.O.	F 19	534 "	1,178 "	2·2
G.M.	M 19	1,159 "	5,922 "	5·1
R.B.	M 18	1,353 "	1,784 "	1·3
S.D.	F 22	1,271 "	2,729 "	2·2
M.H.	M 19	176 "	443 "	2·5
J.C.	F 22	1,545 "	1,112 "	0·8
L.A.	F 23	180 "	454 "	2·5
S.H.	F 22	131 "	772 "	5·9
C.S.	F 21	2,712 "	6,855 "	2·5
R.W.	F 20	731 "	3,936 "	5·4
C.M.	F 21	260 "	648 "	2·5
J.O.	M 21	73 "	140 "	1·9

Contd.

Figure 20 (Contd.)

Subject	Age and Sex	Mean of Control Readings	Mean Response to Candida	Index of Response
F.T.	F 20	478 c.p.m.	347 c.p.m.	0·7
H.W.	M 46	287 "	421 "	1·5
P.W.	M 22	475 "	1,334 "	2·8
A.B.	M 47	1,424 "	1,815 "	1·2
M.L.	M 19	1,150 "	1,150 "	1·0
T.D.	M 38	989 "	8,130 "	9·3
C.M.	F 41	921 "	13,017 "	14·1
T.O.	M 20	141 "	683 "	4·9
R.A.	M 25	218 "	215 "	0·9
F.M.	M 46	8,228 "	41,634 "	5·1
V.McA.	F 51	244 "	2,327 "	9·7
A.A.	M 39	108 "	411 "	3·8
V.A.	M 34	138 "	271 "	2·0
G.M.	M 22	90 "	147 "	1·6
G.M.	F 18	99 "	246 "	2·5
H.W.	F 41	1,463 "	367 "	0·3
A.G.	M 58	122 "	180 "	1·5
B.B.	M 47	583 "	4,680 "	8·3
I.H.	M 37	551 "	15,078 "	28·6
C.W.	F 19	167 "	521 "	3·1
M.W.	F 32	687 "	9,154 "	13·3
B.B.	M 27	748 "	494 "	0·7
E.P.	M 41	528 "	305 "	0·6
R.W.	M 29	204 "	270 "	1·3
R.P.	M 38	120 "	290 "	2·2
M.P.	M 21	603 "	354 "	0·6

Figure 21: The frequency distributions of the lymphocyte responses of the normal population to stimulation with Candida antigens.

Index of Response	Number	Percentage
0 < 1	7	12·7
1 < 2	11	20·0
2 < 3	13	23·6
3 < 4	5	9·2
4 < 5	5	9·2
5 < 6	4	7·3
6 < 7	0	—
7 < 8	0	—
8 < 9	1	1·8
9 < 10	3	5·5
10 < 11	0	—
11 < 12	1	1·8
13 < 14	1	1·8
14 < 15	1	1·8
15 < 16	1	1·8
28 < 29	1	1·8
31 < 32	1	1·8
TOTAL	55	≡ 100

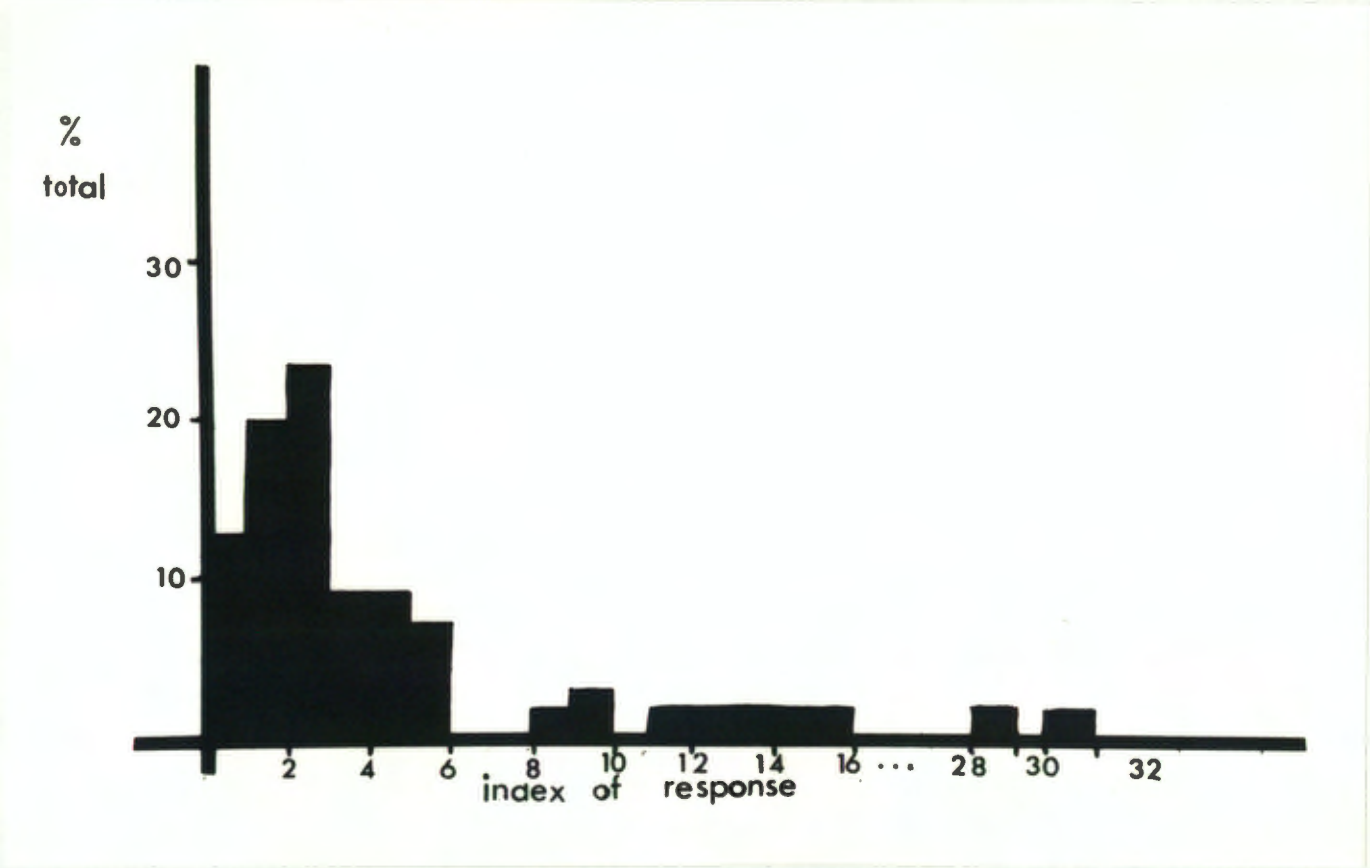

Figure 22: The frequency distributions of the lymphocyte responses of the normal population to stimulation with Candida antigens. [55 subjects.]

Figure 23: Lymphocyte transformation in response to Candida antigens of 8 patients undergoing intermittent haemodialysis for chronic renal failure.

Patient	Mean of Control Readings	Mean Response to Candida	Index of Response
D.S.	509 c.p.m.	1,849 c.p.m.	3·6
D.H.	308 "	305 "	0·9
P.G.	298 "	158 "	0·5
G.W.	300 "	1,204 "	4·0
J.L.	411 "	161 "	0·4
A.B.	1,423 "	4,562 "	3·2
C.L.	697 "	6,827 "	9·8
H.G.	1,037 "	5,351 "	5·2

Figure 24: Lymphocyte transformation to Candida antigens of 21 patients receiving combined azathioprine and prednisone therapy.

Patient	Mean of Control Readings	Mean Response to Candida	Index of Response
A.F.	284 c.p.m.	142 c.p.m.	0·5
W.C.	5,097 "	2,464 "	0·5
G.E.	179 "	69 "	0·4
J.C.	68 "	196 "	2·9
W.R.	1,302 "	542 "	0·4
P.L.	1,201 "	3,085 "	2·6
T.Q.	2,703 "	8,076 "	2·9
D.S.	220 "	193 "	0·9
D.H.	307 "	254 "	0·9
R.R.	419 "	282 "	0·8
K.M.	230 "	354 "	1·5
D.L.	394 "	321 "	0·8
G.W.	1,405 "	1,316 "	0·9
H.A.	15,455 "	7,916 "	0·5
C.W.	457 "	558 "	1·2
J.L.	139 "	159 "	1·1
A.W.	1,049 "	315 "	0·3
P.H.	110 "	153 "	1·4
D.R.	579 "	501 "	0·9
K.M.	666 "	665 "	1·0
D.E.	441 "	293 "	0·7

Figure 25: Lymphocyte transformation in response to Candida antigens of 17 patients receiving steroid therapy.

Patient	Mean of Control Readings	Mean Response to Candida	Index of Response
E.H.	194 c.p.m.	184 c.p.m.	0·9
A.D.	222 "	270 "	1·2
E.B.	147 "	131 "	0·9
J.A.	1,428 "	2,019 "	1·4
M.D.	423 "	371 "	0·9
M.M.	314 "	119 "	0·4
M.J.	361 "	325 "	0·9
C.C.	389 "	230 "	0·6
F.S.	248 "	327 "	1·3
A.D.	118 "	325 "	2·7
J.B.	212 "	397 "	1·9
B.H.	238 "	176 "	0·7
J.A.	267 "	135 "	0·6
E.D.	205 "	306 "	1·5
J.A.	506 "	361 "	0·7
J.O.	1,720 "	168 "	0·1
J.D.	3,792 "	667 "	0·2

Figure 26: Lymphocyte transformation in response to Candida antigens of 6 patients receiving cyclophosphamide therapy.

Patient	Mean of Control Readings	Mean Response to Candida	Index of Response
C.R.	188 c.p.m.	376 c.p.m.	1·9
A.W.	146 "	94 "	0·6
B.W.	240 "	172 "	0·7
J.P.	540 "	478 "	0·9
J.R.	397 "	213 "	0·5
K.D.	3,298 "	1,658 "	0·5

Figure 27: The index of response of lymphocytes stimulated by P.H.A.; a comparison of 24 normal subjects and 11 patients receiving long-term steroid therapy.

NORMAL SUBJECTS (24)		PATIENTS RECEIVING STEROIDS (11)	
Name	Index of Response	Name	Index of Response
M.T.	16·7	E.H.	18·2
G.R.	8·4	A.D.	4·2
A.S.	90·0	E.B.	24·5
R.S.	56·3	J.A.	41·8
S.F.	94·0	M.D.	109·2
L.A.	27·0	B.H.	3·1
S.H.	64·1	M.M.	20·8
C.S.	2·9	C.C.	16·5
G.M.	18·3	J.A.	203·0
R.W.	18·3	A.D.	25·9
C.M.	25·5	S.B.	50·9
F.T.	23·2		
H.W.	21·4		
P.W.	222·7		
A.B.	29·6		
A.P.	78·3		
M.L.	72·6		
F.M.	22·5		
H.W.	2·2		
B.B.	106·0		
I.H.	156·4		
R.W.	46·3		
R.P.	29·0		
M.P.	94·2		

B) RESULTS OF AGGLUTINATING AND IMMUNOFLUORESCENT ANTIBODY INVESTIGATIONS

Introduction

There is general agreement at the present time that the relationship of Candida antibodies in the blood to any increased resistance is inadequately understood (Longbottom, Murray and Pepys, 1968). From the clinical viewpoint, those diseases characterised by humoral immunity - antibody deficiency syndromes - do not appear to be associated with an increased incidence of Candida infection (Soothill, 1968). Experimentally, Dobias (1964) attempted to protect laboratory animals from the lethal effects of Candida with agglutinating antibodies to Candida. No such protection was demonstrable. This was attributed to a toxin produced by Candida which was not affected by the agglutinins.

On the other hand, there can be little doubt that the serum factors which are responsible for *in vitro* agglutinating, complement-fixing, precipitating, and fixation of anti-antibody labelled with fluorescein with Candida antigen are indeed antibodies in nature. They increase in titre in the face of active infection, they are found in higher titres in subjects suffering from Candida infection than in normal subjects, and they have been shown by cellulose chromatography fractionation to be located in the immunoglobulin fraction of the serum proteins (Lehner, 1970).

There is little documented regarding the effects of immunosuppressive and steroid drug regimes on humoral immunity to Candida. Brody and Finch (1960) studied levels of Candida-reacting antibodies in the serum of patients with lymphomas and related disorders. Antibody to Candida was measured by a quantitative immune-adherence technique. Their findings suggested that the antibody-producing mechanisms to Candida are extremely resistant to the adverse effects of neoplastic growth and chemotherapeutic agents, and their conclusions were that "the lack of correlation between

serum antibody titre and mycotic infection is in accord with the concept that humoral antibody is not absolute evidence of immunity".

Rifkind, Marchioro, Waddell and Starzl (1964) showed that infectious diseases associated with renal homotransplantation, which were frequently fungal in nature, were generally associated with hypogammaglobulinaemia due to immunosuppressive therapy. This view was the opposite to that of Brody and Finch. They did recognize, however, that such immunoglobulin depletion, and the steroid-induced diabetes and granulocytopenia which frequently antedated the development of such fungal infections, may merely have served as measurable parameters of more subtle defects.

Eibl and Thump (1970) showed by serial measurement of isoagglutinin, pertussis, diphtheria and measles antibodies that no significant fall occurred in twenty patients with rheumatoid arthritis treated with long-term maintenance chlorambucil therapy. With more intensive therapy there was a fall in antibody titre, but this rose to normal levels again with reduction of dosage to previous maintenance levels.

It was against this background that the following experiments were designed, to establish whether patients whose cellular immunity to Candida had been impaired by chemotherapy had depressed humoral immunity as well. In addition, a prospective study was undertaken in experimental mice.

Experiment XI : The effect of steroid and of immunosuppressive drug therapy on serum levels of agglutinin to Candida.

Introduction

The titre of serum agglutinins to Candida of subjects receiving drug therapy with steroids, long-term azathioprine and prednisone, and cyclophosphamide was compared with the titres of agglutinins of fairly well matched normal controls. All the treated patients studied had been shown

by lymphocyte transformation testing to have depressed cellular immunity to Candida.

Method

The method of quantitative estimation of serum agglutinins to Candida was detailed in Chapter 2. Patients were studied from the following treatment groups:

1. Long-term steroid therapy: 8 patients.
2. Azathioprine and prednisone therapy: 14 patients.
3. Long-term cyclophosphamide therapy: 4 patients.

These results were compared with those of sixteen normal subjects.

All sera were stored in a refrigerator at - 20°C. prior to use.

Results

The results of this investigation are tabulated in figures 28, 29, 30 and 31 and are represented graphically in figure 32. The data indicate that long-term steroid therapy and combined therapy with azathioprine and prednisone had no significant effect on agglutinin titres to Candida compared with the titres of normal control subjects. The serum agglutinin levels of the small group of patients treated with cyclophosphamide were, however, depressed.

Experiment XII : The effect of steroid and of immunosuppressive drug therapy on the serum levels of antibody to Candida measured by the indirect immunofluorescent antibody technique.

Introduction

The objectives of this experiment were precisely the same as those of the previous experiment; the case selection was according to the same principles, and the control subjects were the same subjects.

Method

The indirect quantitative immunofluorescent antibody technique used in this experiment was described in Chapter 2. The following treated and normal subjects were studied:

1. Normal subjects: 16.
2. Long-term steroid therapy: 14 patients.
3. Azathioprine and prednisone therapy: 18 patients.
4. Long-term cyclophosphamide therapy: 4 patients.

All sera were stored in a refrigerator at - 20°C. prior to testing, and none was older than three months.

Results

A normal result is represented in figure 33. The results obtained in this investigation are tabulated in figures 34, 35 and 36. The results of this investigation are represented graphically in figure 37.

With the exception of cyclophosphamide therapy, there was no significant difference in titre between treated patients and normal control subjects. In the small group of cyclophosphamide-treated patients there appeared to be significant depression of serum antibody levels detected by this technique.

Experiment XIII : A prospective study of the effect of immunosuppressive and steroid drug regimes on the serum agglutinin levels to Candida of experimental mice.

Introduction

The majority of normal adult female laboratory mice were found to have serum agglutinins to Candida albicans to a titre of 1:8 to 1:16. A prospective study over a two month period was undertaken to assess whether long-term steroid, azathioprine and combined azathioprine and prednisone

therapy significantly altered the titre of serum agglutinins compared with control mice.

Method

Details of the laboratory mice used, the doses of the drugs used, and the method of estimation of agglutinins were given in Chapter 2.

Seventy normal adult female laboratory mice were studied. Ten served as controls. Groups of twenty received daily treatment with azathioprine, cortisone, and combined azathioprine and cortisone respectively.

At two-weekly intervals five mice from each group were killed, and blood was taken from the carotid artery. Serum was obtained from the blood after centrifugation. The sera were stored in a refrigerator at - 20°C., and all the tests were done at the same time.

Results

No change in the titre of agglutinating antibody to Candida was produced over an eight week period by the various steroid and immunosuppressive drug regimes described (figure 38).

C) AN INVESTIGATION OF SERUM ANTICANDIDAL ACTIVITY

Experiment XIV : A study of the anti-Candida factor in normal human serum.

Introduction

The work of Louria and Brayton (1964) was investigated in this study.

Method

The method used was described in Chapter 2.

The serum of fifty-two normal subjects (blood donors) was used. All sera were stored in a refrigerator at - 20°C. prior to use. No serum was stored for more than six weeks prior to use.

Results

A log 1 (10 ×) fall in Candida census over a six hour period was taken to be a significant fall. Such a fall in Candida census was observed with forty-six out of the fifty-two normal sera studied. The findings of Louria and Brayton for normal persons were thus confirmed.

In the last twenty-four tests, however, direct microscopical examination in addition to viable counting was performed. The findings in all twenty-four tests were of mycelial formation and clumping of the Candida organisms at the six hour interval. This phenomenon is demonstrated in figure 39. This clumping effect was found to have been induced by the serum within two hours.

It was felt that the clumping phenomenon precluded sufficient reliance being placed on this assay system as representing serum candidacidal activity.

A detailed investigation of the effect of steroid and immunosuppressive drug therapy on this serum factor was therefore abandoned.

Comment

The experimental system described by Louria and Brayton (1964) was reproduced and their findings with normal human sera were confirmed. However, clumping of the Candida organisms at the six hour period suggested that a technique of viable counting by pour-plate technique or by the method described by Miles and Misra (1938) was largely invalidated, and that the information to be obtained from such an assay system was limited.

Figure 28: Quantitative estimation of agglutinating antibodies to Candida of normal subjects (16).

Subject	Agglutinating Titre					
	1:2	1:4	1:8	1:16	1:32	1:64
P.F.	+	+	+	+	±	-
R.P.	+	+	+	+	+	-
S.R.	+	+	+	+	±	-
R.F.	+	+	+	-	-	-
C.D.E.	+	+	+	+	+	-
J.S.	+	+	+	-	-	-
H.W.J.	+	+	+	+	-	-
W.D.	+	+	+	+	-	-
G.H.	+	+	+	+	±	-
E.W.	+	+	+	+	-	-
F.P.	+	+	+	+	-	-
R.W.	+	+	+	±	-	-
R.B.	+	+	+	+	-	-
E.G.	+	+	+	+	±	-
F.D.	+	+	+	+	-	-
M.E.	+	+	+	-	-	-

Figure 29: Quantitative estimation of agglutinating antibodies to Candida of patients receiving steroids (8).

Subject	Agglutinating Titre					
	1:2	1:4	1:8	1:16	1:32	1:64
J.O.	+	+	+	+	-	-
E.D.	+	+	+	+	+	-
C.C.	+	+	+	+	-	-
M.D.	+	+	+	+	+	-
J.B.	+	+	+	+	-	-
A.D.	+	+	+	+	±	-
A.D.	+	+	-	-	-	-
E.H.	+	+	+	+	-	-

Figure 30: Quantitative estimation of agglutinating antibodies to Candida of patients treated with azathioprine and prednisone (14).

Subject	Agglutinating Titre					
	1:2	1:4	1:8	1:16	1:32	1:64
A.F.	+	+	-	-	-	-
A.T.	+	+	+	-	-	-
S.G.	+	+	+	+	-	-
W.C.	+	+	+	+	-	-
K.B.	+	+	+	+	±	-
K.M.	+	+	+	+	+	-
J.C.	+	+	+	+	-	-
J.B.	+	+	+	+	-	-
M.R.	+	+	+	+	-	-
R.R.	+	+	+	+	±	-
M.R.	+	+	+	-	-	-
D.L.	+	+	+	-	-	-
P.L.	+	+	+	-	-	-
D.H.	+	+	+	+	-	-

Figure 31: Quantitative estimation of agglutinating antibodies to Candida of patients treated with cyclophosphamide (4).

Subject	Agglutinating Titre					
	1:2	1:4	1:8	1:16	1:32	1:64
J.R.	+	+	-	-	-	-
B.W.	+	-	-	-	-	-
C.R.	+	-	-	-	-	-
P.G.	+	+	-	-	-	-

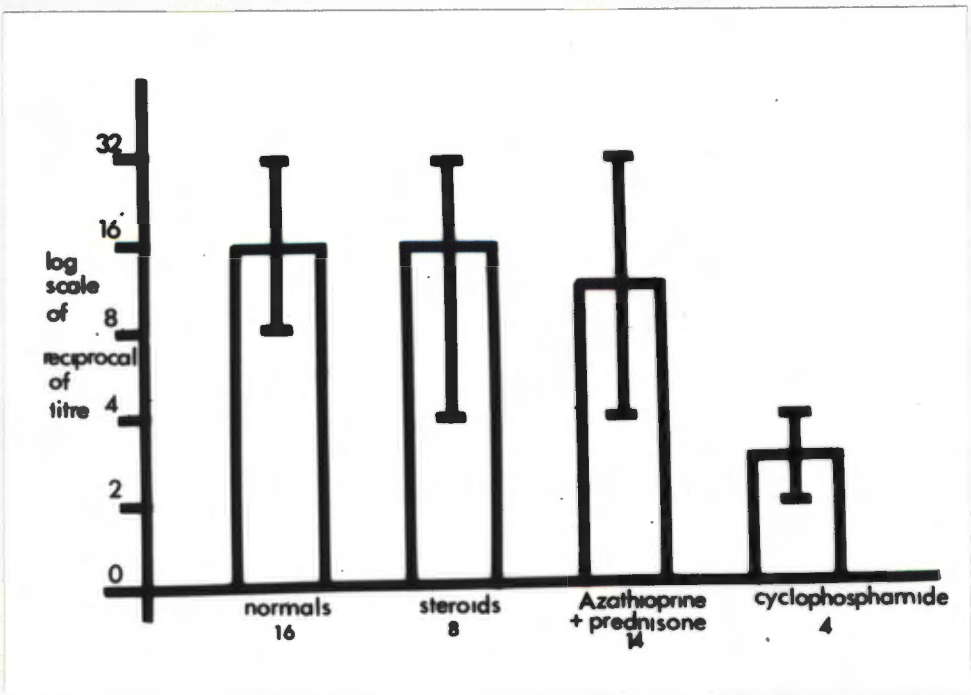

Figure 32: Quantitative estimation of agglutinating antibodies to Candida of normals and treated patients.

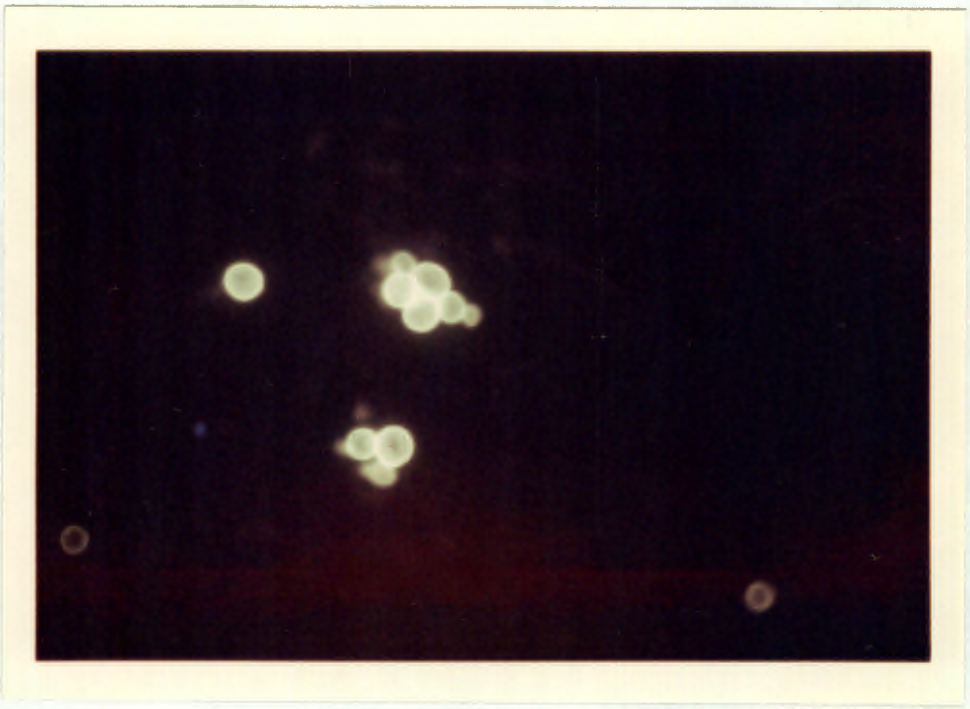

Figure 33: Antibodies to C. albicans demonstrated by the "indirect fluorescent antibody technique". (× 63)

Figure 34: Quantitative estimation of antibodies to Candida by the indirect immunofluorescent technique. Normals (16).

Subject	+ve titre					
	1:2	1:4	1:8	1:16	1:32	1:64
P.F.	+	+	+	+	±	-
R.P.	+	+	+	+	+	-
S.R.	+	+	+	+	-	-
R.F.	+	+	+	+	-	-
L.D.E.	+	+	+	+	+	-
J.S.	+	+	+	±	±	-
H.W.J.	+	+	+	+	±	-
W.D.	+	+	+	+	±	-
G.H.	+	+	+	+	+	-
E.W.	+	+	+	-	-	-
F.P.	+	+	+	+	-	-
R.W.	+	+	+	-	-	-
R.B.	+	+	+	+	-	-
E.G.	+	+	+	+	±	-
F.D.	+	+	+	+	-	-
M.E.	+	+	+	-	-	-

Figure 35: Quantitative estimation of antibodies to Candida by the indirect immunofluorescent technique.

Steroid-treated patients (14). Cyclophosphamide therapy (4).

Subject	+ve titre					
	1:2	1:4	1:8	1:16	1:32	1:64
steroids:						
J.O.	+	+	+	+	-	-
E.D.	+	+	+	+	+	-
C.C.	+	+	+	+	-	-
M.D.	+	+	+	+	-	-
J.B.	+	+	+	-	-	-
A.D.	+	+	+	+	±	-
A.D.	+	+	-	-	-	-
E.H.	+	+	+	+	-	-
E.B.	+	+	+	+	-	-
J.A.	+	+	+	+	+	-
M.J.	+	+	+	+	+	-
F.S.	+	+	+	+	-	-
B.H.	+	+	+	+	-	-
J.A.	+	+	+	+	-	-
cyclophosphamide:						
J.R.	+	+	+	-	-	-
B.W.	-	-	-	-	-	-
C.R.	+	-	-	-	-	-
P.G.	+	+	-	-	-	-

Figure 36: Quantitative estimation of antibodies to Candida by the indirect immunofluorescent technique.

Patients treated with azathioprine and prednisone (18).

Subject	+ve titre					
	1:2	1:4	1:8	1:16	1:32	1:64
A.F.	+	+	+	-	-	-
A.T.	+	+	+	±	-	-
S.G.	+	+	+	+	-	-
W.C.	+	+	+	+	-	-
K.B.	+	+	+	+	±	-
K.M.	+	+	+	+	+	-
J.C.	+	+	+	-	-	-
J.B.	+	+	+	+	-	-
M.R.	+	+	+	+	-	-
R.R.	+	+	+	+	±	-
M.R.	+	+	+	+	-	-
D.L.	+	+	+	+	-	-
P.L.	+	+	+	+	-	-
D.H.	+	+	+	+	±	-
G.E.	+	+	+	+	-	-
W.R.	+	+	+	+	-	-
T.Q.	+	+	+	+	-	-
D.S.	+	+	+	+	-	-

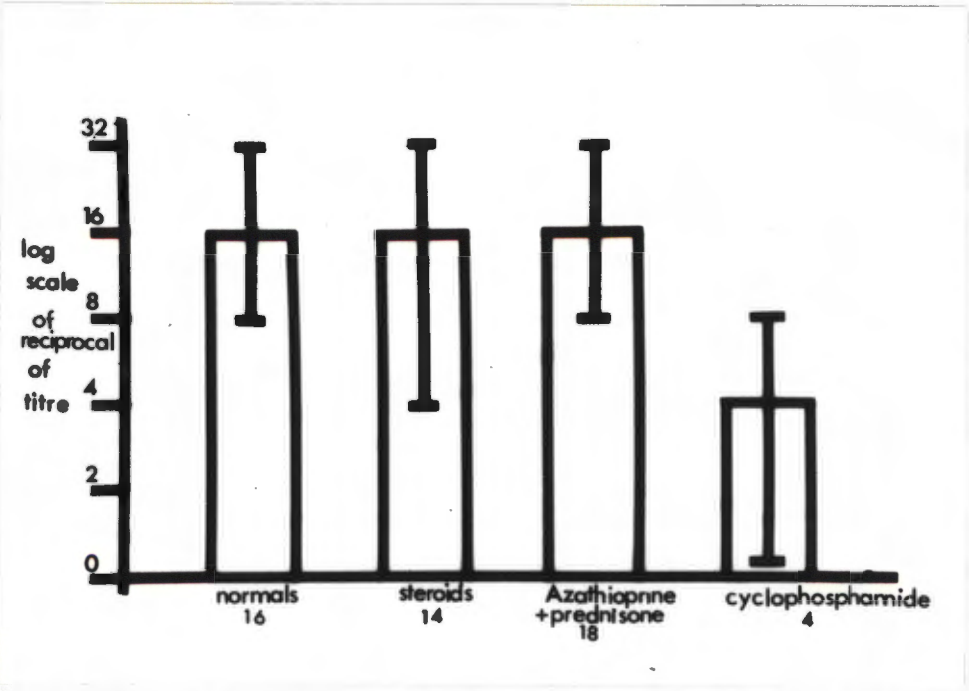

Figure 37: Quantitative estimation of antibodies to C. albicans by the indirect immunofluorescence technique.

Figure 38: Results of a prospective study of the effect of immunosuppressive and steroid drug regimes on the serum agglutinin levels to Candida of experimental mice.

Time	Mice	Reciprocal of titre of serum agglutinins	Mean (approx.)
0 weeks	Normals	8 ; 8 ; 8 ; 8 ; 16 ; 16 ; 16 ; 16 ; 16 ; 16	8-16
2 weeks	Treated with		
	- azathioprine	8 ; 16 ; 16 ; 16 ; 16	16
	- cortisone	8 ; 16 ; 16 ; 16 ; 16	16
	- azathioprine + cortisone	16 ; 16 ; 16 ; 16 ; 16	16
4 weeks	Treated with		
	- azathioprine	8 ; 16 ; 16 ; 16 ; 16	16
	- cortisone	8 ; 8 ; 16 ; 16 ; 16	16
	- azathioprine + cortisone	8 ; 16 ; 16 ; 16 ; 16	16
6 weeks	Treated with		
	- azathioprine	8 ; 8 ; 16 ; 16 ; 16	16
	- cortisone	16 ; 16 ; 16 ; 16 ; 16	16
	- azathiorpine + cortisone	8 ; 16 ; 16 ; 16 ; 16	16
8 weeks	Treated with		
	- azathioprine	16 ; 16 ; 16 ; 16 ; 16	16
	- cortisone	8 ; 16 ; 16 ; 16 ; 16	16
	- azathioprine + cortisone	8 ; 8 ; 16 ; 16 ; 16	16

Figure 39: Mycelium formation and clumping of C. albicans induced by serum over a 6-hour period.

D) RESULTS OF THE CANDIDA ALBICANS MOUSE-THIGH LESION STUDIES

Introduction

Selbie and O'Grady (1954) considered that the tuberculous mouse-thigh lesion closely resembled localised tuberculous disease in man in its natural history, and that this experimental lesion was therefore of considerable value in the assessment of the effects of anti-tuberculous therapy on localised infection. The same sentiment was expressed when Candida infection was produced in mice in a similar manner (O'Grady and Thompson, 1958).

There are, however, certain limitations to the analogy of the murine and human infections. With intravenous or intraperitoneal administration of organisms to the experimental model generalisation of infection takes place much more acutely than in natural human infection. Even good specific immunity might not prevent the death of some animals in which acute generalised candidiasis is produced by an injection of a massive inoculum of organisms directly into the blood stream. An experimental model in which systemic candidiasis in animals would be produced in a similar manner as in man, namely by dissemination through invasion of the blood stream from the gastrointestinal tract, has not been designed. Animals fed with Candida by mouth cannot be infected regularly (Dobias, 1964).

Nevertheless, with the limitations of the experiment borne in mind, it was considered to be of interest to investigate the effects, if any, of cortisone, azathioprine and mouse anti-lymphocyte serum on the natural history of the experimental Candida albicans mouse-thigh lesion. The cumulative mortality rate, local size of the lesion, tendency to disseminate and the histological changes at the site of the lesion were studied.

The results are reported in the following experiments.

Experiment XV : Cumulative mortality studies.

Method

Four groups of mice were studied to compare the cumulative mortality over a fourteen day period in mice administered 15×10^6 Candida albicans cells into the thigh, and the effects on mortality rate of treatment with azathioprine and cortisone.

The method of administration of Candida into the thigh, and the doses and routes of administration of the drugs given, were detailed in Chapter 2.

Post mortem examination, which included detailed macroscopic examination of all organs, and histological examination of kidneys, liver, spleen and endocardium was performed on all the mice that died.

A group of twelve mice were given the same number of Candida organisms directly intravenously. Two groups of control mice were treated with azathioprine and with cortisone respectively, but no Candida lesion was produced.

Results

The cumulative mortality and the percentage mortality of the mice in each group given Candida are shown in figure 40. It was clear that the various forms of drug therapy did not appreciably affect the mortality rate compared with that of the control mice.

Post mortem examination generally revealed that death of the mice was associated with limited disseminated infection, and that when the infection did disseminate it was the kidney that was the target organ (figure 41).

None of the mice in the two control drug-treated groups died over the fourteen day period.

Direct intravenous injection of Candida in numbers equal to those given locally into the thigh was invariably fatal within twenty-four hours of administration.

Comment

Treatment of mice with Candida albicans-thigh lesions with azathioprine or cortisone did not significantly affect either the mortality rate or the tendency of the infection to disseminate. Drug therapy alone did not produce fatalities, and therefore did not appear to be toxic in its own right.

Treatment with azathioprine or with cortisone did not tend to convert a local lesion to one which resembled the disease produced by direct intravenous injection.

Experiment XVI : The effect of azathioprine and of cortisone on the Candida mouse-thigh lesion.

Method

15×10^6 Candida organisms were injected into the thigh of mice in the usual way. Physiological 0.9% sodium chloride was injected into the thigh of control mice. The maximum diameter of the thigh of each mouse was measured daily for the following seven days.

The mice injected with Candida were divided into the following groups:

1. Control mice, i.e. no treatment administered: 14.
2. Mice treated with azathioprine: 14.
3. Mice treated with cortisone: 10.
4. Mice treated with mouse anti-lymphocyte serum: 10.

For each group the mean ($\bar{X}$) and the standard error of the mean (S.E.) of the readings of each day was calculated, and the calculation of the statistical significance of the difference of the means (t) of the various treated groups from those of the control mice was from the formula

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{(S.E.\ \bar{X}_1)^2 + (S.E.\ \bar{X}_2)^2}}$$

The significant levels of t for a significance level of 0.05 for the total number of readings made minus 2 was obtained from Student tables.

A full blood count (haemoglobin, white blood cell count, and differential count) was performed on four mice from each group at two-day intervals.

Results

The means, together with the standard errors of the means, of the daily readings of the control group and of each of the treatment groups are tabulated in figure 42 and represented graphically in figure 43. Calculations showing levels of significance for difference between the size of the thigh in controls and those given azathioprine and cortisone are shown in figures 44 and 45 respectively.

Treatment with both azathioprine and with cortisone significantly depressed the local swelling of the thigh over the seven day period (with the exception of days 3, 4, 5 in the cortisone-treated group).

The results of the full blood count done at two day intervals on mice from each treatment group showed that there was no depression of total white blood cell count, lymphocyte count or haemoglobin with any of the forms of treatment. The modifying effect on the local lesion of the various drug regimes was thus shown not to be associated with any drug-induced haematological aberration.

Mouse anti-lymphocyte serum, given in doses large enough to eliminate normal lymphocyte function in mice (Medawar, 1969), did not depress local swelling in the acute phase. This result suggested that any diminution in the size of the thigh lesion could not be attributed to depression of lymphocyte function caused by drugs. It was of interest that latterly, at the time of development of more chronic inflammatory changes in response to infection, local swelling was reduced in the group treated with M.A.L.S. compared with controls.

Experiment XVII : Morbid anatomical studies.

Method

Following-on from the information obtained from the two previous investigations, a thorough morbid anatomical study was undertaken in an effort to establish what pathological modifications of the local lesion, if any, were produced by the various drug regimes. A systematic post mortem examination, which included histological examination of the thigh lesion, both kidneys, the liver, spleen and endocardium of left ventricle and aortic valve was performed in each case.

Twenty-four mice were divided into the following four groups:

1. Six mice were infected with local (thigh) candidiasis; 15×10^6 viable, pathogenic Candida organisms were injected in the usual manner. No drug therapy was administered to this group.

2. Six mice were given the same local thigh lesion, and treated daily with subcutaneous cortisone.

3. Six mice were given the same local thigh lesion, and treated daily with intraperitoneal azathioprine.

4. Six mice were given the same local thigh disease, and treated with a combination of azathioprine and cortisone by intraperitoneal injection daily.

Two mice from each group were killed on days 1, 4 and 11 following the local injection of Candida, and each mouse was subjected to post mortem examination.

Results

Sections of local lesions of untreated and of treated mice are shown in figures 46, 47, 48 and 49. The conclusions drawn from this series of experiments may be summarised as follows:

a) Injection of Candida into the thigh of normal mice excited an acute inflammatory response with abscess formation. There was no consistent histological difference between the control and the various drug-treated groups. The abscess formed within twenty-four hours. The Candida yeast forms had produced pseudo-hyphae by that time.

b) Very early organisation of the abscess wall could be seen by about the fourth day, although Candida were still present.

c) By the eleventh day the abscesses of all mice were well localised and an abscess wall of cellular fibrous tissue and granulation tissue had formed. The abscess centre was largely necrotic although some pus and Candida could still be seen.

d) Only one mouse showed metastatic abscesses (in one kidney). This was a control mouse (no drugs) at day 4.

In summary, the clear-cut differences in size of the mouse-thigh in the treated mice were not associated with any apparent histological differences. The differences in size are attributed to local inflammatory chemical "effector" factors, in the broadest sense of that term.

Figure 40: Cumulative mortality studies in mice with experimental candidiasis.

Treatment	No. of mice	Cumulative mortality (days) 1.	2.	3.	4.	5.	6.	7. // 14.		% Mortality
Local Candida; no treatment	20	1	1	1	1	1	3	4	4	20%
Local Candida; treated with azathioprine	14	0	2	2	2	2	2	2	2	15%
Local Candida; treated with cortisone	10	2	2	2	2	2	2	2	2	20%
Local Candida; treated with azathioprine + cortisone	10	0	2	2	2	2	2	2	2	20%
Direct intravenous Candida (15×10^6)	12	12	12	12	12	12	12	12	12	100%

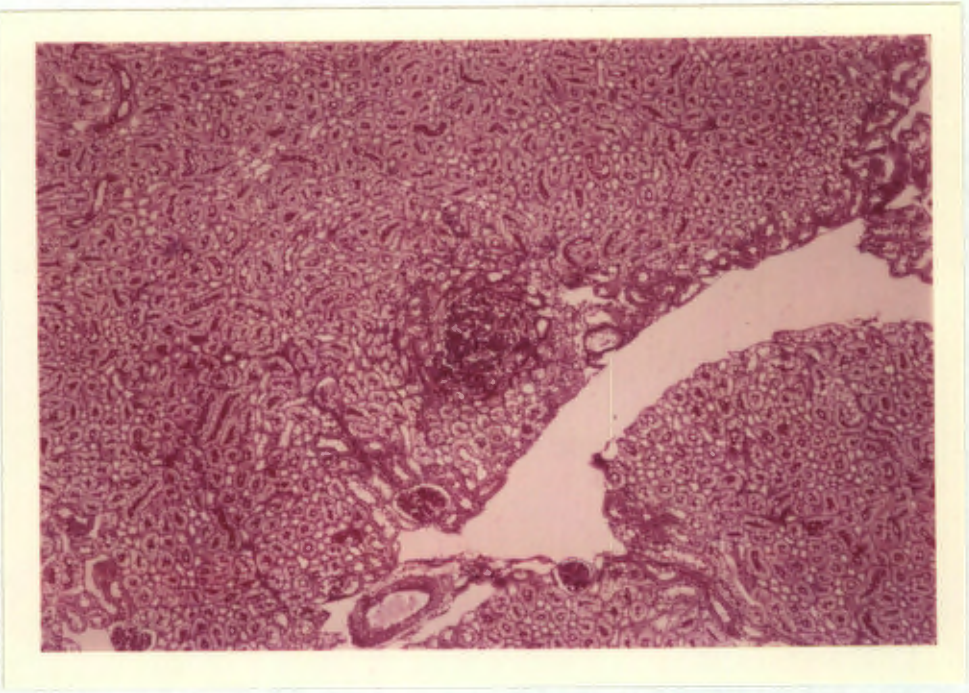

Figure 41: Micro-abscess in the kidney of a mouse due to limited dissemination of Candida from an experimental thigh lesion.

Figure 42: The effect of treatment regimes on the size of the C. albicans mouse-thigh lesion.

Form of treatment	Days 0	1	2	3	4	5	7
Local Candida; no drug treatment. (14)	5·7 (± 0·4)	8·9 (± 0·3)	9·0 (± 0·2)	7·9 (± 0·3)	8·0 (± 0·3)	8·4 (± 0·3)	8·1 (± 0·2)
Local Candida; treatment with azathioprine. (14)	5·4 (± 0·3)	7·8 (± 0·3)	7·9 (± 0·4)	7·3 (± 0·1)	6·9 (± 0·1)	7·1 (± 0·2)	6·2 (± 0·1)
Local Candida; treatment with cortisone. (10)	5·5 (± 0·2)	7·0 (± 0·3)	7·1 (± 0·4)	7·5 (± 0·5)	7·2 (± 0·6)	7·0 (± 0·7)	6·4 (± 0·5)
Local Candida; treatment with M.A.L.S. (10)	5·6 (± 0·1)	8·4 (± 0·2)	7·9 (± 0·2)	7·6 (± 0·2)	7·4 (± 0·3)	7·6 (± 0·3)	7·1 (± 0·3)

Readings are given in mm.; standard errors of the mean are in parentheses.

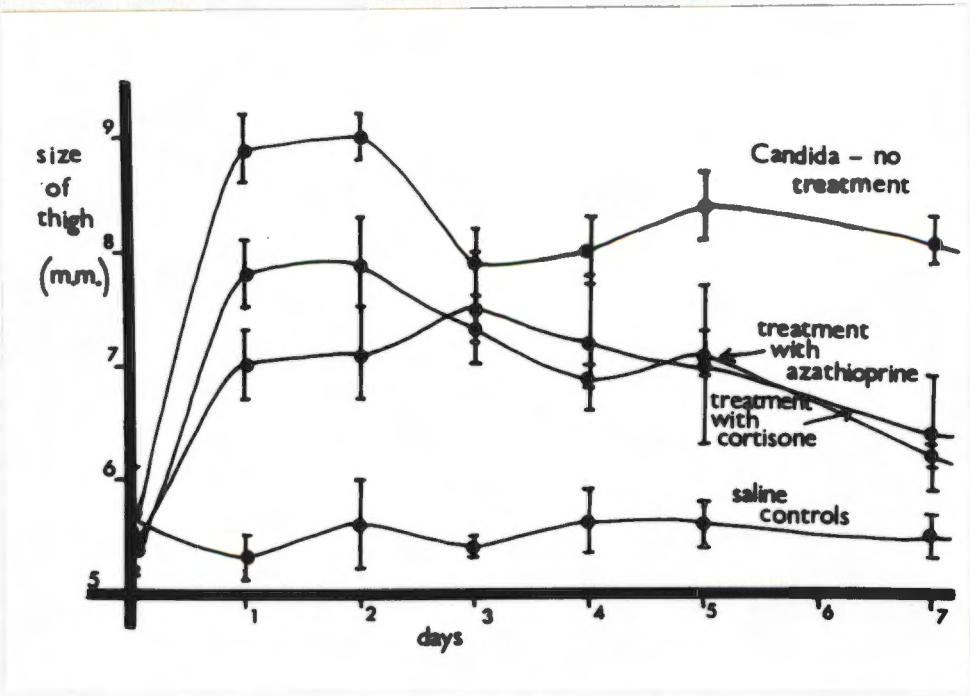

Figure 43: Graphical representation of the effect of treatment regimes on the size of the C. albicans mouse-thigh lesion.

Figure 44: The statistical significance of the differences of the means of the sizes of the mouse-thigh lesions produced by Candida. The control group is compared with the azathioprine-treated group.

The statistical significance of the differences between two means (t) was calculated from the formula

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{(S.E.\ \bar{X}_1)^2 + (S.E.\ \bar{X}_2)^2}}$$

Day	Controls (14)	Azathioprine Group (14)	t
1	8·9	7·8	2·4728
2	9·0	7·9	2·0850
3	7·9	7·3	2·0987
4	8·0	6·9	2·9919
5	8·4	7·1	3·7113
7	8·1	6·2	7·5332

The significant level of t for 26 readings at 0·05 is t = 2·06 (from Student tables).

Figure 45: The statistical significance of the differences of the means of the sizes of the mouse-thigh lesions produced by Candida. The control group is compared with the cortisone-treated group.

The statistical significance of the differences between two means (t) was calculated from the formula

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{(S.E.\ \bar{X}_1)^2 + (S.E.\ \bar{X}_2)^2}}$$

Day	Controls (14)	Cortisone Group (10)	t
1	8·9	7·0	3·7760
2	9·0	7·1	4·0401
3	7·9	7·5	0·7712
4	8·0	7·2	1·1160
5	8·4	7·0	1·9682
7	8·1	6·4	2·9362

The significant level of t for 22 readings at 0·05 is t = 2·07 (from Student tables).

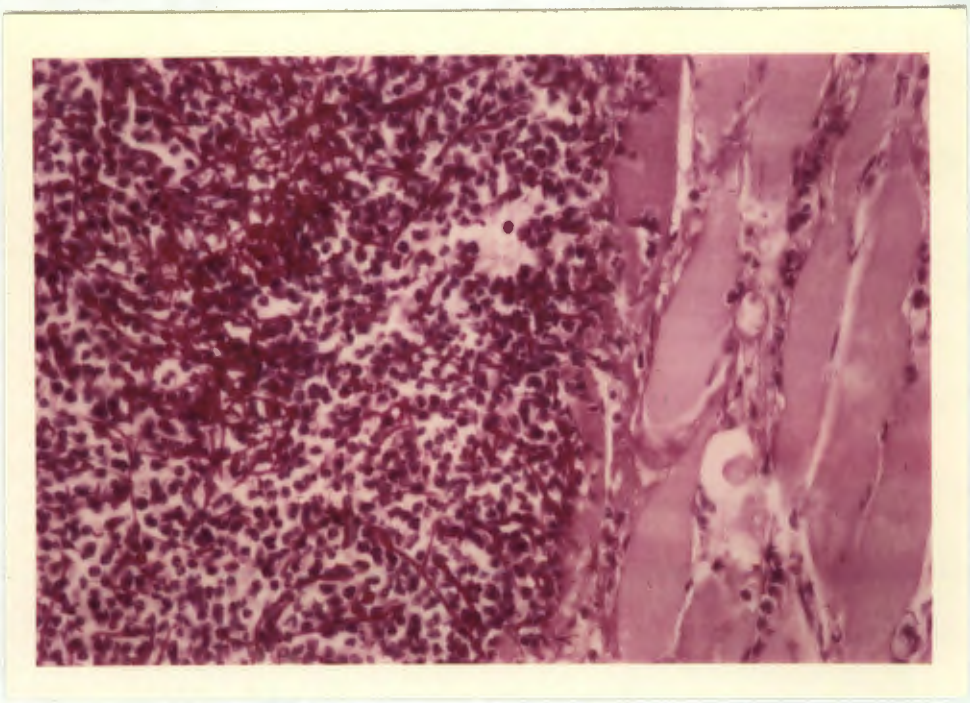

Figure 46: Mouse-thigh candidiasis: day 1.: acute inflammatory reaction; Candida in the mycelial form; surrounding layer of fibrin. (Control group.) [Haematoxylin and eosin.]

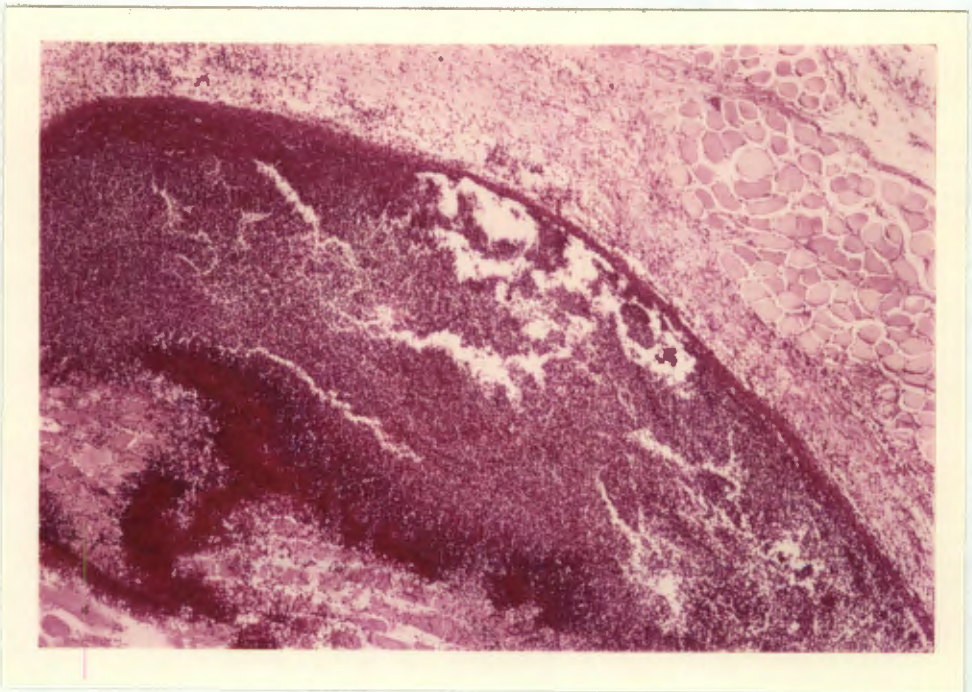

Figure 47: Mouse-thigh candidiasis: day 1.: acute inflammatory reaction, surrounding fibrin layer and clumps of Candida in mycelial form are seen. (Azathioprine group.) [Haematoxylin and eosin.]

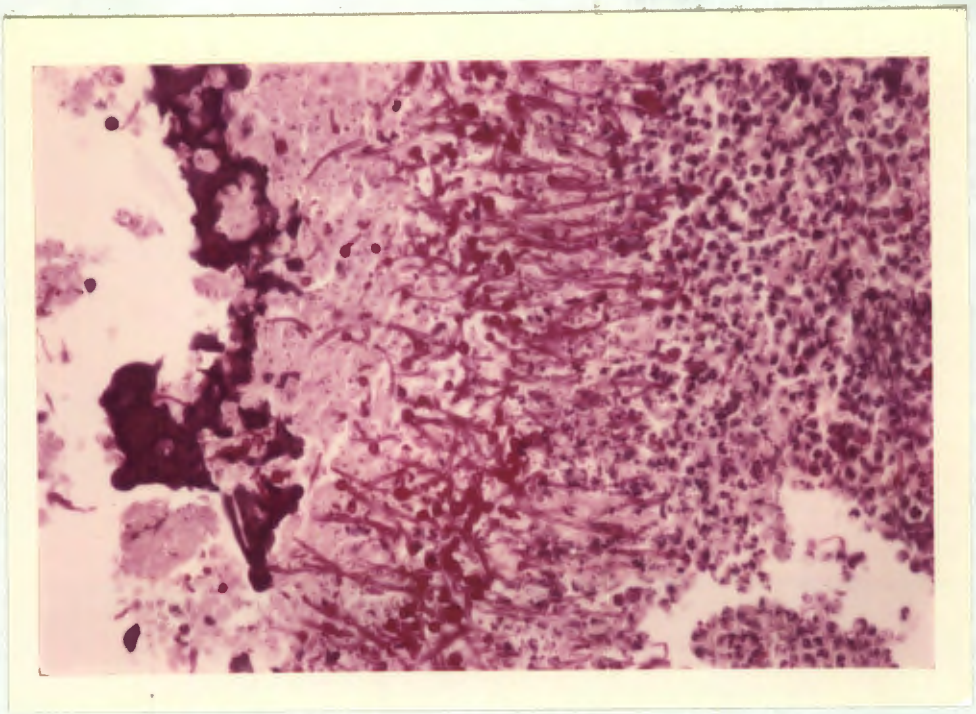

Figure 48: Mouse-thigh candidiasis: day 11. Control group. Early, fairly striking calcification was a feature. [Haematoxylin and eosin.]

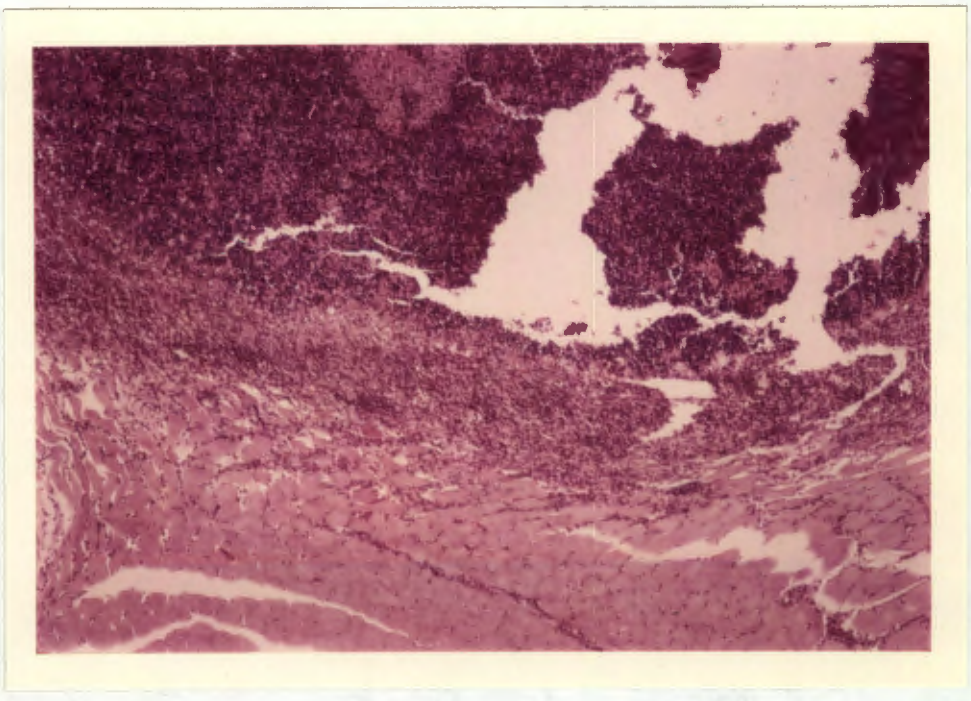

Figure 49: Mouse-thigh candidiasis: day 11. Steroid-treated group. Early granulation tissue is clearly visible. [Haematoxylin and eosin.]

CHAPTER 4 : DISCUSSION

At an International Symposium on Opportunistic Fungal Infection in Man, in 1962, it was noted in the summing up that, in the problem of fungus infection complicating the diseased state, the basic mechanisms underlying the predisposing factors were not understood, and that the avenues for further study in this area were limitless (Chick, 1962).

The ecological relationship between man and Candida organisms has become altered in recent years, so that occasionally the organism achieves a degree of pathogenicity greater than has been known in the past. The statistical evidence is good that this alteration of man-fungus relationship is closely related to recent patterns of drug therapy (Symmers, 1964a). The objective of the investigations reported in this thesis was to make use of recent advances in immunological knowledge and investigative techniques in an attempt to understand this phenomenon.

In general, infections become established in man only if the organism responsible is present in sufficient numbers, or has the opportunity to proliferate, and if the organism gains a portal of entry to the body, and then is able to overcome the resistance of the host (Symmers, 1965).

These principles apply to the particular case of Candida infection. From an analysis of the literature, reported in Chapter 1, it appears that drug therapy of different kinds may theoretically operate at one or more of the following sites, to predispose to local candidiasis, generalised candidiasis, or both:

1. At the level of the gastro-intestinal tract, either by facilitating local proliferation, or by undermining the integrity of the local defence mechanisms, and thus enabling the organisms to penetrate the intestinal wall.

2. By interference with normal "cellular-immunity", i.e. the immunity to micro-organisms which is intrinsic to the normal function of lymphocytes, macrophages and lymph nodes, and their normal inter-relationships (Turk, 1969).

3. By interference with normal humoral defence mechanisms to the organism. The descriptions of potent, lethal activity of normal serum against Candida organisms (Louria and Brayton, 1964) suggested powerful inhibitory activity to the survival of the organism when once it had gained access to the blood stream. This natural resistance might conceivably be impaired by pharmacological agents. Besides an effect on specific anti-Candidal activity of the serum, it is theoretically possible that drugs impair normal antibody activity against Candida. However, although immunoglobulins of all main classes (IgM, IgG and IgA) rise in titre in the serum in subjects with Candida infection (Lehner, 1970) there is no satisfactory evidence that these antibodies exert any protective effect (Murray, 1970).

4. By impairing the process of the normal inflammatory response to the organism when once it has gained access to the tissues.

Incontrovertibly, broad-spectrum antibiotic therapy predisposes to local infection by Candida in the gastrointestinal tract (Cawson, 1969) and there is experimental evidence that certain antibiotics can induce penetration of the intestinal wall by Candida organisms (Beemer et al., 1954). A large amount of work has already been done on the inter-relationships of Candida and antibiotic therapy (Seelig, 1966), and this was not taken further in this study.

Attention was focussed on Candida infection as it complicates steroid and immunosuppressive drug therapy. These drugs are increasingly used in the modern day. The investigations were orientated towards elucidating

the possible effects of these drugs on the cellular factors, the humoral factors and the inflammatory factors which are thought normally to protect against infection.

The integrity of cellular immune mechanisms to Candida *in vivo* was assessed in this study by the *in vitro* lymphocyte transformation phenomenon. It was shown in Chapter 3 that there is very good evidence that this test serves as a reliable parameter of the competence of cellular immunity. It appears to mirror accurately impairment of cellular immunity when this occurs in certain conditions such as Hodgkin's disease, chronic lymphatic leukaemia, sarcoidosis, miliary tuberculosis, lepromatous leprosy and the rare congenital conditions of thymic dysplasia (Marshall et al., 1970). Cellular immunity to Candida seems to be critical to defence against the infection, as it is those diseases in which there is general depression of cellular immune competence which are particularly prone to being complicated by Candida infection (Soothill, 1968).

The technique of lymphocyte transformation, as described by Coulson and Chalmers (1967) for the measurement of the response of human blood lymphocytes to tuberculin PPD in tissue culture, was adapted and standardised for measurement of lymphocyte responses to stimulation by Candida antigens (Chapters 2 and 3). Fifty-five normal adult subjects were investigated for their lymphocyte responses to Candida antigen and it was shown that about 65% of this sample of the normal population had a positive response. This fairly high percentage of positives in the normal population is probably due to either clinical or subclinical infection in the past, or to previous exposure and sensitisation to organisms with a similar antigenic structure to Candida.

It was shown that chronic renal disease with uraemia, treated by twice-weekly intermittent haemodialysis, but not by drug therapy, was not characterised by depression of normal lymphocyte stimulation by Candida.

These findings were in accordance with those of Ling (1968), who reported that chronic renal failure did not depress lymphocyte responses to PHA.

However, combined azathioprine and prednisone therapy, as used to prevent rejection in patients who have previously undergone renal homotransplantation, long-term steroid therapy with prednisone or prednisolone in doses greater than 7.5 mgm. daily, in the treatment of various diseases, and cyclophosphamide therapy, were shown to depress the normal lymphocyte response to Candida to a considerable degree.

The highly complex antigenic structure of the cell wall of the Candida organism and its remarkable resistance to mechanical and chemical disruption in the laboratory (Faux, 1968), would suggest that humoral factors would be ineffective in protection against the fungus. Weight is added to this opinion by the clinical evidence that those rare congenital and acquired diseases which are characterised by pure humoral antibody deficiency do not become complicated by Candida infection (Hermans et al., 1969).

On the other hand, factors in the serum capable of producing agglutination and precipitation with Candida antigens, and capable of producing in vitro phenomena such as indirect fluorescent-labelled gammaglobulin fixation and complement fixation, have been shown to be present in higher titre in persons infected with Candida and to rise in titre in serum in the presence of active infection (Lehner, 1970). These serum factors are immunoglobulin in nature (Lehner, 1970). They are thus antibodies in the accepted sense of that term. Whether or not these Candida antibodies exert a protective function is quite unknown. Evidence obtained from experimental investigation of the possible protective function of Candida antibodies is inconclusive (Dobias, 1964).

With the immediate qualification that these antibodies might not exert any protective function at all, two tests were selected, namely, the

capacity of the serum to agglutinate Candida organisms in vitro and the indirect immunofluorescent-labelled antibody technique, to study the effect of steroid and immunosuppressive drug therapy on humoral immunity. These two tests were taken to represent parameters of humoral immunity to Candida. They were quantitated by titrating to an end-point, with serial dilutions of the sera tested. Subjects in the drug-treated groups were tested, who had already been shown by the lymphocyte transformation testing to have impaired cellular immunity to Candida. Reasonably well-matched normal control subjects were tested at the same time for comparison.

Drug therapy with prednisone or prednisolone or with a combination of azathioprine and prednisone did not affect the titres of antibodies to Candida measured by these two techniques. It was of interest that the titres of Candida antibodies were reduced in the four patients tested who were receiving cyclophosphamide therapy. However, no significance can be attached to these findings as they stand because of the small sample of patients investigated in this treatment group. A prospective study of mice over an eight-week period showed that treatment with cortisone, azathioprine and with a combination of these two did not reduce circulating levels of Candida agglutinins in the blood.

The work of Roth and Goldstein (1961) and of Louria and Brayton (1964) suggested that normal serum generally has a "candidacidal" effect. The inference was that a powerful inhibitory influence was exerted on the viability of any Candida organisms which might succeed in penetrating epidermal or mucosal barriers and thus gain access to the blood stream and tissues. Further, it was postulated that diseases such as leukaemia, Hodgkin's disease, myeloma, etc., which are commonly complicated by Candida infection, are characterised by a deficiency of this serum candidacidal activity.

In this investigation, the experimental system described by Louria et al., (1967), was used, and their findings that the sera of the majority of the normal population caused a significant (log 1) fall of Candida census over a six-hour period, were confirmed. However, the estimation of Candida census by viable counting was theoretically open to the criticism that a single colony counted after a period of incubation need not necessarily represent a single original organism. Indeed, by direct microscopical examination at the six-hour period, in addition to viable counting, it was seen that a fall in Candida census was invariably associated with microscopic evidence of fairly pronounced clumping and mycelial formation of the Candida organisms. In the face of such evidence a technique of viable counting of single colonies after pour-plating and incubation was not thought to be valid. It was quite possible that both single organisms and clumps of cells and hyphae might produce single colonies after incubation. The serum "candidacidal" factor was thought rather to be a clumping factor and it was concluded that the value of such an assay system was strictly limited. Subsequent to the completion of this experiment, the same sentiment was expressed by Lehrer and Cline (1969).

The use of ultrasonification or of chemical means in order to disrupt clumps of organisms and mycelia was not thought to be feasible, both because such techniques could not be guaranteed to be infallible and because of the impairment of the cell viability which these methods would be likely to produce.

In the light of these considerations a detailed study of the effect of drug therapy on this serum activity was not undertaken.

Finally, the effects of steroid and of immunosuppressive drug therapy on the normal inflammatory response to Candida were studied. Here, use was made of the excellent experimental model initially devised by Selbie

and O'Grady (1954) for the observation of the natural history of localised tuberculosis and the effects on the lesion of anti-tuberculous treatment. The model was subsequently adapted for the study of the effects of drugs on the experimental candidiasis by O'Grady and Thompson (1958). The organism was injected into the thigh muscles of the laboratory mouse in sufficient numbers to excite an active local response, without significantly affecting the general condition of the animal. The course of the infection was monitored by cumulative mortality studies, daily measurements of the size of the local swelling, regular haematological investigation, blood cultures, serial local histological examination, and by detailed post mortem examination.

The course of the infection in normal mice was compared with the course of the infection in mice treated with cortisone, azathioprine and with mouse anti-lymphocyte serum respectively.

The findings were that in the normal course of events, the local infection was not fatal but that a marked local inflammatory response was elicited at the site of the injection of the organisms. The local inflammation was characterised by swelling of the limb to almost 100% its normal size and by histological evidence of an acute inflammatory response. This local response was demonstrable within 24 hours of the start of the disease and it persisted for a further three to four days before settling. At the eleven-day stage there was evidence of an early chronic inflammatory response with a diminution of the number of Candida organisms. With one exception, there was no evidence of dissemination of the infection as judged by daily blood cultures and by subsequent autopsy examination.

The effect of treatment with azathioprine and with cortisone was to reduce significantly the local swelling, but not to affect any of the other features of the disease. In some instances it was thought that local oedema as judged by histological appearances was diminished by drug therapy

compared with the untreated mice, but this was not a consistent finding. Mouse anti-lymphocyte serum did not reduce the local swelling in the early stages of the disease, but it did do so in the more chronic phases of the infection. Treatment with the various drug regimes did not appear to inhibit the ability of the tissues to phagocytose and to remove the Candida organisms.

The conclusions reached were that the drug regimes did not modify local "cellular" aspects of acute inflammation in response to Candida in the morbid anatomical sense, but rather that the reduction of swelling consequent on the use of these drugs was likely to be accounted for by some inhibitory effect on local cell-derived chemical mediators of tissue reaction to injury (Houck, 1968). Precisely how these drugs might operate at this level of the inflammatory response is beyond the scope of this thesis. The broad-spectrum anti-inflammatory activity of these drugs no doubt operates at this local "effector" level. Whatever the exact nature of this local drug action, the overall natural history of the infection did not appear to be impaired.

The findings reported in this series of investigations suggest that it is those aspects of resistance to Candida infection which are primarily concerned with normal cellular immune function, that are of critical importance. Drug therapy with steroids, azathioprine, cyclophosphamide and combinations of these, appears to predispose to Candida infections by virtue of impairment of normal cellular immunity. Evidence was not obtained that humoral immunity to Candida was of the same degree of importance or that serum antibodies were inhibited in any way by these various drug regimes.

It may be questioned why, in view of the profound depression of cellular immunity to Candida which has been demonstrated to result from the use of these pharmacological agents, Candida infection is not more

commonly seen in such circumstances. Presumably, factors influencing the numbers of Candida present at a particular site and facilitating penetration of the fungus of normal anatomical barriers, are crucial in tipping the balance in the predisposed subject. In addition, other humoral and/or cellular factors not known at the present time, may be of importance in this regard.

Several points arising in the course of the series of investigations reported in this thesis may have relevance to wider biological and medical considerations, not directly related to problems of Candida infection.

The selective depression by steroids of lymphocyte transformation in response to Candida antigens with relative sparing of the normal lymphocyte stimulation by P.H.A. of the same subjects suggests that the mechanisms of activation of lymphocytes by Candida and by P.H.A. are different. A complex multi-stage biochemical activating system is postulated in the induction of lymphocyte transformation (Ling, 1968). It would seem that Candida antigens and P.H.A. operate at different sites along this activating system. Conceivably, different drugs, or different groups of drugs, might exert their inhibitory effect at different phases of the biochemical pathway. The theoretical possibility that thalidomide blocks activation of developing cells of the foetus, and of lymphocytes stimulated by P.H.A., by enzymic or biochemical inhibition along an activating system, has been suggested by Coulson, Summers and Imman (1969).

The blanket depression of lymphocyte transformation noted with azathioprine and the more selective depression seen in steroid-treated subjects seems to add weight to this hypothesis.

Turk (1969b) has stated that from the points of view of comparative pathology and of evolutionary development, cellular immune mechanisms are probably more primitive than humoral defence mechanisms. Man, it seems, has been exposed to Candida organisms for much of his evolutionary development. It is thought from the findings reported in this thesis that cellular immunity is of critical importance in resistance to Candida and that humoral factors are of lesser (if any) importance. Perhaps it is those potentially pathogenic organisms to which man has been exposed from early in his evolutionary existence which are handled primarily by cellular immune mechanisms. In this regard, the recent findings of Hall (1969) may be of relevance. He has shown that peripheral, circulating lymphocytes, after activation by antigen, very rapidly accumulate in several reticulo-endothelial organs of the body, notable amongst which is the gastro-intestinal tract. The intestinal tract must be a vulnerable site for the establishment of infection in primitive animal life, and it represents the first line of defence for man himself in his encounters with Candida.

How the inference from the pathological studies that steroids and azathioprine therapy appear to modify inflammatory responses to Candida at a chemical "effector" level may be correlated with the inhibitory effect of these drugs on the phenomena of lymphocyte stimulation, is not clear. The two phenomena, need not, of course, be related. However, the recent work of Dumonde, Wolstencroft, Panayi, Matthew, Morley and Howson (1969) which showed that lymphocyte stimulation is largely activated by chemical mediators ("lymphokines") released by a few "primed" cells when these are re-exposed to antigen, suggests a possible unifying

theory. Further discussion at the present time as to the similarity of the two chemical-mediated systems can only be speculative.

It is possible that some of the principles discussed in this thesis as relating to Candida infections, have some application to other "opportunistic" fungal infections. Aspergillosis, nocardiosis and the phycomycetoses resemble candidiasis in the natural history of these infections, their invariably poor prognosis when disseminated through the body, some of the morbid anatomical changes produced, some of the immunological characteristics of the infections and in their tendency to complicate similar forms of drug therapy, (Symmers, 1962). Moreover, multiple infections with these organisms may be seen in the predisposed patient (Symmers, 1964b).

In the field of transplantation surgery, where candidiasis is an especial problem (Rifkind et al., 1967), a lead has been given by Bach, Dardenne and Fournier (1969) which may result in more effective postoperative immunosuppressive therapy. By using a technique of in vitro evaluation of immunosuppression, such as one described by these workers, it may be possible to achieve effective immunosuppression with doses of drugs less than have been used up to the present time. It is most likely that excessively intense immunosuppressive regimes have been used in the past. It will be fortunate if doses of drugs just required to produce immunosuppression are such that the problems of infectious complications are reduced.

It is hoped that the analysis of the literature, the investigative techniques and experimental findings, and the correlation of these, which have been presented in this thesis, have justified for Candida infection

the contention made by Professor Ryle in "The Natural History of Disease" (Ryle, 1948) that:

> "there are circumstances which compel a new review of old and familiar diseases."

CHAPTER 5 : CONCLUSIONS

The organism of the genus Candida, and notably Candida albicans, has achieved a greater occasional pathogenicity for man in recent years. This has been shown in several careful statistical studies to be associated with modern patterns of antibiotic, corticosteroid and immunosuppressive drug therapy.

In this thesis the following conclusions have been reached which are thought to be of relevance to this problem:

1. About 65% of the sample of the normal British population which was studied showed a significant positive response when their lymphocytes were cultured in vitro in the presence of Candida antigens. Evidence is given that this investigation serves as a reliable parameter of normal cellular immunity to specific antigenic stimulation. Experimental data are provided to show that the lymphocyte transformation phenomenon was carefully adapted and standardised for the study of lymphocyte responses to Candida.

2. The normal cellular immunity to Candida, as measured by the lymphocyte transformation phenomenon, was found to be significantly depressed by drug therapy with steroid therapy, cyclophosphamide therapy, and long-term combined azathioprine and prednisone therapy.

3. No such depression of humoral immunity to Candida was demonstrated, as judged from the effects of these various drug regimes on the normal levels of serum agglutinins to Candida and antibodies to the organism demonstrable by the indirect immunofluorescent antibody technique. However, the limitations of this last inference are recognised in that these antibodies have never been shown to exert a protective effect against Candida infection.

4. The natural history of experimental candidiasis in mice was shown to be modified in the acute stages of the disease by cortisone and by azathioprine by virtue of the anti-inflammatory properties of these drugs. The drugs were not shown to have a significant effect on the mortality rate, or on the tendency to disseminate from an established lesion.

The observation which was made that steroid therapy is capable of selectively depressing lymphocyte responses to Candida antigen to a greater extent than to phytohaemagglutinin, suggests that the mechanism of activation of lymphocytes by specific antigen and by P.H.A. is different. This lends weight to the hypothesis that lymphocyte stimulation is activated by a multi-stage biochemical system. It is likely that different drugs which are capable of inhibiting the lymphocyte stimulation phenomenon may do so at different sites in this activating system.

The similarities which Candida infection bears to other fungal infections such as Aspergillosis, Nocardiosis and the phycomycetoses suggest the possibility that some of the principles applicable to Candida infection as discussed in this thesis may have bearing on these diseases as well.

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