This is to certify that the

dissertation entitled

DEVELOPMENT OF LIPOSOMAL AMPHOTERICIN B BEARING ANTICANDIDAL ANTIBODY AND USE OF THIS PREPARATION IN THE THERAPY OF A MURINE MODEL OF CANDIDIASIS

presented by

Duane Russell Hospenthal

has been accepted towards fulfillment
of the requirements for

Doctor of Philosophy degree in Medical Mycology

Major professor

Date 15 Feb 1989

DEVELOPMENT OF LIPOSOMAL AMPHOTERICIN B BEARING ANTICANDIDAL ANTIBODY AND USE OF THIS PREPARATION IN THE THERAPY OF A MURINE MODEL OF CANDIDIASIS

By

Duane Russell Hospenthal

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Botany and Plant Pathology

1989

ABSTRACT

DEVELOPMENT OF LIPOSOMAL AMPHOTERICIN B BEARING ANTICANDIDAL ANTIBODY AND USE OF THIS PREPARATION IN THE THERAPY OF A MURINE MODEL OF CANDIDIASIS

By

Duane Russell Hospenthal

Systemic candidiasis and other disseminated mycoses account for appreciable morbidity and mortality in patients with hematologic malignancies and other debilitating conditions. The treatment of choice for most of these mycotic infections is the antifungal agent, amphotericin B (AMB). While AMB has been shown to produce a wide range of antifungal effects, it has also been called the most toxic antimicrobial agent in current usage today. Due to this toxicity, new formulations have been explored for the deliver of this drug. One of the most interesting of these approaches has been the encapsulation of AMB in phospholipid vesicles or liposomes.

Liposomal amphotericin B (LAMB) was produced in this study by a reverse-phase evaporation procedure. Employing this procedure, LAMB was also produced which bore antibody specific to Candida albicans on its surface (LAMB-Ab). This new formulation, LAMB-Ab, was shown to possess external, C. albicans specific antibody. Toxicity of

LAMB-Ab and LAMB to human erythrocytes *in vitro* was much less than that of free AMB (fAMB). Anticandidal activity of these three compounds was comparable *in vitro*.

Therapeutic effect of LAMB-Ab was compared to other AMB containing preparations *in vivo* in a murine model of systemic candidiasis. In this model, mice infected by intraperitoneal injection received therapy in both prophylactic and treatment studies. LAMB-Ab improved the survival rates of mice over LAMB, which itself improved these rates over fAMB. Therapy with additional preparations provided evidence that increased survival afforded by LAMB-Ab was due to the attachment of *C. albicans* specific antibody.

LAMB-Ab produced similar results in the therapy of a model of candidiasis initiated via intravenous inoculation. In this second model of disseminated murine candidiasis, the infection was followed and characterized by organ homogenization counts of viable yeasts.

Targeting of liposomal AMB via the attachment of anticandidal antibody was shown to be experimentally possible in this study. This targeting, in addition to the reduced toxicity of liposome encapsulation, may allow therapeutic use of amphotericin B to become more safe and effective in the future.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to all those who provided support and guidance to me in this undertaking. To my committee, special thanks for their ongoing guidance of this project from start to finish. To Dr. Alvin Rogers, I wish to extend my thanks for serving as my major professor, for guiding my introduction into the world of research, and for all his help in medical mycology along the way. I wish to express my appreciation to Dr. Everett Beneke for all his help and special insight in this project and all the other problems which I brought to him. Also special thanks to Dr. Beneke for serving as my major professor during the studies which culminated in the awarding of my Master of Science degree. Special thanks to Dr. Gary Mills for help in getting this project off the ground through his help and insight in biochemistry and proper research technique. In addition I would like to express my indebtedness to Dr. Karen Klomparens for her wisdom in the art of electron microscopy and the help of her staff at the Center for Electron Optics. Thanks to Dr. Ronald Patterson for his guidance in the field of immunology.

In addition to thanks bestowed my committee, I would like to extend my appreciation to Karl Gretzinger for his assistance in the murine study and to Dr. Dennis Gilliland for his interpretation of the statistical method which was used.

Finally, I wish to express my undying love and appreciation to my wife Carol, and thank her and my parents, brothers, sister, and the rest of my family for their patience and support over all the years.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES, CONT'D.

INTRODUCTION

Fungal disease in its wide diversity of forms has plagued humans throughout the ages. From athlete's foot (tinea pedis) to valley fever (coccidioidomycosis), fungi have assailed the human species in numerous presentations. The severity of mycoses range from the cosmetic nuisance of tinea versicolor to the often life-threatening systemic mycoses. Of all fungal disease afflicting humans, Candida albicans, a yeast usually present as normal flora, is by far the most common etiologic agent.

Treatment of systemic fungal infection has long been unreliable and even dangerous to the human host. Prior to the introduction of amphotericin B in the early 1960's, treatment for the majority of disseminated mycoses was virtually nonexistent. The discovery of amphotericin B in 1956 heralded the introduction of this polyene antifungal agent which, to date, is the drug of choice for most disseminated mycoses including aspergillosis, candidiasis, coccidioidomycosis, cryptococcosis, histoplasmosis, and mucormycoses. Although it has remained the most reliable and widely used systemic antifungal in the past twenty five years, amphotericin B has many toxic properties

which make this antifungal a difficult and often dangerous drug to administer. Due to the toxic properties of this compound, researchers in the past two decades have searched for drugs to replace amphotericin B or methods to deliver this antibiotic in a less toxic form.

The new family of azole antifungals, including ketoconazole, itraconazole, and fluconazole have shown promise in the replacement of amphotericin B in some applications. Semisynthetic amphotericin derivatives are also being studied due to their reduced toxicities. While final analysis of these new antifungals remains incomplete, amphotericin B remains the drug of choice for all life-threatening systemic mycoses. Adjuvant therapies with other compound, such as 5-fluorocytosine, hydrocortisone sodium succinate, aspirin, diphenhydramine, prochlorperazine, sodium bicarbonate, mannitol, etc., have shown reduction in some, but not all aspects of amphotericin B treatment toxicity. Presently, the most promising attempt to reduce the toxic effect of amphotericin B on the host, is by the encapsulation of this antifungal compound in phospholipid liposomes.

Liposome incorporation of amphotericin B, though still largely experimental, has been shown to greatly enhance the therapeutic effect of the drug by allowing much larger dosages to be employed with dramatically reduced toxic effect. Currently, under a FDA Investigational New Drug

permit, liposomal amphotericin B therapy is producing excellent results in human hematologic malignancy patients with disseminated mycoses.

Antibody targeting of other liposomal compounds has been shown in laboratory studies to aid in the site-specific delivery of these compounds. Targeted delivery of amphotericin B to specific sites of infection could lead to increased efficacy and decreased toxicity to the host tissue and therefore to the host. The goal of this project was to produce such liposomes and test the therapeutic efficacy of these vesicles.

Production of liposomes which encapsulate amphotericin B and bear antibody specific to C. albicans on their surfaces was the first objective of this project. These lipsomes, once formulated, were compared to both commercial and liposomal forms of amphotericin B. Comparison of these formulations included observing their in vitro effect on the growth of C. albicans and their toxic effect on human red blood cells. The therapeutic effect of antibody-bearing liposomal amphotericin B was compared to that produced by the liposomal drug without antibody and the free unencapsulated antifungal in two murine models of systemic candidiasis. Disseminated candidiasis models employed in these studies were produced via either intravenous or intraperitoneal injection of C. albicans.

LITERATURE REVIEW

Candida albicans and Candidiasis

Candidiasis is an acute or chronic, superficial or systemic infection produced by members of the genus *Candida*. Species of this genus considered the most virulent (in order of decreasing virulence) include *Candida albicans*, *C. tropicalis*, *C. stellatoidea*, *C. krusei* and *C. parapsilosis* (Hurley, 1980). *Candida albicans* is by far the most common species of the genus which produces candidiasis, accounting for greater than ninety percent of infections (Roberts et. al., 1984). *C. albicans* is the only member of the genus which is thought to regularly produce a fatal disease in both humans and animals (Mourad and Friedman, 1961). In addition to being the cause of most cases of candidiasis, *C. albicans* is the etiologic agent for the vast majority of all fungal infections. This organism is the cause of over eighty percent of all mycoses (Lopez-Berestein et. al., 1983).

C. albicans is a dimorphic fungus belonging to the form-phylum Deuteromycota, the form-class Blastomycetes, and the form-family Cryptococcaceae. Commonly this fungus occurs as an asporogenous yeast which reproduces by

budding, but it can also form hyphae, pseudohyphae and chlamydoconidia. The telomorph state of this fungus is currently believed to belong in the basidiomycete genus Leucosporidium (Rippon, 1988).

Candida albicans is considered an obligate animal saprotroph which can reside as normal flora in the throat, buccal mucosa, intestine, or vagina (Carmo-Sousa, 1969). The incidence of the yeast in normal humans as flora has been reported to be from ten to fifty percent (Seelig and Kozinn, 1982). Taschdjian et. al. (1973) reported isolation of Candida from normal subjects in the order of twenty to fifty nine percent from the alimentary tract and eleven to seventeen percent from the vagina. Isolation of C. albicans from the vagina of pregnant women or women on oral contraceptives was approximately double that of normal healthy females. Odds (1988) derived similar numbers from compiling many isolation studies and weighing them by number of subjects studied. In this study he reported mean frequencies of recovery of C. albicans in the mouths, feces and vaginas of normal subjects as 17.7, 15.0 and 12.7 percent, respectively. Mean recovery of this yeast from hospitalized patients was calculated to be 40.6 percent from the oral cavity and 26.4 percent in the feces. Women patients with vaginitis and without vaginitis had mean isolation rates of 25.9 and 17.8 percent, respectively. In a recent study,

Burford-Mason et. al. (1988) reported an oral carriage rate of thirty percent in healthy subjects. This group also presented a correlation of Candida carriage with blood group O and the non-secretion of blood group antigens. Rippon (1988) states that C. albicans comprises a small, yet constant population in the normal alimentary tract. C. albicans is also common flora in bird and mammal alimentary tracts (Carmo-Sousa, 1969; Odds, 1988). C. albicans has the reported ability to survive on skin, sand, water, and food, and thus has no problem transferring between hosts (Rippon, 1988).

Candidiasis is an opportunistic infection which can affect almost any area of the body. The mycosis may be localized as a cutaneous infection of the skin or nails, as a mucocutaneous infection of the mouth, throat, bronchi, lungs, vagina, or gastrointestinal tract, or it can present as a systemic infection as a septicemia, endocarditis, or meningitis. Candida species can also cause allergic disease in their hosts. Rippon (1988) cites five conditions in which Candida can leave its normal flora niche and enter into a pathogenic interaction with its host. These conditions are:

1. **Extreme youth.** During the establishment of normal flora Candida may overgrow and produce infections such as thrush and diaper rash.

2. **Physiologic change.** Factors affecting a change in the yeast's environment which allow its overgrowth. These include changes such as those seen in pregnancy, steroid therapy, and endocrine dysfunctions such as diabetes.

3. **Prolonged administration of antibiotics.** The removal or alteration of other flora which suppress Candida can lead to candidiasis.

4. **General debility and the constitutionally inadequate patient.** This category includes a wide range of host problems from slight avitaminosis to severe immune defects. Almost any disease, defect, or therapy which can allow a breach of the host's immunologic defense can lead to candidal infection.

5. **Iatrogenic and break-barrier.** Medical procedures which are invasive, as well as any trauma, can allow a portal to invasion for Candida species.

Systemic infection by C. albicans is the most life-threatening of the candidiases. These severe mycoses have become all too common in postoperative and immunosuppressed patients. The prevalence of disseminated candidiasis has come from a rare occurrence prior to 1960 to an important hospital-acquired infection. In a recent study Candida was reported to be the fifth most common isolate from blood in nosocomial septicemias and the forth most common isolate from all blood cultures (Edwards et. al., 1978). Reingold et. al. (1986) reported a fifty percent increase in candidiasis acquired in the hospital and/or by immunocompromised patients between 1976 and 1980-1982. This increase in prevalence is believed to be coincident with the increased use of antibiotics, immunosuppressants, hyperalimentation fluids, catheters, invasive monitors, heroin abuse and surgical procedures including organ transplants and prosthetic heart valve replacements. Reingold et. al. (1986) also attributed the rise in candidiasis to the increasing life spans now afforded to immunocompromised patients by modern therapy. Candidiasis is also prevalent and not a rare cause of fatality in burn victims. In a study reported by MacMillan et. al. (1972) 15 of 385 children admitted to a burn unit died of candidiasis. C. albicans is also a cause of mortality in myeloproliferative and other organic immunocompromising disorders. It has been reported that over twenty percent

of leukemia patients and thirteen percent of lymphoma patients die of candidal infection (Feld et. al.,1974; Inagaki et. al., 1974).

Systemic or deep candidiasis is an infection which may present as a focal involvement of an organ system or as a disseminated disease. Disseminated candidiasis is used widely as a synonym of systemic candidiasis due to the fact that the majority of these infections are initiated via a hematogenous transmission of the yeast (Odds, 1988). The exceptions to this rule are infections of the respiratory, digestive and urogenital tracts which are believed to be initiated directly by candidal flora. These infections are considered by many to belong in the mucocutaneous group of Candida infections due to their origin and location. Systemic infection is believed to be most commonly produced by the spread of C. albicans by the blood from the gastrointestinal tract and barrier-break procedures, especially intravenous catheters (Roberts et. al., 1984). C. albicans has been documented to infect almost every organ and body compartment. The fungus can be the cause of meningitis, cerebral candidiasis, endocarditis, myocarditis, pericarditis, peritonitis, endophthalmitis, arthritis, osteomyelitis, pyelonephritis, cortical renal infection, as well as septicemia (candidemia) (Emmons et. al., 1977; Odds, 1988; Rippon, 1988; Seelig and Kozinn, 1982).

Candidal septicemia and disseminated candidiasis are the most overwhelming forms of the systemic disease and can lead to rapidly fatal outcomes. These infections are usually confined to severely compromised patients, especially those with hematological malignancies. Candidemia can lead to rapid death directly due to shock and coma (Dennis et. al., 1968; Stone, 1974), or via disseminated intravascular coagulation or other disseminated pathologies (Phillippidis et. al., 1971). The heart and kidneys are the most common sites of disease in disseminated candidiasis with cerebral, meningeal, bone and joint involvement occurring less often (Seelig and Kozinn, 1982). The kidney has been reported to be the primary target organ of the genus Candida in disseminating infections (Louria et. al., 1962). Pathology of all the organs affected include multiple microabscesses and a predominance of neutrophils (Kauffman and Jones, 1986). In murine studies deaths from high inoculum counts are associated with interstitial myocarditis and those with decreased inoculum are related to renal failure (Ryley et. al., 1988).

Acute disseminated candidiasis closely resembling the human disease is produced by the intravenous or intraperitoneal injection of C. albicans into mice or rabbits. Guinea pig or rats may also be used, but with a decreased reproducibility of results (Odds, 1979). Intravenous

inoculation of C. albicans leads to the production of lesions in the kidneys, lungs, liver, heart, brain, spleen, and other organs in mice (Louria et. al., 1963). Injection of 10^6 colony-forming units via the caudal veins of a mice leads to one hundred percent mortality within a week (Adriano and Schwarz, 1955). At lower inoculation doses, only C. albicans recovered in the kidneys has been shown to be producing a progressive infection (Hurley and Winner, 1963). Rogers and Balish (1976) reported that C. albicans persisted in the lungs, spleen and liver of mice for up to thirteen days after infection, but the yeast did not multiply within these organs. This group supported results of Louria et. al. (1963) that indicated that the kidneys were the only organ in which this chronic disease progresses in this model. Kidney involvement in the murine model has been shown to be produced asymmetrically involving the invasion by C. albicans of the renal tubular lumen. Accompanying this kidney involvement is a decrease in kidney function and a marked diuresis (Ryley et. al., 1988).

Results simular to these are produced in mice intraperitoneally employing inoculum at a ten-fold increase over that of the intravenous model (Young, 1958). Intraperitoneal injection of C. albicans produces an almost immediate transformation from a yeast to a filamentous form followed by growth within the body

cavity. The filamentous form, thought to be produced to evade phagocytic cells, then invades abdominal organs, primarily the pancreas. By twenty four hours, C. albicans enters the blood vessels of the pancreas and is disseminated to other parts of the body including the kidneys (Young, 1958). As in the intravenous models the kidney has been shown to be the only organ in which infection persists.

Amphotericin B and Antifungal Therapy

Therapy of systemic mycoses dates back only as far as 1903 when the beneficial effect of potassium iodide was discovered (Drouhet, 1970). This therapy proved to be only beneficial against most, but not all cases of sporotrichosis. It wasn't until almost fifty years later that antifungal chemotherapy made its next advance. In 1949, Hazen and Brown (1951) discovered nystatin, which they originally named fungicidin. Nystatin, the metabolic product of a actinomycete, heralded the discovery of more than 60 other antibiotics produced by the actinomycetes which are called polyenes (Drouhet, 1970). Of this large number of polyene antibiotics discovered in the early 1950's (including candicidin, ascosin, eulicin, trichimycin and amphotericins A and B), only amphotericin B proved to be absorbed well enough and have a toxicity which was acceptable enough to be used systemically

(Hildick-Smith et. al., 1964). The other polyenes discovered during this era have been clinically limited to the therapy of cutaneous and mucocutaneous mycotic infections. Amphotericin B stands out in the polyene antibiotics as the only member of this group which can be readily administered into the body fluids in a therapeutic concentration without causing the host great injury (Hildick-Smith et. al., 1964).

Amphotericin B is currently, and has been for the past twenty five years, the drug of choice for all life-threatening mycoses. In systemic fungal infections, it is the most reliable and widely used antifungal agent (Graybill and Craven, 1983). Amphotericin B is currently recommended in the treatment of the aspergillosis, blastomycosis, disseminated candidiasis, coccidioidomycosis, cryptococcosis, trichosporonosis, histoplasmosis, mucormycosis, and systemic sporotrichosis (Beneke et. al., 1984). In the treatment of systemic candidiasis, Seelig and Kozinn (1982) name this antifungal agent the most effective drug available.

Amphotericin B was isolated and first described by Gold et. al. in 1956 as a metabolic product of Streptomyces nodosus M4575, a soil actinomycete isolated in Venezuela. Synonyms for this antifungal include Amphozone, Fungizone, Fungilin and Ampho-Moronal.

Amphotericin B is a 924.11 molecular weight polyene with a chemical formula of $C_{47}H_{73}NO_{17}$ as depicted in Figure 1 (Merck & Co., Inc., 1983). Amphotericin B is characterized by having a macrolide ring closed by the formation of an internal ester and bearing eleven hydroxyl groups (Medoff and Kobayashi, 1980). The antifungal compound has both hydrophobic and hydrophilic aspects to its structure and is very insoluble in water. The seven double bounds of amphotericin B form the hydrophobic part, while the

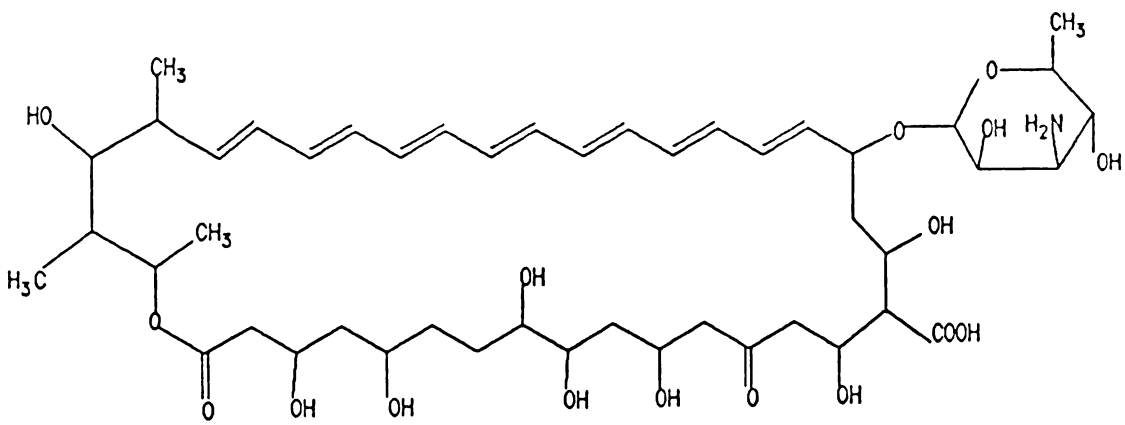


Figure 1. Chemical Structure of Amphotericin B

hydroxyl groups and the mycosamine moiety form the hydrophilic portion (Medoff et. al., 1983). In the commercial form, Fungizone, amphotericin B is delivered with deoxycholate to allow suspension of the drug in saline. The deoxycholate allows the amphotericin B to be delivered in micelles formed by this compound.

Amphotericin B, both in the commercial form and in its native form, appears as a yellow compound which is sensitive to inactivation by exposure to air and light (Merck & Co., Inc., 1983).

The reported effect of amphotericin B, along with the other polyene antifungals, is to promote leakage of cellular components leading to cell death. This effect is dependent on the attachment of these polyene compounds to sterols in cell membranes (Medoff and Kobayashi, 1980). Amphotericin B produces both a fungicidal and fungistatic response depending on the concentration delivered to the fungus (Brajtburg et. al., 1980). At low concentrations, amphotericin B causes small pores or channels to leak small components such as potassium and magnesium ions, and is reversible. The damage is more severe and irreversible at higher concentrations, causing the fungicidal effect. This effect has also been shown in human erythrocytes with their release of potassium ions when exposed to low concentrations of the drug and hemoglobin release at increased amounts of amphotericin B (Teerlink et. al., 1980). Recently it has been shown that amphotericin B produces its lytic and lethal effects on Candida albicans by oxidative damage (Sokol-Anderson et. al., 1986). Antifungal activity of amphotericin B comes from the increased affinity of the compound for ergosterol, which is found in fungal cell membranes, over cholesterol, the

common mammalian cell membrane sterol (Kotler-Brajtburg et. al., 1974). In addition to these primary effects, amphotericin B has also been reported to effect specific membrane enzymes of fungal cells and act as an immuno-adjuvant toward the host. Surarit and Shepherd (1987) have shown greater than seventy five percent inhibition of Candida albicans cell membranes enzymes ATPase, glucan synthase, adenyl cyclase and 5'-nucleotidase in situ. Vecchiarelli et. al. (1986) reported an in vivo augmentation of resistance to C. albicans provided by amphotericin B which correlated in vitro with increased anticandidal activity of macrophages from mice in the study. Amphotericin B has also been shown to activate macrophages in vitro to kill bacteria (Lin et. al., 1977), parasites (Olds et. al. 1981), and tumor cell lines (Chapman and Hibbs, 1978). At higher concentrations the antifungal becomes toxic to cells and causes decrease in chemotaxis of neutrophils and phagocytosis and killing in macrophages (Hauser and Remington, 1982).

Although amphotericin B has been shown to preferentially bind to ergosterol, it also binds to cholesterol found in mammalian cell membranes. This single fact provides much of the answer as to how and why amphotericin B causes the many toxic effects with which it is associated. Human patients treated with the antifungal drug present a wide range of acute and chronic toxic effects.

The most common of these are the association of chills and fever with the intravenous infusion and the varying degree of nephrotoxicity seen after this treatment (Medoff and Kobayashi, 1980; Pratt, 1977; Speller, 1980). Short term effects begin usually within four to six hours after the beginning of infusion (Graybill and Craven, 1983). These can commonly include vomiting, hypotension and delirium in addition to fever and chills. Other symptoms which occasionally occur are nausea, abdominal pain, headache, anorexia, phlebitis and rarely cardiac arrhythmias (Pratt, 1977). Treatment over an extended period of time with slow infusion rates is performed to avoid the acute cardiotoxicity of amphotericin B which can progress to cardiovascular collapse and death. After a few weeks of therapy a reversible normocytic, normochromic anemia appears in most patients. This is due to a suppression of erythrocyte production (Brandriss et. al., 1964). In rare instances leukopenia and thrombocytopenia will accompany this anemia. Other rare toxic manifestations include hepatic dysfunction and allergic reactions.

The major toxic effects of amphotericin B therapy are those which affect the kidney. Glomerular filtration rate has been shown to be decreased approximately forty percent in almost all patients treated with amphotericin B (Medoff et. al., 1983). During treatment, nephrotoxicity is monitored via blood urea nitrogen and creatinine serum

levels. In one study of amphotericin B therapy, ninety three percent of patients had elevated blood urea nitrogen levels and eighty three percent had elevated creatinine values (Butler et. al., 1964). Histopathology of amphotericin B treated kidneys show tubular degenerative changes with intratubular and interstitial calcium deposits (Wertlake et. al., 1963). Renal acidosis can also be present in individuals being treated with amphotericin B (McCurdy et. al., 1968). This acidosis is believed to be associated with the nephrocalcinosis and renal loss of potassium ions seen in these patients. The combination of these renal effects of the drug lead to varying degrees of permanent kidney damage. This damage may be severe enough to cause renal failure and even death in the patient before amphotericin B can eradicate the fungal disease being treated (Butler et. al., 1964).

Even with its toxic effects, amphotericin B remains the single most reliable drug in the treatment of most life-threatening mycoses (Taylor et. al., 1982). Many other systemic antifungals have been introduced since amphotericin B in 1956, yet at this time these drugs have either proven to be less effective or are still experimental. These other antifungal compounds include the pyrimidine analogue 5-fluorocytosine, the azoles (e.g.

ketoconazole, itraconazole and fluconazole), semisynthetic derivatives of amphotericin B and chitin-synthesis inhibitors.

Flucytosine (5-fluorocytosine) is a synthetic antifungal agent effective against many disease-causing yeasts and the dematiaceous fungi which cause chromoblastomycoses. It is believed to work by inhibiting protein synthesis after incorporation into RNA. The use of flucytosine is limited by the large number of organisms which are resistant or become resistant to this antifungal during treatment (Roberts et. al., 1984). 5-fluorocytosine can produce dose-related thrombocytopenia and neutropenia, as well as nausea, diarrhea and rash. In current usage, flucytosine is the recommended drug for chromoblastomycosis and cryptococcosis (in combination with amphotericin B) (Beneke et. al., 1984).

The azoles are a group of synthetic compounds which appear to work as antifungal agents by inhibiting ergosterol biosynthesis. Clinical resistance to the azoles is a rarity, as is clinical resistance to amphotericin B and the other polyenes (Hitchcock et. al., 1987; Smith et. al., 1986). The azoles currently used against systemic fungal infections are miconazole, ketoconazole, itraconazole and fluconazole. Miconazole is poorly absorbed orally, intravenously produces side effects including phlebitis, pruritus, nausea, fever or

chills and rash, and has had very limited clinical testing (Heel et. al., 1980). This antifungal agent also has been reported to cause hyponatremia and hematological abnormalities (Stevens, 1977). For these reasons and the advent of ketoconazole, which has a longer half-life in serum and is less toxic (Brass et. al., 1982), miconazole is regarded as a second choice drug (Roberts et. al., 1984). Ketoconazole is the most widely used of the systemically effective azoles. It is recommended in the treatment of chronic and disseminated candidiasis, chromoblastomycosis, paracoccidioidomycosis, and some forms of coccidioidomycosis (Beneke et. al., 1984). Some serious side effects of this antifungal include hepatitis, gynomastia and impotence. Other toxic effects include rash, nausea, anorexia and gastrointestinal disturbances (Graybill and Craven, 1983). These effects are either rare (hepatitis is estimated to occur in 1:10,000 patients) or mild in their presentations as compared to miconazole or amphotericin B (Roberts et. al., 1984). For this reason ketoconazole is often the first choice in the treatment of systemic mycoses that are not life-threatening. This reduced toxicity and the fact that ketoconazole is orally prescribed has also led to it being widely studied as a possible prophylaxis for fungal infection in neutropenic cancer patients (Meunier-Carpenter, 1984; Young, 1982). New additions to the azole

family of antifungal agents, itraconazole and fluconazole, are presently classified as FDA Investigational New Drugs in the United States. Itraconazole and fluconazole are effective against a wider range of fungi and are better absorbed than ketoconazole. Itraconazole has been shown to be absorbed eight times better than ketoconazole and to be one hundred times more active against Aspergillus strains (Marichal et. al., 1985). Both itraconazole and fluconazole are cleared more slowly than ketoconazole and thus therapeutic levels are easier to reach with these drugs in the patient (Graybill and Ahrens, 1984). Fluconazole has also been shown to be more active than ketoconazole. Troke et. al. (1985) reported an example of the superior performance of fluconazole in a murine model of systemic candidiasis. Most importantly, fluconazole is water soluble and has the ability to reach therapeutic levels in the brain via oral administration (Graybill et. al., 1986). In a recent study of human coccidioidal meningitis patients fluconazole was shown to produce substantial penetration into the cerebrospinal fluid with only minimal toxicity (Tucker et. al.,1988). Initial results of this therapy were encouraging and prompted Tucker et. al. (1988) to state that fluconazole may become the drug of choice for coccidioidal meningitis.

The semisynthetic derivatives of amphotericin B include the experimental drugs amphotericin B methyl ester

hydrochloride and N-D-ornithyl amphotericin B methyl ester. These two derivatives have been shown to be less toxic and more efficacious than amphotericin B in laboratory animals (Parmegiani et. al., 1987). However, both of these compounds have been shown to produce neurologic effects at increased dosages. In subchronic toxicity studies conducted by Massa et. al. (1985), N-D-ornithyl amphotericin B methyl ester proved to cause behavioral and morphological brain damage in dogs at doses of 2.5 and 10 mg of the drug per kilogram of animal. Treatment with large cumulative doses of amphotericin B methyl ester hydrochloride over prolonged periods have also been shown to produce neurologic problems. In clinical studies this antifungal agent produced a distinctive neurologic syndrome and injury to human white matter (Ellis et. al., 1982).

Peptidyl nucleoside antibiotics known to inhibit chitin synthesis are yet another experimental class of antifungal agents being explored. This group of compounds, which includes nikkomycins and polyoxins, have been reported to produce very low or inapparent toxic effects in laboratory mice (Isono et. al., 1965). Nikkomycin, the most potent of the group, has proven itself effective in a murine model of disseminated candidiasis (Becker et. al., 1988). The problem encountered in this study was that when treatment was

stopped, the candidal infection reestablished in the nikkomycin-treated mice and ultimately led to their death.

Paralleling the search for new, effective antifungal drugs has been the search for safer methods of presenting amphotericin B to the mammalian patient. The two most common approaches to the task of reducing toxicity have been to administer amphotericin B concurrently with other drugs or substances or to encapsulate the antifungal in phospholipid vesicles (liposomes). Synergistic effect of amphotericin B with 5-fluorocytosine have been shown to reduce the toxicity of amphotericin B by reducing the amount used. This reduction has proven effective in only a few of the systemic mycoses, such as crytococcosis (Speller, 1980). Experimentally, mycolase has been studied as a compound to potentiate amphotericin B treatment (Chalkley et. al., 1985). This mixture of enzymes, which includes a chitinase, glucanases and exoglycosidases, has shown some enhancement to amphotericin B therapy. Many compounds have been and/or are presently being used in the attempt to reduce the toxic symptoms of amphotericin B therapy. To reduce chills and fever, hydrocortisone sodium succinate is added to the infusion of the antifungal drug. Premedication with aspirin, diphenhydramine or meperidine hydrochloride has also been employed to control chills and fever (Graybill and Craven, 1983; Medoff and Kobayashi, 1980). Nausea

associated with amphotericin B therapy is often treated with prochlorperazine (Medoff et. al., 1983), Gouge and Andriole (1971) have reported reduction in nephrotoxicity in rats co-treated with sodium bicarbonate. Mannitol administered concurrently with amphotericin B has been shown to reduce blood urea nitrogen levels to normal in dogs (Hellebusch et. al.,1972). This effect has not proven to reflect any reduction in renal dysfunction in patients treated in this manner (Bullock and Bathona, 1976). The most recently applied method to reduce amphotericin B toxicity has been to encapsulate this compound in liposomes. This encapsulation has been shown to be effective in the reduction of toxicity of amphotericin B in a large number of laboratory and clinical studies. A U.S. patent has recently (1988) been issued to Liposome Technology Incorporated of Menlo Park for their formulation of liposomal amphotericin B.

Liposome Technology

Liposomes (lipid bodies) is the term coined to describe vesicles consisting of phospholipid bilayers assembled into closed membrane systems (Bangham, 1980). These phospholipid vesicles were initially used as model systems for biological membranes. Much of the action of amphotericin B and its interaction with sterols has been investigated using liposomes as model membranes (Bolard

et. al., 1984; Clejan and Bittman, 1985; Cohen, 1983; Cybulska et. al., 1986). Recently, liposomes have been explored as potentially important drug delivery systems due to their property to entrap compounds in their internal aqueous or lipid compartments and sequester these compounds from direct contact with the host (Lopez-Berestein, 1986). Liposomes can carry polar drugs in their aqueous compartment and nonpolar drugs in both this and their lipid membrane compartment (Juliano and Stamp, 1979; Stamp and Juliano, 1979). Liposomal encapsulation prevents the reaction of compounds with cellular blood constituents and reduces the clearance rate of many encapsulated substances (Gregoriadis and Neerunjun, 1975). This allows reduction in allergic and toxic reactions caused by unencapsulated forms of many compounds. The contents of liposomes are believed to be transferred to cells directly by fusion or via the endocytosis of the entire liposomes by cells (Scheider, 1985). The actions of liposomes have been shown to be modified by physical and chemical properties of their construction. An example of this is the tendency of negatively charged liposomes to accumlated preferentially in the spleen (Schneider, 1985). Much research has been focused on the encapsulation of toxic antitumor and other drugs which have been limited in their usage because of toxicity problems (Juliano and Stamp, 1978).

Besides drug delivery, liposome transport of many other substances has been studied. Liposomes have been studied as vaccine carriers to produce immunity to many viruses, bacteria, parasites and various proteins including birth control antigens (Alving, 1987; Ostro, 1987a). The induction of the immune system is another area of exploration. Liposomes incorporating a polyribonucleotide have been shown to increase interferon production in cell culture (Straub et. al., 1974). The property of liposomes that lends their ability to interact preferentially with host immune cells is their tendency to accumulate in the reticuloendothelial system of the patient upon intravenous injection (Hsu and Juliano, 1982; Juliano and Stamp, 1975; Kimelberg and Meyhew, 1978; Pagano and Weinstein, 1978; Richardson, 1983). Other compounds carried in liposomes include hormones, such as bovine somatotrophic hormone (to increase milk production) and epithelial growth factor (to aid in wound healing), and tear components (Ostro, 1987). Liposomes are also being used as emollients in cosmetics and as diagnostic tools such as the recently introduced immunoassay test kit for Streptococcus species (Schach, 1987).

Liposomes are produced in three classes or categories depending on their relative size and lamellar nature. These three categories are small unilamellar (SUV), large unilamellar (LUV) and multilamellar (MLV) vesicles. These

classes have associated with them differing physical properties and thus differing applications. Multilamellar vesicles were the first liposomes produced. Bangham et. al. described the first and most popular method to produce these liposomes in 1965. In this procedure the lipids used are dissolved in an organic solvent. This solvent is then evaporated leaving the lipids as a film on the inner surface of the preparation flask. Drugs or other compounds to be encapsulated are then added to the flask in aqueous solution. The flask is then swirled by hand to produce large MLVs. These large multilamellar liposomes can be up to fractions of a millimeter in diameter and have the concentric appearance of a sliced onion when observed by electron microscopy (Pagano and Weinstein, 1978). These liposomes are very responsive to osmotic gradients and can swell or shrink in response. Large multilamellar liposomes have a capture volume of about 4 ul/mg phospholipid (Poznansky and Juliano, 1984).

The second class of liposomes produced were the small unilamellar vesicles. These phospholipid vesicles usually have a diameter in the twenty to fifty nanometer range and single bilayer membranes and aqueous compartments. These small liposomes have the smallest capture volume of the three classes of vesicles, a mere 0.5 ul/mg lipid (Poznansky and Juliano, 1984). SUVs are insensitive to osmotic pressures and display different physical chemical

characteristics than MLVs (Pagano and Weinstein, 1978). These microscopic vesicles also present the advantage of an increased half-life in blood over the larger classes of liposomes (Schneider, 1985). These small liposomes are commonly produced by ultrasonic dispersion. This may be accomplished via probe sonication or in an ultrasonic cleaning bath. Other less common methods include forcing phospholipid mixtures under high pressure through a needle or other small orifice, or by preparing lipid mixtures in detergents and then removing the detergents by dialysis.

The third and final class of liposome preparations is large unilamellar vesicles. These large, single bilayered membranes are usually between sixty nanometers and several micrometers in diameter. Characteristically these liposomes are two hundred to one thousand nanometers and a capture volume of approximately 14 ul/mg lipid (Poznansky and Juliano, 1984). These liposomes have been produced by the slow hydration of phospholipid film into distilled water and through the injection of an ether solution of lipid into warm aqueous media (Pagano and Weinstein, 1978). The most common preparation method of LUVs is via reverse-phase evaporation as described by Szoka and Papahadjopoulos (1978). In this method material to be encapsulated, in aqueous mixture, is added to phospho-lipids in organic solvent and then the liposomes are

formed by the evaporation of these solvents under reduced pressure. Sonication of this product can also be used to produce small unilamellar vesicles.

The ability of liposomes to deliver compounds in vivo was first examined by Gregoriadis and Ryman in 1972. In this study liposomes were shown to deliver albumin into the livers of rats. Depending on the targeted site of a particular liposome-encapsulated compound, liposomes may be introduced in vivo by intravenous, intraperitoneal, intracerebral, or localized injections or by oral administration (Kimelberg and Mayhew, 1978). Local intramuscular injections lead to the accumulation of encapsulated material in the lymphatic system associated with the area (Gregoriadis, 1977). Oral administration of liposomal insulin has been proven to be a possible method to deliver insulin in normal and diabetic rats (Gregoriadis, 1977). Factor VIII has been reported to be absorbed by Hemophilia A patients given this procoagulant orally in liposomes (Schneider, 1985).

The most common method, intravenous injection, provides a route to administer compounds to a large area of the host via the circulatory system. Circulating liposomes are preferentially taken up by organs rich in reticuloendothelial cells. Accumulation in liver, spleen, lung and bone marrow is the rule in both mice and humans (Lopez-Berestein et. al., 1984b and 1984c). Liposomes of

diameters equal to or smaller than one micron circulate freely in the mammalian patient. Hunt et. al. (1979) reported that those larger tend to accumulate in the lungs of mice. This presents a problem with large liposomes delivering drugs unless the lungs are indeed the target organ. Papahadjopoulos (1986) introduced the projected study of liposomal treatment of Pneumocystis pneumonia in acquired immunodeficiency syndrome patients (AIDS) as an example of when the lungs would be targeted. Liposomes can be altered in size after they are produced by many methods. Multilamellar vesicles in particular are usually large and heterogenous by nature. These liposomes can be fractionated by centrifugation or separated by filtering. Olson et. al. (1979) presented a procedure to make defined size liposomes by a process of extrusion through polycarbonate membranes.

Liposomal Amphotericin B

The first study of the encapsulation of amphotericin B in liposomes was conducted by New et. al. (1981). While exploring liposomal leishmaniasis therapies this group discovered that amphotericin B toxicity to the host was reduced when delivered in liposomes. In the following year Taylor et. al. (1982) published a study which employed liposomal amphotericin B in the treatment of murine histoplasmosis. Liposomes produced by this group

were of the MLV class and included ergosterol. These vesicles were shown to produce a nine-fold reduction in toxicity of amphotericin B. The study also showed that encapsulation increased the tissue concentration and decreased the serum concentration of the drug. Members of this group then went on to explore the use of liposomal amphotericin B in the treatment of murine cryptococcosis (Graybill et. al., 1982). This study indicated liposomal amphotericin B to have reduced toxicity and better localization than the commercial preparation of the antifungal. The liposomes and the disease-producing yeast cells accumulated in the reticuloendothelial system as expected. In what seems to contradict previous studies, liposomal amphotericin B was shown to reduce intracerebral counts of cryptococci following intravenous therapy.

Using the same liposome formulation, Ahrens et. al. (1984) explored this novel antifungal therapy in a murine model of candidiasis. Liposomal amphotericin B showed increased efficacy by allowing larger doses to be used. At equal doses with the commercial drug preparation, liposomal amphotericin B in this formulation proved less effective. Later studies have reported that liposomes containing sterols have inherent toxic aspects which can diminish the apparent efficacy of amphotericin B encapsulated within them (Juliano et. al., 1985). Hopfer et. al. (1984) reported that with the incorporation of

sterols twelve and fifty times as much amphotericin B was needed to kill strains of Candida albicans when liposomes included cholesterol and ergosterol, respectively. Other groups have also studied liposomal amphotericin B in the treatment of candidiasis and leishmaniasis reporting lowered toxicity in all cases (Panosian et. al., 1984; Tremblay et. al., 1984 and 1985). In order to produce more stable amphotericin B liposomes some groups have prepared polymerized phospholipid vesicles (Mehta et. al., 1986). Unlike the previously mentioned studies, polymerized liposomes produced thus far do not reduce amphotericin B toxicity.

The most thoroughly explored and promising encapsulation of amphotericin B in liposomes is the formulation introduced by the Lopez-Berestein group of Houston (Lopez-Berestein et. al., 1983; Juliano et. al., 1983). Liposomes produced by this group are multilamellar vesicles consisting of a seven to three molar ratio of dimyristoyl phosphatidylcholine (DMPC) and dimyristoyl phosphatidyl-glycerol (DMPG) prepared in a manner similar to the original Bangham et. al. (1965) procedure previously discussed. This formulation has been shown to produce no abnormal findings in blood chemistry or histology of animals injected with it (Lopez-Berestein et. al., 1983). Liposomal amphotericin B prepared in this manner produced none of the nephrocalcinosis and renal

parenchymal edema that the free (commercial) amphotericin B produced in mice. The liposomal drug proved as effective as the free drug in equal concentrations in a murine model of disseminated candidiasis (Lopez-Berestein et. al., 1983). Though the maximum tolerated dose of free drug was 0.8 mg per kilogram of mouse (in this study), 12 milligrams of liposomal amphotericin B per kilogram was well tolerated by the mice. This allowed much larger doses of liposomal drug to be used and thus a higher therapeutic index achieved by the encapsulation of amphotericin B.

The pharmacologic effects of this liposome formulation (without amphotericin B) in cancer patients has been examined (Lopez-Berestein et. al., 1984b). Liposomes carrying radiolabel were shown to produce tissue retention predominately in the reticuloendothelial cells of the patients. No toxic side effects were reported and the conclusion was made that effective targeting of drugs to treat pathologic conditions involving organs rich in reticuloendothelial cells could be afforded via liposomes. The additional finding that liposomal amphotericin B accumulated in the reticuloendothelial cells even more in mice infected with candidiasis also kindled hopes of more effective therapy of fungal infection (Lopez-Berestein et. al., 1984c). Many patients afflicted with systemic candidiasis or other fungal infections have hematologic

malignancies or other underlying conditions which render them neutropenic and immunocompromised. Knowing this, the Lopez-Berestein group next studied the effect of liposomal amphotericin B in the treatment of candidiasis in neutropenic mice, as well as the effect of this compound on immune and other circulatory cells.

Liposomal amphotericin B prophylaxis in neutropenic mice was reported to provide protection against candidiasis (Lopez-Berestein et. al., 1984). Empty liposomes or free amphotericin B showed no prophylactic efficacy in these studies. In treatment studies of disseminated candidiasis in neutropenic mice liposomal amphotericin B proved again to be more efficacious due to the increase in dosage that this preparation allowed (Lopez-Berestein et. al., 1984a). In addition, liposomal drug therapy was shown to be effective five days after initiation of the disease while free drug therapy had to be initiated within three days to provide any effect. Liposomal amphotericin B improved survival time and reduced renal impairment in the neutropenic mice.

Liposome-encapsulation of amphotericin B has been shown <u>in vitro</u> to greatly reduce the toxic effect of the drug on mammalian cells. The effect of liposomal amphotericin B produced by the Lopez-Berestein group on human blood cell was reported by Mehta et. al. (1984). They showed that while both free and liposomal drug killed

C. albicans just as effectively, free amphotericin B lysed erythrocytes at one microliter per milliliter buffer, but the liposomal drug produced no appreciable lysis at one hundred times this concentration. A later study by Juliano et. al. (1987) showed that lack of toxicity in these liposomes is due to the use of saturated acyl chain phospholipids. Liposomal amphotericin B produced with unsaturated acyl chain phospholipids have been shown to be as toxic as the free drug itself. They also pointed out that the toxicity or lack of toxicity produced by amphotericin B preparations is due to the lipid composition of the membranes that they contact. Juliano et. al. (1987) put forth the theory that amphotericin B is selectively transferred from liposomes to cells of the host and not slowly leaked from the liposomes as previously believed. They purport that this diffusion is regulated by properties of both the liposome and cell membranes. Szoka et. al. (1987) tested the toxicity and organ distribution of various liposomal and free amphotericin B preparations and came to a somewhat different conclusion. They concluded that liposomal preparations of amphotericin B showed no change from the free drug in organ distribution and only lessened or slowed its toxic effect. They theorize that liposomal encapsulation reduces toxicity of amphotericin B by slowing its transfer rate to sensitive target cells.

Mehta et. al. (1985a) reported that the 7:3 DMPC to DMPG liposomal amphotericin B formulation reduced the immunosuppressive effects seen in free amphotericin B therapy. The liposomal drug was shown not to reduce macrophage production of superoxide anion or differentiation markers *in vitro* like the free drug does. While free amphotericin B inhibited T cell blastogenesis at high concentrations, this proved not the case for the liposomal form of the antifungal. Both forms of the drug did however inhibit antibody production *in vivo*.

The liposomal amphotericin B formulation of the Lopez-Berestein group is, as stated above, currently being investigated clinically under a FDA Investigational New Drug permit. Results of the initial clinical trials of liposomal amphotericin B have been quite promising. The first published results reported remission in eight of twelve patients treated with the liposomal compound (Lopez-Berestein et. al., 1985). Included in this group were seven persons with aspergillosis, three with candidiasis, one with mucormycosis, and one suffering from histoplasmosis. The twelve patients, all immunosupressed and nine granulocytopenic, were all previous nonresponders to free amphotericin B as well as other antifungals. Liposomal amphotericin B infusion produced mild or moderate temperature increases or chills in two patients, but no other toxic responses. In fact, three

patients with liver or kidney dysfunction actually showed improvement in the respective affected organs during the course of treatment.

In a similar group of patients, all with hematologic malignancies, fungal infections, and previous amphotericin B therapy, liposomal amphotericin B also proved to be efficacious (Shirkhoda et. al., 1986). Six of the eight patients with disseminated mycosis of the liver, most also having spleen involvement, showed improvement after liposomal therapy. In another report of nine patients with hepatosplenic candidiasis, Lopez-Berestein et. al. (1987) have reported cures produced by the liposomal drug in eight cases. The remaining patient showed improvement during therapy. One of the eight cured patients in this study had a history of anaphylactic reaction to free amphotericin B.

Besides the study of liposomal amphotericin B, other liposomal strategies for the therapy of systemic fungal infection have and are currently being explored. These include studies of the encapsulation of other antifungal compounds and studies of combined treatment of liposomal amphotericin B with other liposome-encapsulated materials. The study of liposome-encapsulated nystatin in the treatment of candidiasis has shown that intravenous injection of this drug may be possible (Mehta et. al., 1987 and 1987a). Another study by Mehta et. al. (1985)

showed that combined treatment of liposomal amphotericin B and a liposome-encapsulated macrophage activator (6-O-stearoyl- N-acetylmuramyl- L-alpha-aminobutyryl-D-isoglutamine) provided an additive effect as compared to either of these compounds alone. This macrophage activator itself has been shown to be an effective prophylactic liposomal therapy against candidiasis in mice (Fraser-Smith et. al., 1983; Lopez-Berestein et. al., 1983a). These results may also be reflected in human macrophage activation. Mehta et. al. (1982) showed that macrophage activator delivered in liposomes was phagocytized by human peripheral blood monocytes in vitro.

Targeting of Liposomes

Liposomes delivered intravenously are localized in the reticuloendothelial system after a period of circulation in the plasma. The duration of this circulation is dependent upon various characteristics of each particular liposome preparation including size, composition and surface charge (Gregoriadis et. al., 1985 and 1977). Small liposomes remain in the circulatory system for much longer periods than large multilamellar vesicles (Gregoriadis, 1977). During the circulatory life of the liposomes, a chance for specific targeting of these vesicles to specific cells exists. Employing this idea, and using various molecules on the surface of liposomes,

researchers have explored this theoretical concept of specific delivery. Molecules which have been bound to the surface of liposomes include glycoproteins, glycolipids, antibodies and other cytophilic molecules. Initial results from these studies indicate that specific liposomal targeting is indeed a realistic approach to deliver compounds to cells in proximity of the circulatory system.

Glycolipid targeting of liposomes to the liver of mice has been reported by Nozawa et. al. (1986). Galactose-containing ligands on liposome surfaces were reported to be highly specific for hepatocytes. Bachhawat and Dasgupta (1986) also targeted hepatocytes with the glycolipid, monosialoganglioside with success. A glycolipid sulfatide has been shown by Yagi and Naoi (1986) to allow liposomes access across the blood-brain barrier to the brain.

Polysaccharide-coated liposomes have been shown to be effective in the treatment of Legionnaire's disease in guinea pigs (Sunamoto, 1986). Sunamoto states that this use of sisomycin encapsulated in O-palmitoylamylopectin-coated liposomes is the first successful treatment of a bacterial infection by targeted liposomal drug.

Antibody and fragments of antibody are by far the most studied of the liposome-targeting molecules. A multitude of methods have been devised to add active antibody to the

surface of liposomes. The simplest of these methods, detailed by Gregoriadis and Neerunjun in 1975, involves adding antibody to the aqueous solution to be encapsulated. Huang and Kennel (1979) later reported that the sonication employed in this procedure was responsible for the external expression of a proportion of the antibody. This group showed that sonication of preformed liposomes with antibody led to the externalization of some antibody with retained activity.

Covalent coupling of antibody was reported in 1979 by Torchlin et. al. This group "activated" preformed liposomes containing Indium-111 chloride with glutaraldehyde. These "activated" liposomes were then allowed to react with antimyosin antibody overnight. These liposomes were shown to cause the localization of Indium-111 chloride in the infarcted area of a dog's heart, the targeted site.

Leserman (1981) found fault with both of the above mentioned formulations of antibody-targeted liposomes. He stated that the sonication insertion method was too inefficient and also likely to produce Fc-mediated binding. He was unable to repeat the glutaraldehyde formulation results in his laboratory. The method introduced by Leserman involved covalently modifying both the amine end of the antibody and the phosphatidylethanolamine used in the liposomes. This modification added the

cross-linking reagent N-hydroxy succinimidyl -1 -3-(2 pyridilyldithio) propionate, also denoted SPDP, to both of these components. Following production of liposomes with SPDP linked phosphatidylethanolamine, modified antibody is added and allowed to react forming the covalent connection of the two. Specificity of these liposomes was reported by Leserman et. al. (1983) in a study involving localization of these vesicles in mice. The study also reported that antibody bound to liposomes failed to induce antiidiotypic responses. The localization of this preparation of targeted liposomes was also studied by Gregoriadis et. al. (1985) with similar results.

The addition of $F(ab')_2$ to liposomes by oxidation-reduction reaction has been reported by Heath et. al. (1980). This preparation entailed first oxidizing the surface of preformed liposomes with periodate, followed by the addition of antibody fragment and the reducing agent sodium cyanoborohydride. Binding of liposomes to human erythrocytes increased two hundred times when this method was employed to add conjugated antierythrocyte $F(ab')_2$ to these vesicles. This method was also reported to be effective in the binding of whole antibody to liposomes (Heath et. al., 1984). Recent work performed by this group shows a shift from this formulation to the preparation presented by Leserman above using SDSP cross-binding.

Employing this new formulation, they have shown in vitro that the toxic antitumor agent methotrexate-gamma-aspartate can be delivered safely and specifically to cancer cell lines by antibody-directed liposomes (Heath et. al., 1983; Paphadjopoulos et. al., 1985).

Huang et. al. (1981) have presented another way of attaching antibody to liposomes. Their method involves the use of previously modified antibody and no oxidization of the liposomes. The advantage of this method, put forth by Huang et. al. (1982), is that the liposomes, and more importantly the often chemically fragile liposomal contents, are not subjected to the potential damage that the previously described coupling methods may produce. In this formulation antibody is covalently modified by the addition of fatty acid residues to its Fc end (Huang et. al., 1980). The modified antibody is then allowed to react with slightly destabilized liposomes produced by reverse-phase evaporation (Shen et. al., 1982). The product of this reaction is then stabilized by the removal, by dialysis, of the destabilizing emulsifier and residual organic solvents. The result of this process is the formulation of liposomes which safely encapsulate materials and externalize a specific immunoglobulin.

Development of Amphotericin B Liposomes

Bearing Antibody Specific to *Candida albicans*

Duane R. Hospenthal, Alvin L. Rogers and Gary L. Mills

Published in Mycopathologia 101: 37-45 (1988)

Development of Amphotericin B Liposomes Bearing Antibody Specific to Candida albicans.

Duane R. Hospenthal[1], Alvin L. Rogers[1,2], and Gary L. Mills[1]

Departments of [1]Botany and Plant Pathology, [2]Microbiology and Public Health and Medical Technology Program, Michigan State University, East Lansing, MI 48824, USA

Keywords: amphotericin B, Candida albicans, liposome

Summary.

Liposomes expressing external antibody specific for Candida albicans and encapsulating amphotericin B were developed and characterized in this study. Antibody was first modified by the covalent attachment of palmitic acid residues. Liposomes were produced by reverse-phase evaporation and modified antibody was incorporated into these liposomes via the hydrophobic interaction between the palmitic acid and the phospholipids composing the liposomes. The liposomes were characterized as to the amount of amphotericin B by spectroscopy and for the

presence of antibody by protein analysis and secondary immunolabeling by fluorescent and electron microscopic methods. Immunogold labeling showed that the antibody was being expressed externally on the liposomes in the electron microscopic studies and the specificity of these liposomes for C. albicans was observed by secondary immunofluores-cence.

'Address for offprints: Duane R. Hospenthal, Department of Botany and Plant Pathology, Michigan State University, East Lansing, MI 48824, USA'

Introduction

Candida albicans is the causative organism of greater than eighty percent of all fungal infections. It is responsible for thirteen percent of fatal infections in lymphoma patients and over twenty percent of those in leukemia patients (3). Although several new antifungal agents are being used, amphotericin B remains the drug of choice for most systemic fungal infections including candidiasis. Until recently the use of this antifungal agent has been limited due to its acute and chronic toxicity. However, it has now been established that encapsulation of amphotericin B in liposomes can reduce the toxic effects of the drug without decreasing its efficacy (6,8). The best characterized and most promising results reported are

from a particular phospholipid liposome formulation (7:3 molar ratio of dimyristoyl phosphatidylcholine to dimyristoyl phosphatidylglycerol) (6,8,9,22). This formulation has been tested successfully in laboratory animals (12,13,14, 16) as well as in a group of hematologic malignancy patients with systemic mycoses which previously have shown no response to amphotericin B treatment (11,19). The results of these and other studies show a large potential for future use of liposomal amphotericin B treatment.

Liposomes containing drugs other than amphotericin B and modified with antibody have also been shown to be useful in the specific delivery of these drugs and other macromolecules to antigenic sites on cell and tissues (5). For example, the cytotoxic action of actinomycin D encapsulated in antibody bearing liposomes is selective towards only those cells bearing the corresponding antigen (4). An important prerequisite for producing liposomes which are directed by antibody is to attach the immunoglobulins to the phospholipid vesicles without damaging or losing the substance which is being encapsulated. With this in mind we have adopted a procedure in which palmitic acid residues are covalently bound to antibody and then these modified immunoglobulins are inserted into the surface of preformed liposomes (7,18).

Materials and Methods

Antibody modification

Adsorbed rabbit antisera to C. albicans (Difco, Detroit, MI) was reacted with N-hydroxysuccinimide ester of palmitic acid (NHSP) to produce antibody with covalently bound palmitic acid residues (Ab-P) as previously described by Huang et al (7,18). The NHSP was prepared by the method of Lapidot et al (10) with 40 uCi 1-^{14}C-palmitic acid per mmol palmitic acid included to follow the reaction. Equal molar concentrations of N-hydroxy-succinimide and palmitic acid were reacted in the presence of dicyclohexylcarbodiimide (present also in a like amount) overnight. A sample of the labeled product along with ^{14}C-palmitic acid were chromatographed on ITLC-SA chromatography medium (Gelman, Ann Arbor, MI) using multiple development with the following solvents: A. isopropyl alcohol: ammonium hydroxide (100:7 v/v) and B. isooctane:diethyl ether:acetic acid (100:5:0.5 v/v/v). The chromatograms were developed twice with solvent A to 10 cm, removed and dried between runs, and finally developed to 17.5 cm with solvent B. The resulting chromatograms were then scanned for radioactivy with a Tracerlab 4-pi Scanner. Quantitative data were derived by the comparison of total peak area obtained from scans. Esterified palmitic acid was visualized with ferric hydroxylamine while free palmitic acid was observed and

localized using H_2SO_4-K_2CrO_4 charring (20). The amount of the product used in the antibody modification was calculated from the percent of radioactivity associated with the chromatographed product peak and the original ratio of radioactivity to palmitic acid. NHSP was reacted with antibody in a molar ratio of 15:1 in phosphate-buffered saline (PBS; 137 mM NaCl/2.7 mM KCl/1.5 mM KH_2PO_4/1 mM Na_2HPO_4) containing 0.5% deoxycholate (DOC) overnight at 37C to bind palmitic acid to the immunoglobulin. The reaction mixture was centrifuged (3400 g) to remove particulates and the supernatant was applied to a Sephadex G-75 column (1.9 X 20 cm). The modified antibody, as determined from protein analysis and associated radioactivity, was eluted in the void volume with PBS. Fractions containing the product (Ab-P) were combined, concentrated by ultrafiltration (Amicon, XM-50) and then frozen in aliquots for future use. Slide agglutination tests (2) were performed using both the recovered product and the original antiserum with C. albicans to determine antibody activity.

Antibody-bearing liposome production

Dimyristoyl phosphatidylcholine (DMPC) and dimyristoyl phosphatidylglycerol (DMPG) were purchased from Sigma (St. Louis, MO). Deoxycholate-free amphotericin B was supplied by Squibb Pharmaceuticals (New Brunswick, NJ). DMPC was

suspended and stored at -12C in chloroform in a stock dilution of 40 mg/ml, while DMPG was diluted to a 20 mg/ml stock solution in chloroform-methanol (2:1 v/v) and stored at -12C. Amphotericin B was suspended in methanol at a concentration of 40 ug/ml and stored covered from light at 4C. Antibody-coated amphotericin B liposomes (LAMB-Ab) were produced by a modification of the reverse-phase evaporation method of Huang et al. (7). Stock solutions containing 35 mg DMPC, 15 mg DMPG, and 500 ug amphotericin B were combined with 2.0 ml PBS and 1.0 ml of chloroform. Organic solvents were evaporated with N_2 and heat (45C) and the solution concentrated to a viscous gel, approximately 1.0 ml. One milliliter of PBS was added and the mixture was then briefly sonicated (2 s, 100 w) and allowed to stand uncovered for 1 h at room temperature. Modified antibody (Ab-P) in 1.0 ml PBS containing 0.15% DOC was then added to the reaction mixture and left at room temperature for 2 h. The solution was dialyzed (Spectrapor 2 dialysis tubing) overnight at 4C with three changes of 500 ml PBS and the product (LAMB-Ab) was recovered by centrifugation (15 m, 30,000 g, 4C). The pellet was washed twice with PBS to remove unbound antibody and suspended in 2.0 ml PBS. Liposomes lacking antibody were prepared with (LAMB) and without (LIPO) amphotericin B using the same technique.

Liposome characterization

Liposomes were analyzed for lipid, amphotericin B and protein. Total lipid for LIPO, LAMB, and LAMB-Ab was based upon dry weight determination after extraction with $CHCL_3$-CH_3OH (2:1v/v). Amphotericin B content of the solvent extract was quantified spectrophotometrically at 405 nm and compared to that of standard dilutions of the drug in chloroform-methanol (2:1 v/v). Protein analysis of the product in PBS was performed using the Folin phenol reagent described by Lowry et al. (15).

Electron microscopy

Immunogold labeling of the prepared liposomes to detect surface antibody was conducted according to the procedure of Bendayan (1). Liposome suspensions were air dried onto parlodion covered, carbon stabilized grids. After the suspension was dry the grids were pretreated with ovalbumin and incubated with gold labeled goat anti-rabbit IgG antibody (Janssen, Beerse, Belgium) for 20 m at 37C. Unbound immunogold was removed by multiple rinsing of the grids in PBS followed by distilled water. The grids were fixed with 2% osmium tetroxide, extensively rinsed in distilled water, and then negative stained (1 m) with 0.5% uranyl acetate. Grids were examined with a Philips 201 transmission electron microscope.

Fluorescent microscopy

Immunofluorescent studies were performed employing the following variation of studies described by Sundstrom and Kenny (21). Liposome or control suspensions were incubated with C. albicans for 30 m at 37C to allow antigen-antibody binding. The resulting mixture was dried onto microscope slides coated with 1% poly-L-lysine to aid in adherence. Three drops of 1:1 PBS diluted fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Sigma) were applied to each slide and allowed to react at 37C for 30 m in a moist chamber. This incubation was followed by extensive washing of the slides with distilled water. Slides were mounted in 10% glycerol and observed for fluorescence with an Olympus BH2 series microscope with reflected light fluorescence attachment (BH2-RFL). Photomicrographs were taken with an automatic Olympus camera system (PM-10ADS) with TRI-X Pan film. Palmitic acid modified antibody at a concentration of 50 ug/ml was treated as above and served as the control.

Yeast cells

Candida albicans MSU-1, a clinically isolated strain maintained at Michigan State University Medical Mycology Laboratory was used in the fluorescent, slide agglutination and antifungal studies. Cells were grown in trypticase soy broth containing 4% glucose at 37C on a rotary

shaker for 14 h to develop the stationary phase. The yeasts were recovered by centrifugation and washed three times in PBS. Candida albicans used in the fluorescent and agglutination studies were fixed in 0.5% formaldehyde (30 m at 4C), rinsed, and then suspended in PBS at a concentration of 2.7×10^7 cells ml^{-1}.

Toxicity

The toxicity of the LAMB-Ab was assessed and compared to that of LAMB and the free drug (free AMB) utilizing a method previously described (17). Two percent freshly washed human red blood cells in PBS were incubated with differing concentrations of amphotericin B in liposomes or as the free solubilized drug. After 45 m at 37C, erythrocyte damage, observed as red color (hemoglobin release) in the supernatant of the centrifuged reaction mixtures, was assessed. Lysis was compared to the control mixture which included distilled water and 2% red blood cells. Free amphotericin B (free AMB) was prepared by solubilizing deoxycholate-free amphotericin B with DOC at a concentration similar to that found in the commercial drug, Fungizone.

Antifungal activity

To assess antifungal activity dilutions of the three amphotericin B-containing mixtures (LAMB-Ab, LAMB, and

free AMB) were incubated for 16 h at 37C with 7 X 10^5 colony forming units (CFU) of C. albicans. After this incubation dilutions were plated on Sabouraud dextrose agar and colony counts were made 24-48 h later to determine viable CFU. This procedure is a modified susceptibility test described by Mehta et al (17).

Results

Formation and characterization of LAMB-Ab

A critical step in the formation of LAMB-Ab is the production of Ab-P. This was accomplished by reacting palmitic acid with N-hydroxysuccinimide in the presence of dicyclohexylcarbodiimide. The product formed (NHSP), as indicated by thin-layer chromatography, represented 90.6% of the added radioactivity, had a Rf of 0.31, and stained positive for esterified fatty acid. In contrast free palmitic acid had an Rf of 0.79 and could only be detected by sulfuric acid charring. The reaction of NHSP with antisera to Candida albicans resulted in the formation of Ab-P. Greater than 90% of the starting protein and 80% of the starting labeled lipid were associated with Ab-P following the removal of unreacted side products and palmitic acid by centrifugation, gel filtration, and ultrafiltration. Based upon the recovered protein and radioactivity it was determined that 5-6 palmitic acid residues were bound to each molecule of antibody.

Incubation of Ab-P with liposomes containing amphotericin B resulted in the lipophilic interaction of the palmitic acid residues of Ab-P with the phospholipid vesicles to yield LAMB-Ab. This product, recovered as a pellet following centrifugation, was extensively washed to remove any unattached Ab-P. Analytical data for five separate LAMB-Ab preparations indicated that the average recovery for protein, lipid and amphotericin B was 11%, 30% and 61%, respectively.

Electron microscopy provided an indication of the external expression of immunoglobulin G on the surface of the liposomes prepared with antibody (Figure 1a), but not on LAMB (Figure 1b) or LIPO (not shown). Background amounts of immunogold were found in the LAMB and LIPO treated as controls with this secondary immunomarker. Electron microscopy also provided a size distribution of the liposomes. The predominant size, shown in Figures 1a and 1b, was 10-60 nm. Size distribution was heterogeneous and liposomal diameter increased with storage. After 4 weeks at 4C a large number of the liposomes were approaching 5 um in diameter.

Fluorescent microscopy indicated that the antibody associated with liposomes showed specificity for the yeast cells of C. albicans (Figure 2a). Free palmitic acid modified antibody in a concentration approximating that in the LAMB-Ab was used as a positive control in Figure 2b.

Microscopically the fluorescent intensity of the yeasts in both of these reactions were approximately equal. However, due to the intensity of the amorphous mass associated with the LAMB-Ab reaction mixture, the exposure time had to be reduced to avoid bleaching of the photograph. Figure 2b was exposed 2.5 m while for Figure 2a, the exposure time was 1.6 m. C. albicans alone and in combination with liposomal amphotericin B without antibody (LAMB), the negative controls in this experiment, were negative for fluorescence.

Cytotoxicity and antifungal activity

Toxicity of LAMB-Ab to human erythrocytes reflected the benefit of liposome encapsulation previously reported for LAMB (8,17) in comparison to free amphotericin B. In the erythrocyte toxicity test LAMB-Ab and LAMB showed no damage in concentrations of 100 ug/ml, almost 10 times the concentration at which free AMB caused lysis (Table 1).

In addition, the antifungal activity of these three mixtures (LAMB-Ab, LAMB and free AMB) was approximately equal at identical concentrations in a series of two-fold dilutions of 0.03-1.00 ug amphotericin B (Figure 3). These data represents the average growth of C. albicans (four experiments done in duplicate) after incubation with the drug in each of the three mixtures. A small increase

in antifungal activity was evident in the liposomal preparations as compared to the free drug.

Discussion

Liposomes bearing antibodies and enclosing amphotericin B were produced in this study. Secondary labeling by immunogold and immunofluorescence show that the modified antibody used in the experiment was being expressed on the surface of these liposomes and that this immunoglobulin is indeed specific for the yeast. The chemical quantification of this antibody was quite difficult to determine once the liposomes were formed. The protein test used to detect antibody in the liposome preparations showed interference (large values in LAMB and LIPO) which the authors assumed to be caused by the turbidity of the solutions and the yellow coloration of amphotericin B. The electron microscopy showed that the size distribution of these liposomes were quite small. This should aid in the reduction of toxicity since larger liposomes may become trapped in small capillaries when in vivo studies are performed. However, it should be noted that during this study that an increase in the size of the liposomes did occur with storage. Use of fresh liposomal batches in future studies will be important. The amphotericin B enclosed within these liposomes (LAMB-Ab and LAMB) showed the reduced toxicity and retained antifungal activity of

previously described liposomal amphotericin B (8,17). Whether this compound will prove to be more effective in the treatment of candidiasis remains to be seen. A murine model of this mycoses is currently being used to initiate the therapeutic study of this new drug delivery system. The potential role of the use of antibody-directed amphotericin B-encapsulating liposomes in the future remains to be explored.

Liposomal amphotericin B has shown an increased therapeutic index in human and animal systemic mycoses. The targeting of this liposome-encapsulated antifungal with specific antifungal antibodies may increase this index to even a greater extent. Increased tolerable doses and better internal directing of this antifungal drug could lead to shorter term, more effective treatment of a majority of the current disseminated mycoses.

Acknowledgements

This project was sponsored in part by funding from the Colleges of Osteopathic Medicine and Natural Science through their NIH Biomedical Research Support Grants for 1986-1987.

References

1. Bendayan M: Protein A-gold electron microscopic immunocytochemistry: Methods, applications, and limitations. J. Electron Microsc. Tech. 1:243-270, 1984.

2. Difco Laboratories: Bacto Candida albicans antisera. In: Difco Manual. 10th Edition. Difco Laboratories Inc, Detroit, 1984, pp. 196-198.

3. Feld R, Bodey GP, Rodriguez V, Luna M: Cause of death in patients with malignant lymphoma. Am J Med Sci 268:97-106, 1974.

4. Hashimoto Y, Endoh H, Minoran S: Chemical methods for the modification of liposomes with proteins or antibodies. In: G Gregoriadis (ed) Liposome Technology Vol. III. Targeted Drug Delivery and Biological Interaction. CRC Press, Boca Raton, Florida, 1984, pp. 41-50.

5. Heath TD, Montgomery JA, Piper JR, Papahadjopoulos D: Antibody-targeted liposomes: increase in specific toxicity of methotrexate-gamma-aspartate. Proc Natl Acad Sci USA 80:1377-1381, 1983.

6. Hopfer RL, Mills K, Mehta R, Lopez-Berestein G, Fainstein V, Juliano RL: In vitro antifungal activities of amphotericin B and liposome-encapsulated amphotericin B. Antimicrob Agents Chemother 25:387-389, 1984.

7. Huang L, Huang A, Kennel SJ: Coupling of antibodies with liposomes. In: G Gregoriadis (ed) Liposome Technology Vol.III. Targeted Drug Delivery and Biological Interaction. CRC Press, Boca Raton, Florida, 1984, pp. 51-62.

8. Juliano RL, Lopez-Berestein G, Hopfer R, Mehta R, Mehta K, Mills K: Selective toxicity and enhanced therapeutic index of liposomal polyene antibiotics in systemic fungal infections. Ann NY Acad Sci 446:390-402, 1985.

9. Juliano R, Lopez-Berestein G, Mehta R, Hopfer R, Mehta K, Kasi L: Pharmacokinetic and therapeutic consequences of liposomal drug delivery: Fluorideoxyuridine and amphotericin B as examples. Biol Cell 47:39-46, 1983.

10. Lapidot Y, Rappoport S, Wolman Y: Use of esters of N-hydrosuccinimide in the synthesis of N-acylamino acids. J Lipid Res 8:142-145, 1967.

11. Lopez-Berestein G, Fainstein V, Hopfer R, Mehta K, Sullivan MP, Keating M, Rosenblum MG, Mehta R, Luna M, Hersh EM, Reuben J, Juliano RL, Bodey GP: Liposomal amphotericin B for the treatment of systemic fungal infections in patients with cancer: A preliminary study. J Infect Dis 151:704-710, 1985.

12. Lopez-Berestein G, Hopfer RL, Mehta R, Mehta K, Hersh EM, Juliano RL: Prophylaxis of Candida albicans infection in neutropenic mice with liposome-encapsulated amphotericin B. Antimicrob Agents Chemother 25:366-367, 1984.

13. Lopez-Berestein G, Hopfer RL, Mehta R, Mehta K, Hersh EM, Juliano RL: Liposome-encapsulate amphotericin B for treatment of disseminated candidiasis in neutropenic mice. J Infect Dis 150:278-283, 1984.

14. Lopez-Berestein G, Mehta R, Hopfer RL, Mills K, Kasi L, Mehta K, Fainstein V, Luna M, Hersh EM, Juliano R: Treatment and prophylaxis of disseminated infection due to Candida albicans in mice with liposome-encapsulated amphotericin B. J Infect Dis 147:939-945, 1983.

15. Lowry OH, Rosebrough NJ, Fall AL, Randall RJ: Protein measurement with the folin phenol reagent. J Biol Chem 193:265-275, 1951.

16. Mehta RT, Lopez-Berestein G, Hopfer RL, Mehta K, White RA, Juliano RL: Prophylaxis of murine candidiasis via application of liposome-encapsulated amphotericin B and a muramyl dipeptide analog, alone and in combination. Antimicrob Agents Chemother 28:511-513, 1985.

17. Mehta R, Lopez-Berestein G, Hopfer R, Mills K, Juliano RL: Liposomal amphotericin B is toxic to fungal cells but not to mammalian cells. Biochim Biophys Acta 770:230-234, 1984.

18. Shen D, Haung A, Haung L: An improved method for covalent attachment of antibodies to liposomes. Biochim Biophys Acta 689:31-37, 1982.

19. Shirkhoda A, Lopez-Berestein G, Holbert JM, Luna MA: Hepatosplenic fungal infections: CT and pathological evaluation after treatment with liposomal amphotericin B. Radiology 159:349-353, 1986.

20. Skipski VP, Barclay M: Thin-layer chromatography of lipids. In: JM Lowenstein (ed) Methods in Enzymology Vol. XIV. Lipids. Academic Press, New York, 1969, pp. 530-597.

21. Sundstrom PM, Kenny GE: Characterization of antigens specific to the surface of germ tubes of Candida albicans by immunofluorescence. Infect. Immun. 43:850-855, 1984.

22. Szoka FC, Milholland D, Barza M: Effect of lipid composition and liposome size on toxicity and in vitro fungicidal activity of liposome-intercalated Amphotericin B. Antimicrob Agents Chemother 31:421-429, 1987.

Figure 1a. Transmission electron micrograph of LAMB-Ab (bar = 0.2 um). Gold-conjugated anti-rabbit IgG (electron dense circles) indicates the presence of antibody on the liposomes.

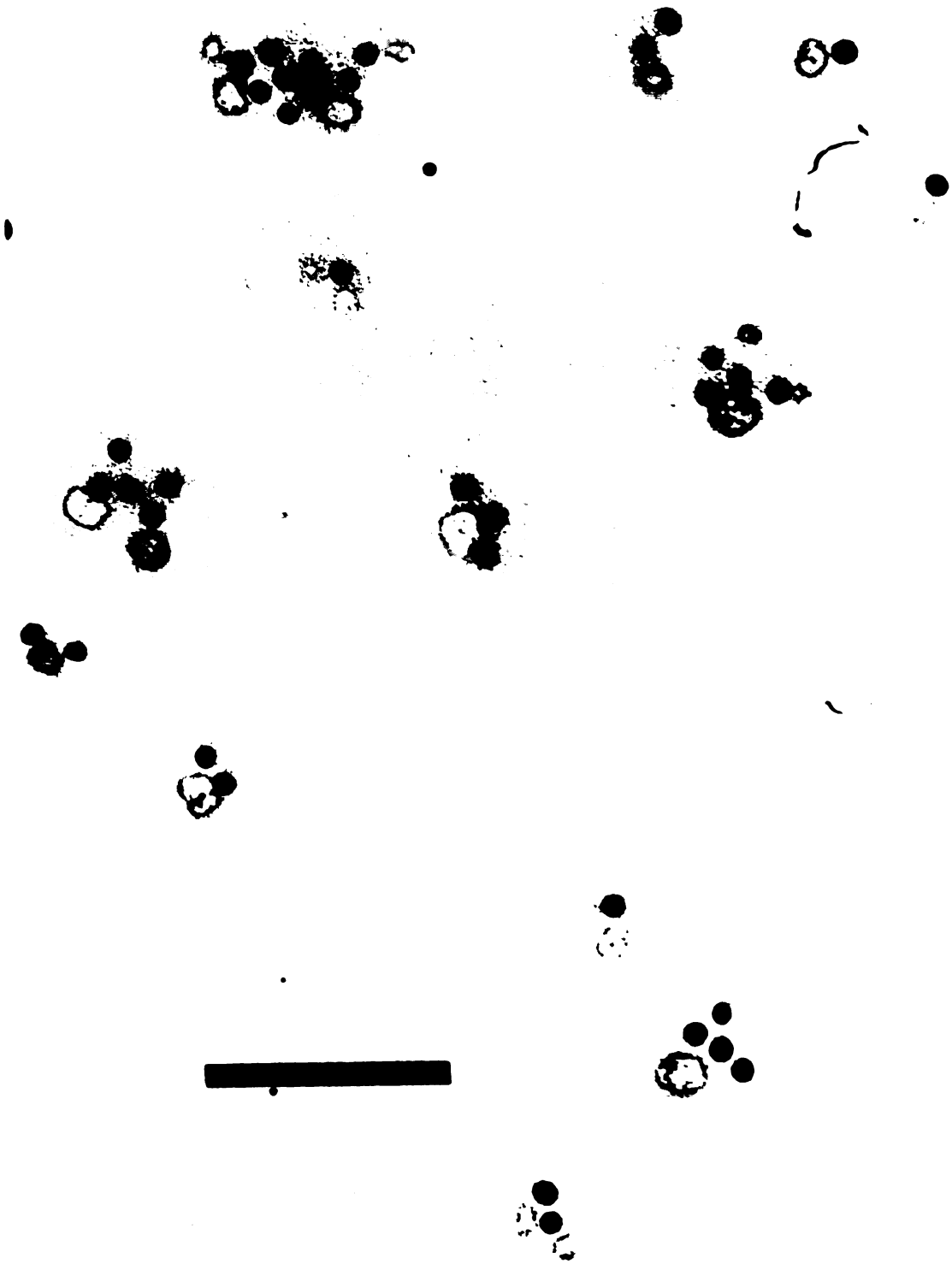


Figure 1a. Transmission electron micrograph of LAMB-Ab (bar = 0.2 um). Gold-conjugated anti-rabbit IgG (electron dense circles) indicates the presence of antibody on the liposomes.

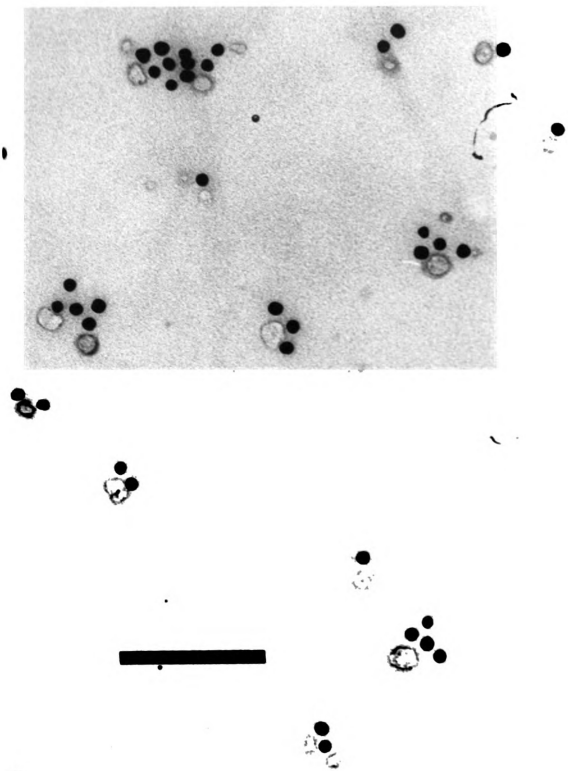


Figure 1b. Electron micrograph of LAMB (bar = 0.2 um). Lack of gold labeling indicates the absence of antibody on these liposomes.

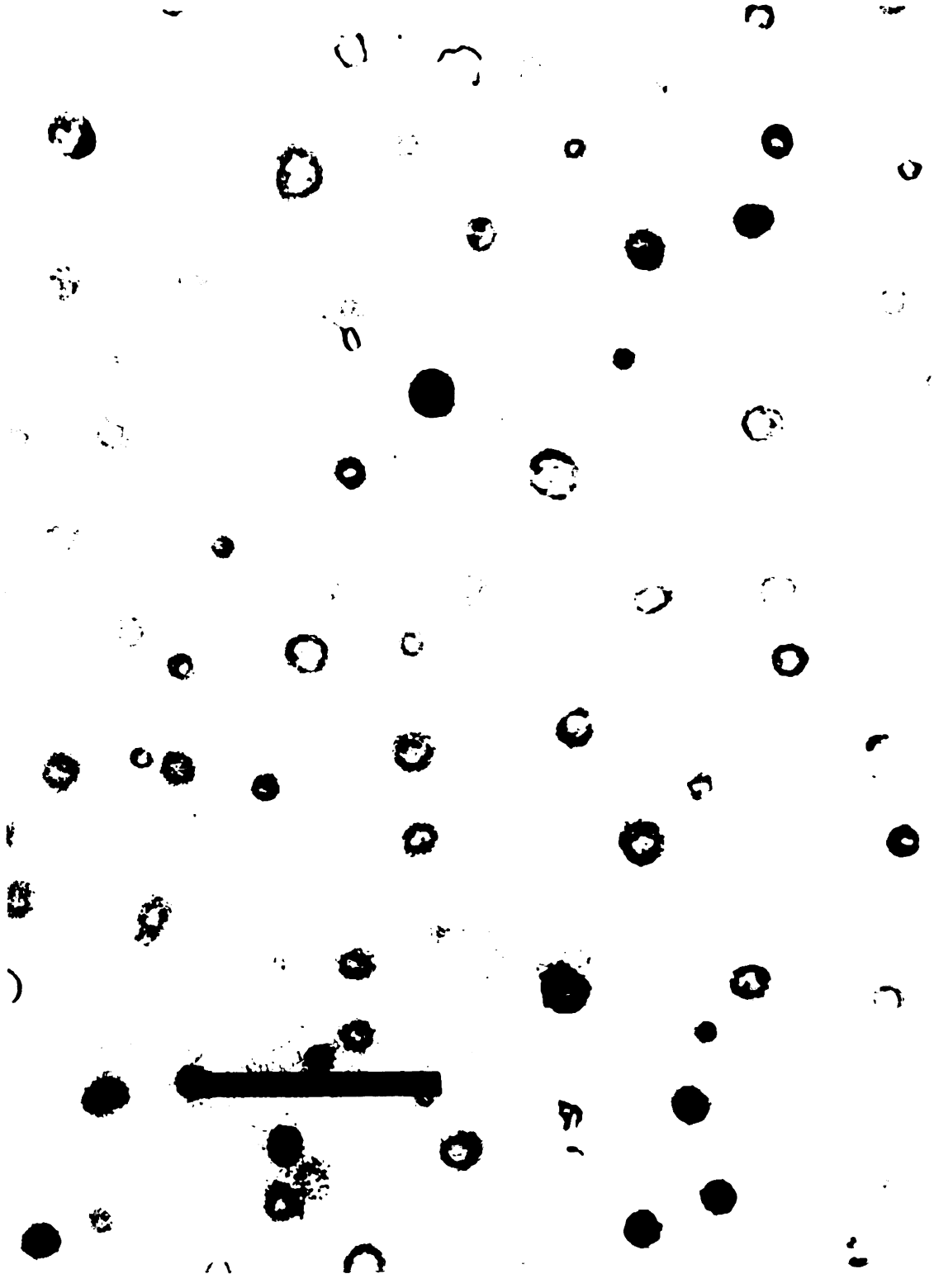


Figure 2a. Micrograph of LAMB-Ab reacted with Candida albicans (bar = 20 um). Bright areas indicate fluorescence of FITC-conjugated anti-rabbit IgG labeling the location of antibody.

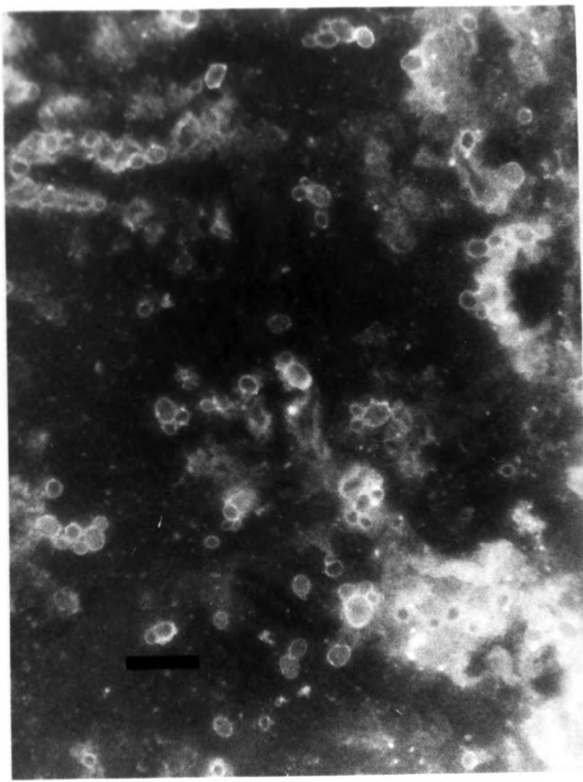
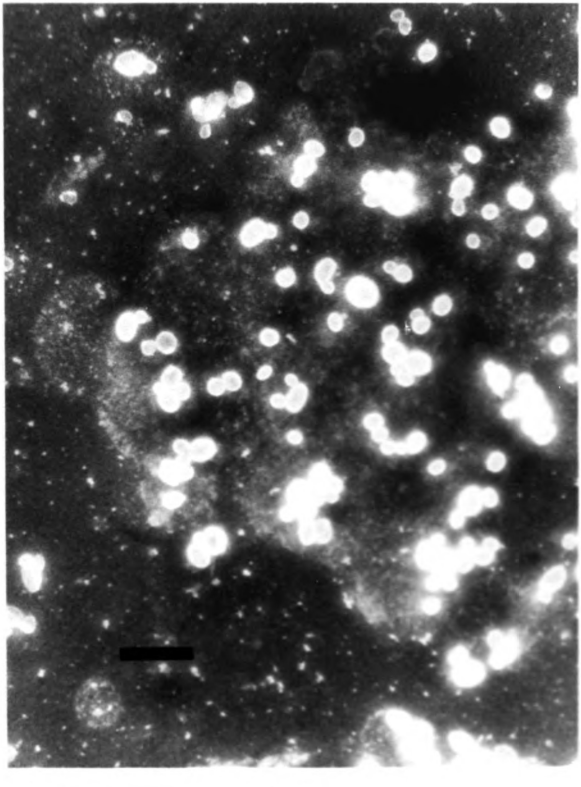


Figure 2b. Fluorescent micrograph of Candida albicans reacted with palmitic acid modified antibody (Ab-P). Yeast cell fluorescence indicates secondary immunolabeling (FITC conjugate) for immunoglobulin G (bar = 20 um).



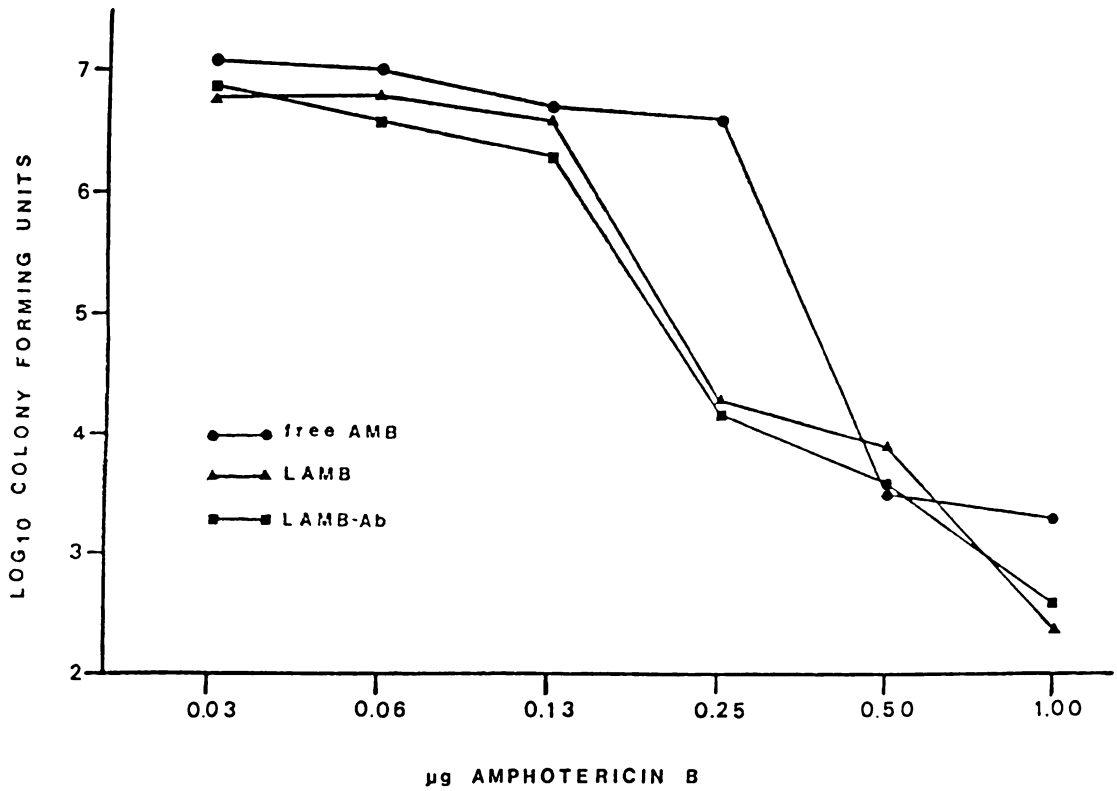


Figure 3. The effect of antibody-bearing amphotericin B-encapsulating liposomes (LAMB-Ab), liposomal amphotericin B (LAMB) and free amphotericin B (free AMB) on the growth of Candida albicans. Initial colony forming units of C. albicans numbered 7 X 10^5 (log_{10} = 5.8) in each test. Untreated controls averaged 1.6 X 10_7 (log_{10} = 7.2) after the 16 h,37C incubation and subsequent plating used in each test.

Table 1. The cytotoxic effect of free AMB, LAMB and LAMB-Ab on a 2% human red blood cell suspension after incubation for 45 m at 37C. Lysis was observed as red color in the supernatant resulting from hemoglobin release. Lysis was considered to be complete (100%) if color matched that of the control (2% red blood cell in distilled water).

LYSIS OF HUMAN RED BLOOD CELLS

	µg AMPHOTERICIN B				
	6.25	12.50	25.00	50.00	100.00
free AMB	–	+	++	+++	+++
LAMB	–	–	–	–	–
LAMB-Ab	–	–	–	–	–

+++ Complete Lysis

– No Lysis

Effect of Attachment of Anticandidal Antibody to the Surfaces of Liposomes Encapsulating Amphotericin B in the Treatment of Murine Candidiasis

Duane R. Hospenthal, Alvin L. Rogers and Everett S. Beneke

Published in

Antimicrobial Agents and Chemotherapy 33: 16-18 (1989)

Effect of Attachment of Anticandidal Antibody to the Surfaces of Liposomes Encapsulating Amphotericin B in the Treatment of Murine Candidiasis

Duane R. Hospenthal,[1*] Alvin L. Rogers,[1,2,3] and Everett S. Beneke[1,2]

Department of Botany and Plant Pathology, College of Human Medicine,[1] Department of Microbiology and Public Health,[2] and the Medical Technology Program[3] Michigan State University, East Lansing, Michigan 48824

* corresponding author

ABSTRACT

The effect produced by antibody specific to Candida albicans when attached to liposomes containing amphotericin B was studied in vivo. Liposomal amphotericin B bearing specific immunoglobulin (LAMB-Ab) was compared with the unencapsulated drug (fAMB) and other liposomal amphotericin B formulations in the short-term survival (21 day) of mice with disseminated candidiasis. Both the treatment and prophylaxis of the murine model of

candidiasis were explored in these trials. LAMB-Ab increased survival rates in the model more than other liposomal preparations containing amphotericin B. Liposomal amphotericin B compounds as a group prolonged survival over fAMB. Liposomal preparations used for comparison included liposomes with attached nonspecific antibody (LAMB-Ab-), liposomes without antibody (LAMB), and liposomes with unattached specific antibody (LAMB+).

INTRODUCTION

Amphotericin B (AMB) is the drug of choice for most disseminated mycoses including candidiasis, the most common mycosis affecting humans (1,2). The acute and chronic toxic effects of this antifungal agent have limited its therapeutic use clinically. Recently, encapsulation of AMB in liposomes has been shown to greatly reduce the toxicity of this drug. Studies done in vitro and in vivo with both murine and human subjects have shown that encapsulation enhances the therapeutic effect of AMB owing to a decrease in toxicity (7,9,10,12). This reduction of toxic effect allows larger dosages of AMB to be used safely. Most of the increase in therapeutic effect is thought to be the result of this decrease in toxicity.

Targeting of specific drugs and other macromolecules to antigenic sites by using antibody-directed liposomes

has been reported previously (4,5,8). Recently, researchers in our laboratory developed a procedure to produce such liposomes that contain AMB and which bear anti-candidal antibody on their surfaces. This liposome preparation was shown *in vitro* to reduce the toxicity while retaining the potency of the antifungal compound (6). Testing to see whether the attachment of specific antibody enhances the beneficial effect of liposomal AMB *in vivo* is the objective of the present investigation.

MATERIALS AND METHODS

Animals and materials. White Swiss mice weighing 18 to 22 g were purchased from Charles River Laboratories, Portage, Mich. Dimyristoyl phosphatidylcholine (DMPC) and dimyristoyl phosphatidylglycerol (DMPG) were purchased from Sigma Chemical Co., St. Louis, Mo. AMB without emulsifier used in liposome preparations was supplied by Squibb Pharmaceutical, New Brunswick, N.J. Emulsified AMB (fAMB), in a formulation similar to that used clinically, was obtained from Sigma. Antibody was obtained from rabbit antisera to *Candida albicans* (Difco Laboratories, Detroit, Mich.).

Yeast cells. *C. albicans* AK785, a clinical isolate obtained from M. Kennedy, The Upjohn Co., Kalamazoo, Mich., was maintained at the Michigan State University Medical Mycology Laboratory. This yeast was used in the

production of nonspecific modified antisera and in the murine model of candidiasis. Cells were recovered from a frozen sample and incubated overnight (37 C) on slants of Sabouraud glucose agar. An inoculum from these slants was then placed in tryptic soy broth containing 4% glucose and incubated at 37 C on a rotary shaker for 14 h to develop the stationary phase. Cells were recovered by centrifugation (2,000 X g, for 10 min) and washed in phosphate-buffered saline three times.

Liposome preparation. The liposomes were produced by reverse-phase evaporation as previously described (6) with a 7:3 molar ratio of the two phospholipids DMPC and DMPG. Liposomal preparations were quantitated with respect to their content of AMB by spectroscopy at 405 nm before being diluted for injection. Four formulations of liposomal AMB were produced for comparison. The first two preparations, which both bore externalized antibody, were produced with antibody which was first modified and then inserted into the surface of preformed liposomes. Modification of antibody by covalent binding of palmitic acid residues to the immunoglobulin and the insertion were performed as previously described (6). The first preparation externalized antibody specific to C. albicans (LAMB-Ab), and the second bore nonspecific antibody (LAMB-Ab^-). Nonspecific antibody used in these liposome preparations was produced though adsorption of previously modified

antisera with C. albicans AK785. This was performed by reacting the modified antiserum product used in producing LAMB-Ab with 5 X 10^6 CFU of C. albicans for 30 min at 25 C and then removing the yeast cells with the bound specific antibody by centrifugation at 2000 X g for 10 min. The procedure was repeated twice, and the supernatant product was then shown to be without anticandidal activity by agglutination testing. The adsorbed antiserum was quantitated for protein content by using the Folin phenol method (11) and used at the same concentration as the anticandidal antisera in LAMB-Ab production. From this analysis, the amount of C. albicans-specific antibody contained in the antisera was calculated to be 10%. Liposomal AMB (LAMB) without any further alteration was the third type of drug encapsulating liposome produced. The fourth liposomal preparation was produced by adding modified anticandidal antisera to LAMB batches in an amount equal to that recovered in LAMB-Ab preparations. This preparation was called LAMB+ to indicate that it contained AMB liposomes with unattached, specific antibody. All liposome preparations which included antibody (LAMB-AB, LAMB-Ab^-, and LAMB+) contained an approximate protein-to-AMB ratio of 1:2 (wt/wt). The liposome formulations, as well as the emulsified drug (fAMB), were diluted with phosphate-buffered saline to concentrations used in

therapy and stored at 4 C. For this study, the liposome preparations were all stored for less than 3 days between production and use.

Therapy and prophylaxis of candidiasis. Disseminated infection in mice was produced by interperitoneal injection of 7 X 10^7 CFU of C. albicans in 0.5 ml of phosphate-buffered saline. In this model, C. albicans spreads to the kidneys and other organs via a hematogenous route by 24 (13) and, at the dosage used, produces death in some mice after 2 days.

Survival of mice in groups of 10 to 12 was compared over a 21 day period. Therapy consisted of single-dose injections of the treatment compounds in quantities determined by the AMB content. Mice were injected via the caudal vein with the AMB preparations 1 day after infection in the treatment groups and 1 day before infection in prophylactic groups. Both liposomal products and the free drug (fAMB) were incubated for 20 min at 37 C before the injections to further reduce the toxicity of the liposomal compounds (12). Dosages at and below 0.8 mg of AMB per kg of mouse body weight were included. A single intravenous injection of 0.8 mg of AMB per kg has been reported to be the maximum tolerated dose of free drug in this size of mouse (10).

Toxicity of liposomes bearing antibody. Groups of 8 to 10 mice were injected intravenously with the two liposomal compounds bearing antibody on their surfaces (LAMB-Ab and LAMB-Ab^-). The groups were observed over a 10-week period for obvious toxicity caused by these liposomes given in single dosages of 0.6, 0.8, and 2.0 mg of AMB per kg.

Statistical analysis. A generalized Wilcoxon test (3) was used to compare differences in the survival distributions recorded in these studies.

RESULTS

Therapy of murine candidiasis. Results of initial studies comparing liposomes bearing specific antibody (LAMB-Ab), liposomes without the antibody (LAMB), and the free drug (fAMB) are shown in Table 1. Treatment and prophylactic dosages of 0.8 mg of AMB per kg improved the 21 day survival of mice in these groups as compared with untreated controls ($P < 0.001$). The prophylactic dose of 0.4 mg of AMB per kg provided no significant increase in survival in any of the three preparations over the control.

Liposomes bearing nonspecific antibody (LAMB-Ab^-) and liposomes with unattached specific antibody (LAMB+) were included in subsequent studies comparing dosages of 0.6 mg of AMB per kg. Survival curves for mice in the liposome

groups without bound specific antibody (LAMB, LAMB+, and LAMB-Ab$^-$) and those treated with fAMB showed no statistical difference in survival compared with untreated controls (Fig. 1). In the treatment groups only LAMB-Ab produced an improved survival ($P < 0.05$) over the control.

Prophylactic experiments at a dosage of 0.6 mg of AMB per kg also produced results which separated LAMB-Ab from the other test compounds (Fig. 2). At this dose, LAMB-Ab significantly improved the survival of mice as compared with the control group ($P < 0.001$). LAMB also produced an increase in survival ($P < 0.003$), whereas the free drug was virtually ineffective at this dose. The other antibody-containing liposomes, LAMB+ and LAMB-Ab$^-$, also increased survival ($P < 0.03$ and $P<0.06$, respectively) over the untreated group. The LAMB-Ab prophylaxis group presented an improved survival rate over both LAMB+ and LAMB-Ab$^-$ ($P < 0.03$).

Toxicity of liposomes bearing antibody. Intravenous administration of liposomal AMB with antibody bound to its surface (LAMB-Ab and LAMB-Ab$^-$) at doses up to 2.0 mg of AMB per kg showed no noticeable toxic effect on mice over 70 days.

DISCUSSION

The attachment of anticandidal antibody to the surfaces of liposomes containing AMB (LAMB-Ab) was observed

to enhance the therapeutic effect of the drug in these experiments. This compound was tested at dosages less than optimum for treatment of the murine candidiasis model. We wanted to compare this liposomal compound with the free drug (fAMB), which has been shown to exhibit toxic effects at larger doses (10). In initial studies, the survival of mice was increased by LAMB-Ab over both the liposomal drug (LAMB) and fAMB in all but the prophylaxis study at 0.8 mg of AMB per kg. In our present candidiasis model, both LAMB and LAMB-Ab therapies yielded 100% survival over 3 weeks in the former study. These results led to the selection of a dosage of 0.6 mg of AMB per kg to compare the therapeutic effect of liposomes bearing nonspecific antibody (LAMB-Ab^-) or unbound antibody (LAMB+) with fAMB, LAMB, and especially LAMB-Ab. These studies show that both the specificity of the antibody to C. albicans and the binding of this antibody to the liposomes was responsible for the increase in short-term survival provided by LAMB-Ab over LAMB.

No in vivo toxicity was noted at the dosages used in this study over a 70 day period. Previously we showed that even very large concentrations of LAMB-Ab are nontoxic in vitro (6). We expect that this decrease in toxicity will be reflected in vivo at therapeutic concentrations.

Liposomal encapsulation of AMB has been shown to increase the therapeutic effect of this drug chiefly as a result of the reduction of toxicity, which allows the use of larger single dosages (9). Our preparation of LAMB-Ab, with its specific antibody, appears to produce this reduction in toxicity and also show an increase in therapeutic effect over other liposomal AMB compounds. Disseminated organisms susceptible to AMB are known to spread via hematogenous pathways in their course of infection. In the circulatory system, access to the antigenic sites on these organism is available to intravenously injected antibody-bearing preparations. Antibody-directed liposomes may provide a method of reaching the target organisms with reduced toxicity and increased specificity, thus improving the efficacy of the drug. Direction of the AMB in this less toxic form may provide a more effective treatment of the majority of disseminated fungal infections which affect humans.

ACKNOWLEDGEMENTS

This project was sponsored in part by funding from the Michigan State University All-University Research Initiation Grant program and the College of Natural Science through their Public Health Service Biomedical Research Support Grant for 1987 to 1988 from the National Institutes of Health.

LITERATURE CITED

1. Edwards, J. E., Jr., R. I. Lehrer, E. R. Stiehm, T. J. Fischer, and L. S. Young. 1978. Severe candidal infections: clinical perspective, immune defense mechanisms, and current concepts of therapy. Ann. Intern. Med. 89: 91-106.

2. Feld, R., G. P. Bodey, V. Rodriguez, and M. Luna. 1974. Cause of death in patients with malignant lymphoma. Am. J. Med. Sci. 268: 97-106.

3. Gehan, E. A. 1965. A generalized Wilcoxon test for comparing arbitrarily single-censored samples. Biometrika 52: 203-217.

4. Gregoriadis, G., J. Senior, B. Wolff, and C. Kirby. 1985. Targeting of liposomes to accessible cells in vivo. Ann. N.Y. Acad. Sci. 446: 319-340.

5. Heath, T. D., J. A. Montgomery, J. R. Piper, and D. Papahadjopoulos. 1983. Antibody-targeted liposomes: Increase in specific toxicity of methotrexate-gamma-aspartate. Proc. Natl. Acad. Sci. USA 80: 1377-1381.

6. Hospenthal, D. R., A. L. Rogers, and G. L. Mills. 1988. Development of amphotericin B liposomes bearing antibody specific to Candida albicans. Mycopathologia 101: 37-45.

7. Juliano, R. L., G. Lopez-Berestein, R. Hopfer, R. Mehta, K.Mehta, and K. Mills. 1985. Selective toxicity and enhanced therapeutic index of liposomal polyene antibiotics in systemic fungal infections. Ann. N.Y. Acad. Sci. 446: 390-402

8. Leserman, L. D., P. Machy, C. Devaux, and J. Barbet. 1983. Antibody-bearing liposomes: Targeting in vivo. Biol. Cell 47: 111-116.

9. Lopez-Berestein, G., and R. L. Juliano. 1987. Application of liposomes to the delivery of antifungal agents, p.253-276. In M. J. Ostro (ed.), Liposomes, from biophysics to therapeutics. Marcel Dekker, Inc., New York.

10. Lopez-Berestein, G., R. Mehta, R. L. Hopfer, K. Mills, L. Kasi, K. Mehta, V. Fainstein, M. Luna, E. M. Hersh, and R. Juliano. 1983. Treatment and prophylaxis of disseminated infection due to Candida albicans in mice with liposome-encapsulated amphotericin B. J. Infect. Dis. 147: 939-945.

11. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265-275.

12. Szoka, F. C., D. Milholland, and M. Barza. 1987. Effect of lipid composition and liposome size on toxicity and in vitro fungicidal activity of liposome-intercalated amphotericin B. Antimicrob. Agents Chemother. 31: 421-429.

13. Young, G. 1958. The process of invasion and the persistence of Candida albicans injected intraperitoneally into mice. J. Infect. Dis. 102: 114-120.

TABLE 1. Mice surviving systemic candidiasis after treatment or prophylaxis with AMB preparations

Prepn	No. of mice surviving to 21 days/total no. (%) after:		
	Treatment dose (0.8 mg of AMB/kg)[a]	Prophylactic dose[b]	
		0.4 mg of AMB/kg	0.8 mg of AMB/kg
fAMB	58.3% (7/12)	8.3% (1/12)	66.7% (8/12)
LAMB	50.0% (5/10)	8.3% (1/12)	100.0% (12/12)
LAMB-Ab	90.9% (10/11)	16.7% (2/12)	100.0% (12/12)
Control[c]	25.0% (4/12)	8.3% (1/12)	25.0% (4/12)

[a] Treated 1 day after infection with C. albicans. The treatment was given in a single dosage based on the AMB content.

[b] Prophylactic dose given 1 day prior to infection with C. albicans.

[c] No treatment.

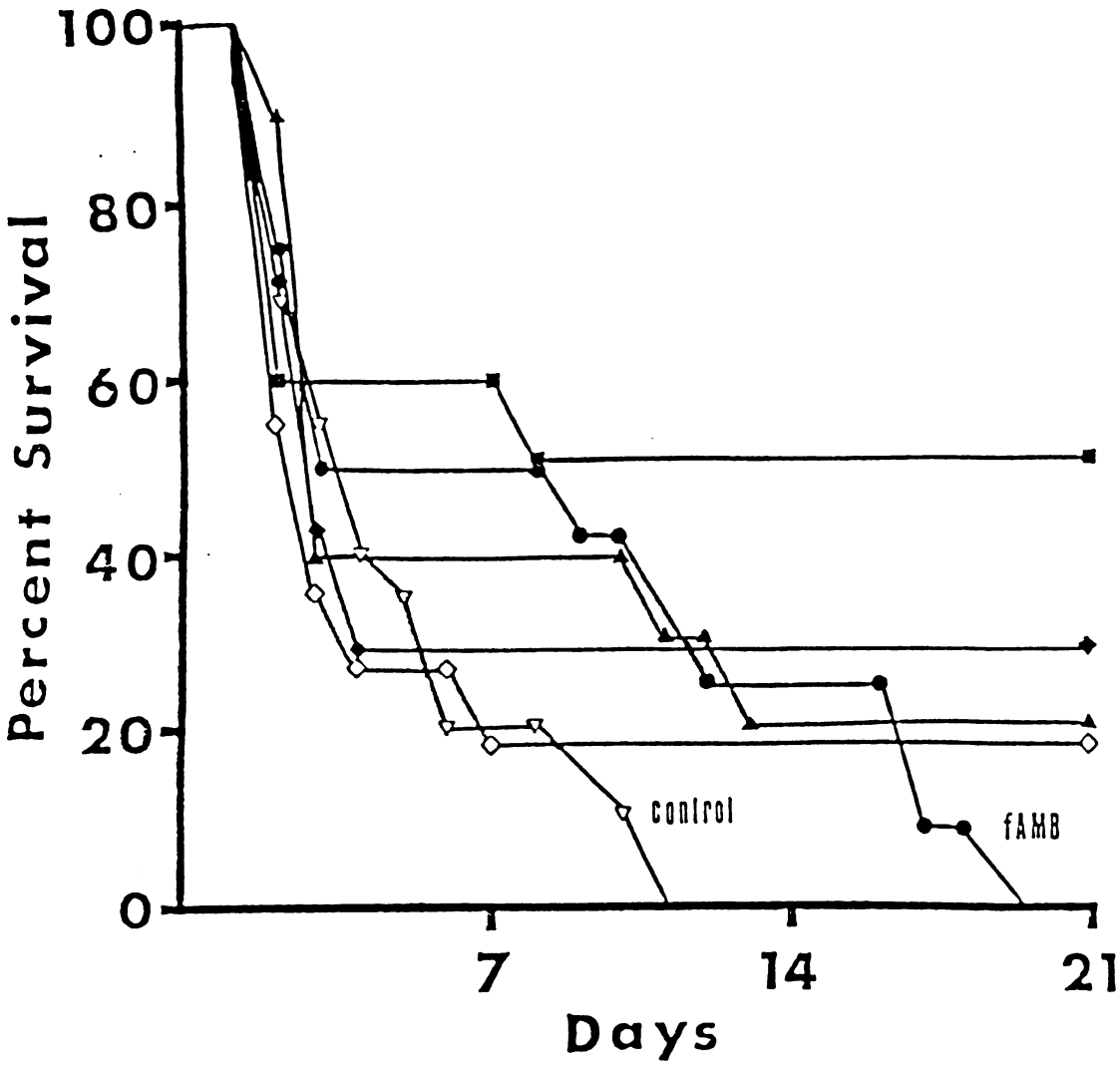


FIG. 1. Effect of liposome-encapsulated AMB bearing antibody specific to C. albicans on survival of mice infected with C. albicans. One day after this infection, the animals were treated with 0.6 mg of AMB per kg. Symbols: ■, LAMB-Ab; ▲, LAMB; ◆, LAMB-Ab⁻; ◇, LAMB+; ●, fAMB; ▽, control (not treated).

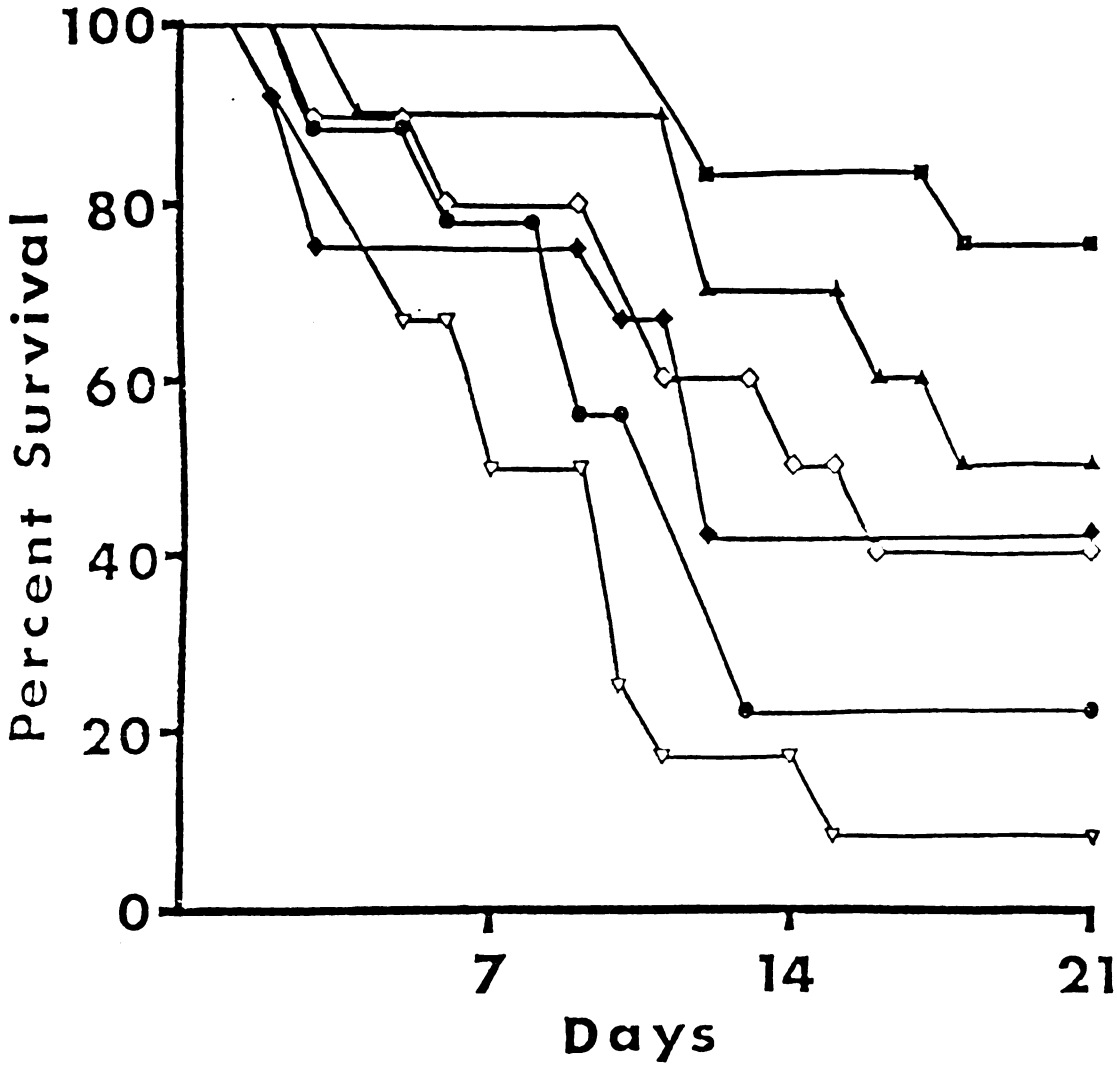


FIG. 2. Effect of liposome-encapsulated AMB bearing antibody specific to C. albicans on the survival of mice infected with C. albicans. One day prior to infection, each animal was given a prophylactic dosage of 0.6 mg of AMB per kg. Symbols: ■, LAMB-Ab; ▲, LAMB; ◆, LAMB-Ab⁻; ◇, LAMB+; ●, fAMB; ▽, control (not treated).

Treatment of a Murine Model of Systemic Candidiasis with Liposomal Amphotericin B Bearing Antibody to *Candida albicans*

D. Hospenthal, K. Gretzinger, and A. Rogers

Submitted to
Journal of Medical Microbiology
January 21, 1989

Treatment of a Murine Model of Systemic Candidiasis with Liposomal Amphotericin B Bearing Antibody to Candida albicans

D. HOSPENTHAL,[1] K. GRETZINGER,[2] and A. ROGERS[1,3]

Department of Botany and Plant Pathology, [1]College of Human Medicine, [2]College of Osteopathic Medicine, [3]Department of Microbiology and Public Health, and Medical Technology Program, Michigan State University, East Lansing, Michigan, U.S.A. 48824

Summary. Survival of mice infected with an intravenous injection of Candida albicans was observed in a short-term (21 day) survival study. Concentration of C. albicans in the kidneys, liver, and spleen was quantitated at various points in the infection model. Treatment of the murine model with the commercial formulation of amphotericin B (fAMB), liposomal amphotericin B (LAMB), and liposomal amphotericin B bearing external antibody specific for C. albicans (LAMB-Ab) was explored by comparing the effect of

these compounds on mouse survival. In single intravenous treatment dosages of 0.6 mg amphotericin B/kg mouse, the liposomal forms of the drug (LAMB and LAMB-Ab) enhanced the survival of mice as compared to mice treated with the unencapsulated antifungal compound, fAMB ($P < 0.03$ and $P < 0.001$, respectively). LAMB-Ab, at this dosage, produced an increase in the survival ($P < 0.007$) of mice over that produced by LAMB. LAMB-Ab treatment caused a greater than 3-fold increase over fAMB. In comparison to LAMB, the percentage of LAMB-Ab treated mice which survived for 21 days was almost double that of the LAMB mice. The increase in survival afforded by this treatment did not however lead to the eradication of C. albicans in all mice which survived to the end of the experiment.

Introduction

Opportunistic infections caused by Candida albicans can be severe, sometimes fatal complications in patients with hematologic malignancies and other immunocompromising conditions (Bodey, 1984). To treat these infections, the antifungal compound amphotericin B (AMB) is administered intravenously in its emulsified commercial form, Fungizone. Clinical use of AMB is limited by its nephrotoxicity, which affects most patients treated systemically with the drug and its other various toxic

effects (Graybill and Craven, 1983). Though this antifungal agent produces many undesirable effects, AMB is currently the drug of choice for most life-threatening disseminated mycoses including candidiasis. Lack of any current antifungal drugs to effectively replace AMB has led to recent research aimed at exploring alternative formulations in which to deliver AMB in a less toxic, yet equally active form. Attempts to produce derivatives of AMB (Bannatyne and Cheung, 1977) or to discover less toxic carriers of the compound (Kirsh et al., 1988; Lopez-Berestein et al., 1983) have been the objective of most of this research. The encapsulation of AMB in liposomes (LAMB) has been shown to produce the desired effects on the antifungal, reducing toxicity with no decrease in activity. Tests of LAMB <u>in vitro,</u> and <u>in vivo</u> in mice have yielded data which suggest that this encapsulation increases the efficacy of AMB by allowing larger doses to be used safely (Juliano et al., 1985). Initial clinical trials by Lopez-Berestein et al. (1987; 1985) have produced encouraging results in hematologic malignancy patients, most of which had shown no previous response to the unencapsulated drug (fAMB).

Liposomes targeted by antibody incorporated into their surfaces have been shown to aid in the specific targeting of compounds to antigen-specific sites (Heath et al., 1983; Leserman et al., 1983). Recently, liposomes

containing AMB and bearing C. albicans antibody have been produced in our laboratory (Hospenthal et al., 1988). These liposomes, designated LAMB-Ab, have been shown in vitro to be as effective against C. albicans, but less toxic to human cells than fAMB. In initial murine studies using an intraperitoneal candidiasis model (Hospenthal et al., 1989), increased survival rates produced by LAMB-Ab were shown to be caused by the attachment of specific antibody to the surface of LAMB. This effect was not produced by the attachment of nonspecific antibody to LAMB, or by the inclusion of unattached specific antibody in the LAMB treatments. The current investigations were performed to establish a new intravenous model of candidiasis for this and future studies, and to conduct further preliminary in vivo tests of LAMB-Ab.

Material and methods

Animals and material

White Swiss mice, each weighing 18 to 22 grams, were purchased from Charles River Laboratories, Portage, Michigan. Phospholipids used in the preparation of liposomes, dimyristoyl phosphatidylcholine (DMPC) and dimyristoyl phosphatidylglycerol (DMPG), were purchased from Sigma Chemical Co., St. Louis, Missouri.

Amphotericin B, both in the commercial preparation, Fungizone (fAMB), and in the nonemulsified form (AMB) used in liposomal formulations, was supplied by Squibb Pharmaceutical, New Brunswick, New Jersey. The antibody preparation used was produced from rabbit antisera to Candida albicans (Difco Laboratories, Detroit, Michigan).

Yeast cells and infection

C. albicans AK785, a clinical isolate, was maintained on frozen slants of Sabouraud glucose agar. Cells recovered from these slants were incubated overnight at 37 C on fresh Sabouraud glucose agar slants. Inoculum from these slants was then transferred to trypic soy broth containing 4% glucose and incubated (37 C) on a rotary shaker for 14 h to produce the stationary phase. Yeast cells were recovered by centrifugation (2000 x g, 10 min) and washed three times in phosphate-buffered saline prior to injection. Systemic candidiasis was produced by the injection of 3×10^5 cfu of the yeast into a lateral caudal vein of each mouse in a single bolus contained in 0.2 ml of phosphate-buffered saline.

Yeast viability

Viability counts of C. albicans in the kidneys, liver, and spleen of individual mice were performed by dilution plating of these organs following homogenization. Mice

included in these counts were either sacrificed by cervical dislocation or had died of the candidal infection. The liver, spleen, and kidneys (each pair prepared as one unit) were removed aseptically and homogenized in sterile distilled water. The homogenates were serially diluted in four, ten-fold dilutions using sterile distilled water. Spleen dilutions were 1:5 to 1:5000, liver 1:20 to 1:20,000, and kidneys 1:100 to 100,000. Dilutions were then plated on Mycosel agar (BBL Microbiology Systems, Cockeysville, Maryland), incubated at 37 C for 48 h, and then the colonies counted. Organ concentrations of C. albicans were recorded in colony forming units (cfu). Only plates which grew between 10 and 500 colonies were used in the calculating of cfu. Using this criteria, yeast concentrations less then 50 cfu ($\log_{10}$ = 1.7) in the spleen, 200 cfu ($\log_{10}$ = 2.3) in the liver, 1000 cfu ($\log_{10}$ = 3.0) in the kidneys were not detected or included in this sudy.

Liposome preparation

Liposomes were produced with a 7:3 molar ratio of DMPC and DMPG by a reverse-phase evaporation procedure previously described (Hospenthal et al., 1988). Quantitation of liposomes was by AMB content, determined by spectroscopy at 405 nm. Liposomes bearing antibody, LAMB-Ab, were produced with rabbit antisera which was

modified by the covalent attachment of palmitic acid residues. This antisera, containing modified antibody, was present in LAMB-Ab in a concentration of 1:2 (w/w) protein to AMB. Previous studies revealed that the C. albicans-specific immunoglobulin content in the modified antisera to be approximately 10% (Hospenthal et al., 1989). Free drug and liposomal preparations (LAMB and LAMB-Ab) were diluted to injection concentrations in phosphate-buffered saline and stored refrigerated (4 C) until the day of treatment. No liposomal preparations were stored for more then one day in this study.

Therapy

Groups of 8 to 10 mice were treated with a single injection two days after the initiation of the infection with C. albicans and observed 21 days for survival. Liposome and free drug preparations were incubated 20 min at 37 C prior to injection into the caudal veins of the mice to further reduce the toxicity of the liposomal compounds (Szoka et al., 1987). Treatment dosages of 0.6 and 1.2 mg AMB/kg mouse of fAMB, LAMB, and LAMB-Ab were compared. These dosages were selected due to the fact that the maximum tolerated dose of fAMB has been reported by Lopez-Berestein et al. (1983) to be 0.8 mg AMB/kg in the mouse size used in this study.

Statistical analysis

The 21 day survival patterns of mice treated with the AMB preparations and untreated controls were compared at each dose employing a generalized Wilcoxon test (Gehan, 1965).

Results

Canidiasis Model

The amount of viable C. albicans in the kidneys, liver, and spleen of untreated mice was examined each day through the first 7 days of the study. Figure 1 shows the results of viability counts in the kidneys and livers of these mice. Spleen homogenates of all mice in the study produced less than seven colonies (usually zero) upon plating of the smallest dilution (1:5) and thus were not included in this figure. Mice which died on days 6 and 7 of the infection had C. albicans concentrations similar to the mice sacrificed on these days, approximately 10^4 cfu in the liver and 10^6 cfu in the kidneys per mouse. The size and gross morphology of kidneys at similar days of the infection (in both matched pairs and between mice) were quite varied, even among the treated mice. Individual kidneys were atrophied, enlarged with or without purulent discharge, nodular with granulomatous

material, or normal appearing. Liver and spleen gross morphology showed much more consistency in this study.

Therapy of candidiasis

The combined 21 day survival pattern of two studies of mice treated with 0.6 mg AMB/kg is shown in Figure 2. All amphotericin B formulations improved the survival of mice over the control at this dose ($P < 0.001$). LAMB-Ab and LAMB increased the survival over the free drug ($P < 0.001$ and $P < 0.03$, respectively). Survival distribution between the LAMB and the LAMB-Ab groups of mice was also significant ($P < 0.007$). Additional mice, which were infected and treated parallel to this study, were sacrificed at days 7 and 14 of the infection. At day 7, all three groups of treated mice (fAMB, LAMB, and LAMB-Ab) produced no recoverable cfu from their spleens or livers, and all had similar recovery values of 10^4 cfu in their kidneys. Day 7 colony counts in the untreated mice are incorporated into the data presented in Figure 1. Fourteen days after infection, LAMB treated mice had 10^7 cfu in their kidneys and 10^5 cfu in their livers. At this time, mice treated with LAMB-Ab had recoveries of 10^7 cfu and 10^4 cfu in their kidneys and livers, respectively. Due to the survival rates of the untreated and fAMB groups (Figure 2), mice from these groups did not survive to be sampled at day 14.

Surviving mice from one 0.6 mg AMB/kg study were examined at day 42 for residual candidal infection. Of this group, which consisted of 1 fAMB, 2 LAMB, and 5 LAMB-Ab mice, all the organs were negative for growth except for two of the LAMB-Ab mice. These two mice had livers which grew 10^3 cfu and kidneys which grew 10^6 cfu.

Experimental therapy studies at the dose of 1.2 mg AMB/kg proved, as previously seen in our peritoneal murine candidiasis model (Hospenthal et al., 1989), to show little separation of the two liposomal compounds. The 21 day survival of mice at this dose was 64.7% for LAMB-Ab and 61.1% for LAMB. The survival of the LAMB-Ab and LAMB groups did prove to better than the fAMB groups ($P < 0.02$ and $P < 0.03$, respectively).

Discussion

Liposomal amphotericin B which bears anticandidal antibody, LAMB-Ab, was shown to produce increased survival in mice infected with Candida albicans at a dose (0.6 mg AMB/kg) which is below the maximum tolerated dose of Fungizone in these mice. Our formulation of LAMB at this dose also produces an increase in survival over the free drug (fAMB), though much less than that of the LAMB-Ab treatment. Murine survival studies of these liposomal amphotericin B compounds at a dosage of 1.2 mg AMB/kg

also show these compounds to be more effective than fAMB. LAMB at this dose increases the survival rate of mice to that of LAMB-Ab. LAMB-Ab at this dose shows no change in survival rate over that which it produces at 0.6 mg AMB/kg. The authors feel this may be due to problems associated with the 90% nonspecific protein content of the antisera which is used in the preparation of LAMB-Ab. Future studies employing a monoclonal anticandidal antibody will be neccessary to explore this detail.

The organ homogenization study of this model of systemic candidiasis shows that at the time when untreated mice begin to die from this infection, six days after infection, they normally have 10^3 cfu in their livers and 10^6 cfu in their kidneys. The normal survival of these mice is 6 to 17 days post infection. Mice sacrificed in the 0.6 mg AMB/kg LAMB and LAMB-Ab groups at 14 days produced cfu counts which averaged 10 times greater than those produced by the untreated control. This may indicate that mice surviving longer, may be more resistant to the infection, and yet may harbor large numbers of the yeast.

The encapsulation of amphotericin B in liposomes has been proven to reduce the toxic effect of this antifungal in numerous studies (Juliano et al., 1987; Mehta et al., 1984; Shirkhoda et al., 1986; Szoka et al., 1987). In these studies, this reduction in toxic effect has allowed

increasingly larger doses of AMB to be employed, and thus has improved the clinical effectiveness of this drug. In our studies, we have shown that our formulation, LAMB-Ab, has increased the survival of mice with disseminated candidiasis as compared to fAMB when administered in single, low dosages. In previous studies we have shown that this compound is less toxic than fAMB when administered to noninfected mice or incubated with human red blood cells. Dissemination of fungal cells which are susceptible to AMB is thought to be largely via the circulatory system. If our compound, or other preparations, can produce antibody-antigen targeting of drugs to infectious agents, then perhaps lower dosages of drugs with toxic side effects can be employed successfully in the treatment of these diseases. The combination of this targeting and the reduction in toxicity that liposomes provide could improve the efficacy of many toxic compounds, including that of amphotericin B in the treatment of life-threatening mycoses.

Acknowledgement

This research was supported in part by a grant from Michigan Health Care Education and Research Foundation, Inc. (grant number: 036-SAP/88-05) under its Student Awards Program.

References

Bannatyne R M, Cheung R 1977 Susceptibility of Candida albicans to amphotericin B and amphotericin B methyl ester. Antimicrobial Agents and Chemotherapy **12**:449-450.

Bodey G P 1984 Candidiasis in cancer patients. American Journal of Medicine **77**:13-19.

Gehan E A 1965 A generalized Wilcoxon test for comparing arbitrarily single-censored samples. Biometrika **52**:203-217.

Graybill J R, Craven P C 1983 Antifungal agents used in systemic mycoses: activity and therapeutic use. Drugs **25**:41-62.

Heath T D, Montgomery J A, Piper J R, Papahadjopoulos D 1983 Antibody-targeted liposomes: increase in specific toxicity of methotrexate-gamma-aspartate. Proceedings of the National Academy of Sciences USA **80**:1377-1381.

Hospenthal D R, Rogers A L, Beneke E S 1989 Effect of attachment of anticandidal antibody to the surfaces of liposomes encapsulating amphotericin B in the treatment of murine candidiasis. Antimicrobial Agents and Chemotherapy **33**:16-18.

Hospenthal D R, Rogers A L, Mills G L 1988 Development of amphotericin B liposomes bearing antibody specific to Candida albicans. Mycopathologia **101**:37-45.

Juliano R L, Grant C W M, Barber K R, Kalp M A 1987 Mechanism of the selective toxicity of amphotericin B incorporated into liposomes. Molecular Pharmacology 31:1-11.

Juliano R L, Lopez-Berestein G, Hopfer R, Mehta R, Mehta K, Mills K 1985 Selective toxicity and enhanced therapeutic index of liposomal polyene antibiotics in systemic fungal infections. Annals of the New York Academy of Sciences **446**:390-402

Kirsh R, Goldstein R, Tarloff J, Parris D, Hook J, Hanna N, Bugelski P, Poste G 1988 An emulsion of amphotericin B improves the therapeutic index when treating sysytemic murine candidiasis. Journal of Infectious Diseases **158**:1065-1070.

Leserman L D, Machy P, Devaux C, Barbet J 1983 Antibody-bearing liposomes: targeting in vivo. Biology of the Cell **47**:111-116.

Lopez-Berestein G, Bodey G P, Frankel L S, Mehta K 1987 Treatment of hepatosplenic candidiasis with liposomal amphotericin B. Journal of Clinical Oncology **5**:310-317.

Lopez-Berestein G, Fainstein V, Hopfer R, Mehta K, Sullivan M P, Keating M, Rosenblum M G, Mehta R, Luna M, Hersh E M, Reuben J, Juliano R L, Bodey G P 1985 Liposomal amphotericin B for the treatment of systemic fungal infections in patients with cancer: a preliminary study. Journal of Infectious Diseases **151**:704-710.

Lopez-Berestein G, Mehta R, Hopfer R L, Mills K, Kasi L, Mehta K, Fainstein V, Luna M, Hersh E M, Juliano R 1983 Treatment and prophylaxis of disseminated infection due to Candida albicans in mice with liposome-encapsulated amphotericin B. Journal of Infectious Diseases **147**:939-945.

Mehta R, Lopez-Berestein G, Hopfer R, Mills K, Juliano R L 1984 Liposomal amphotericin B is toxic to fungal cells but not to mammalian cells. Biochimica et Biophysica Acta 770: 230-234.

Shirkhoda A, Lopez-Berestein G, Holbert J M, Luna M A 1986 Hepatosplenic fungal infections: CT and pathological evaluation after treatment with liposomal amphotericin B. Radiology **159**:349-353.

Szoka F C, Milholland D, Barza M 1987 Effect of lipid composition and liposome size on toxicity and in vitro fungicidal activity of liposome-intercalated amphotericin B. Antimicrobial Agents and Chemotherapy **31**:421-429.

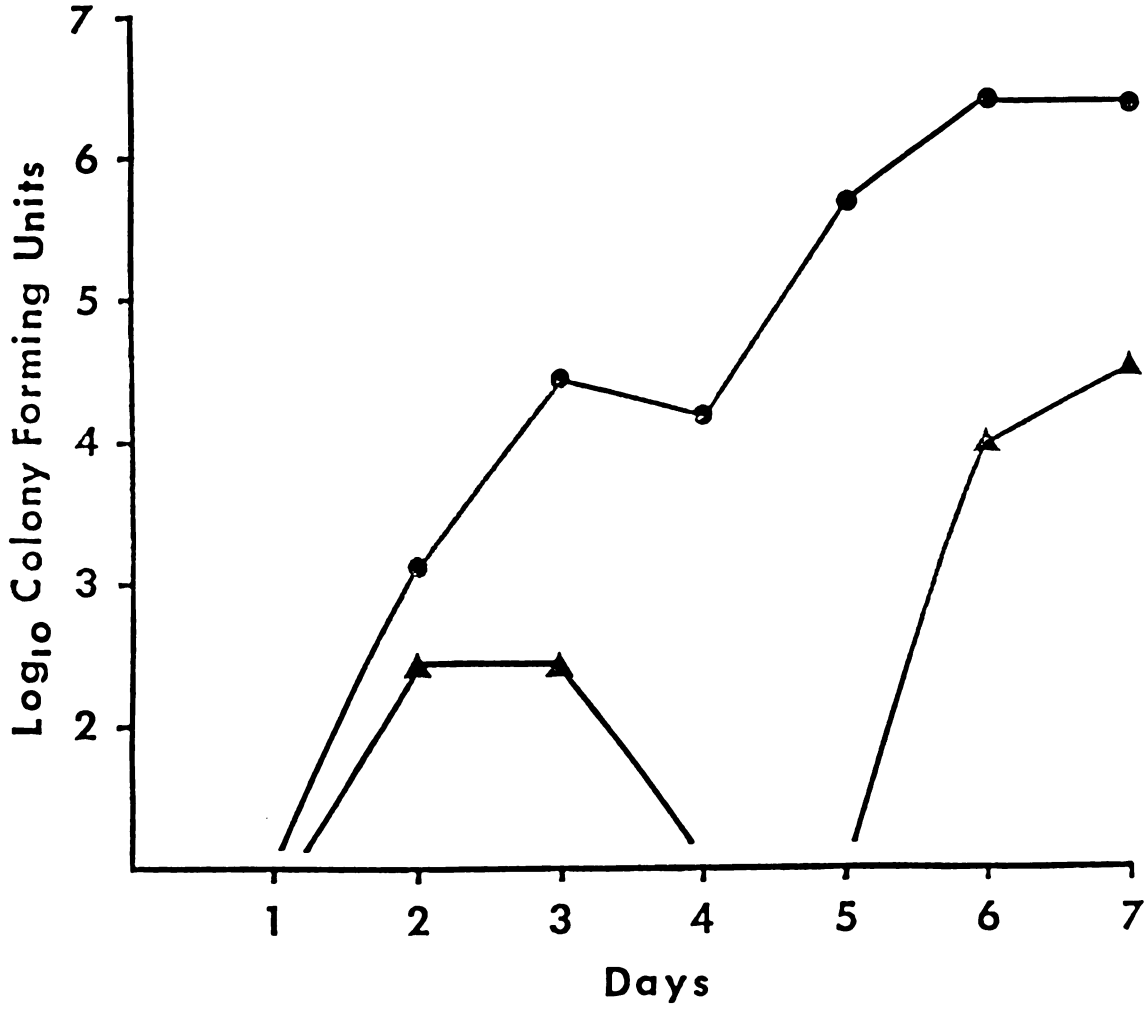


Figure 1. Candida albicans present in the kidneys (●) and livers (▲) of untreated mice sacrificed during the first 7 days of infection. Animals were infected with 3 X 10^5 cfu of the yeast. Yeast concentrations less than $\log_{10}$ cfu of 2 were below the sensitivity of the study design.

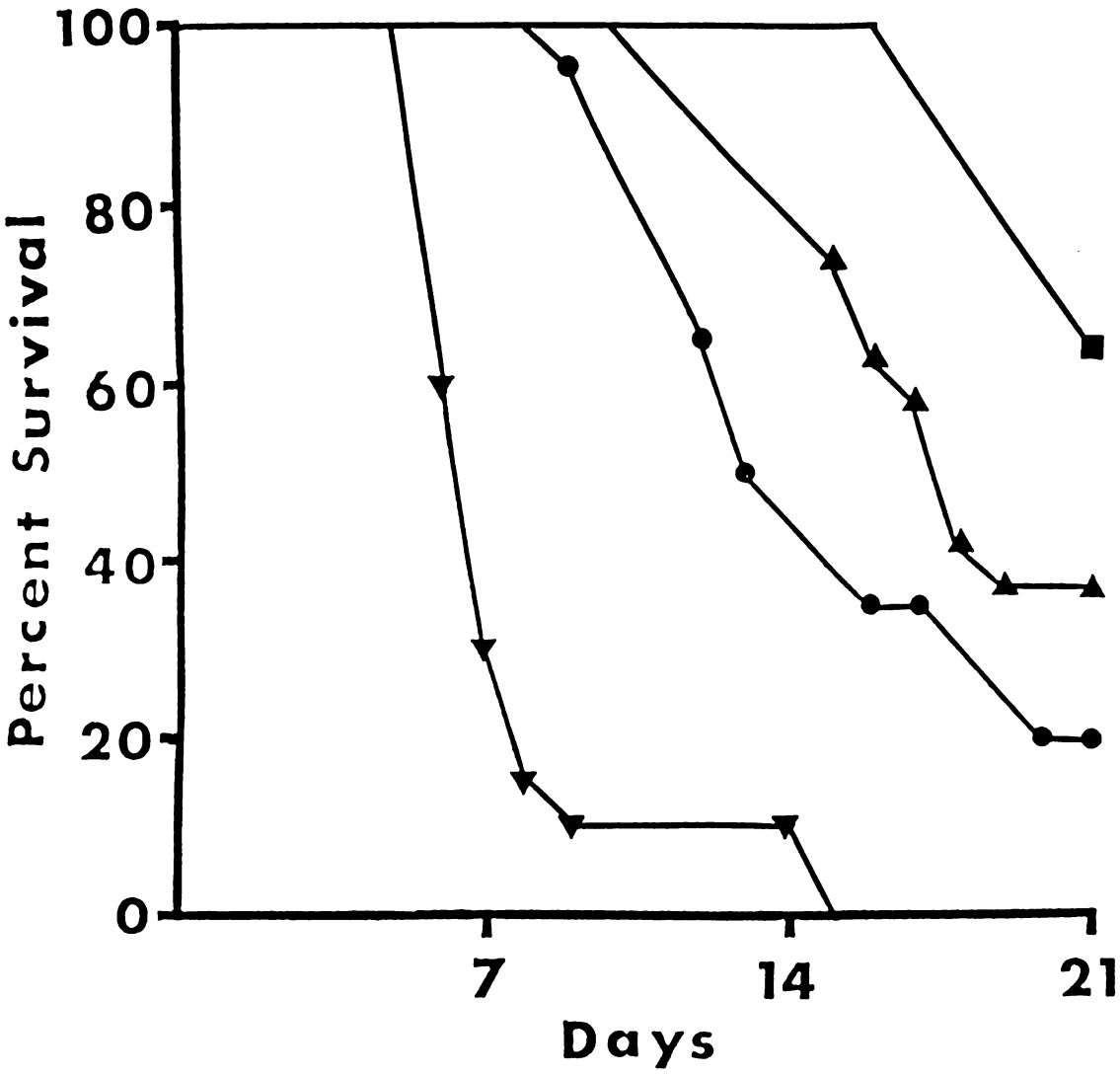


Figure 2. The effect produced by anticandidal antibody bearing liposomal AMB on the survival of mice infected with Candida albicans. Two days after initiation of the infection the animals were treated with a single dose of 0.6 mg AMB/kg as LAMB-AB (■), LAMB (▲), fAMB (●), or not treated (▼).

SUMMARY

The initial aim of this project was to determine if the toxicity of amphotericin B could be reduced and the therapeutic effect increased by the encapsulation of this polyene antifungal agent in phospholipid vesicles which bear antibody to etiologic agents of deep mycoses. The fungal agent choosen for this project was Candida albicans, the most common of the fungal pathogens which infect humans. The liposomal amphotericin B which bore anticandidal immunoglobulins was tested both in vitro, and in vivo in two murine models of candidiasis.

Formulation of the liposomal drug bearing antibodies was influenced primarily by work of Lopez-Berestein et. al. (1984; 1983a) in the encapsulation of amphotericin B within liposomes and that of Huang et. al. (1982; 1981; 1980) and Shen et. al. (1982) in the modification and incorporation of antibody onto the surfaces of liposomes produced by reverse-phase evaporation. Combining features of liposome technology presented by these groups with various original modifications, including

methodology to produce much smaller vesicles, liposomes encapsulating amphotericin B and bearing external antibody to C. albicans were produced.

In Article I, formulation and in vitro testing of this preparation, LAMB-Ab, was reported. This preparation was visualized and shown to bear external antibody by the use of transmission electron microscopy with secondary immunogold labeling. Secondary immunofluorescence was employed to visualize that the antibody which was externalized on LAMB-Ab was still active against C. albicans at the end of preparation. Toxicity and antifungal activity of LAMB-Ab in vitro was compared with the free drug (fAMB) and the liposomal drug without antibody (LAMB). No toxicity to human red blood cells was apparent at concentrations of 100 ug/ml of the two liposomal preparations whereas fAMB lysed these erythrocytes at a concentration of 12.5 ug/ml. The anticandidal effect of these three compounds was essential the same, with LAMB-Ab showing a slight advantage over LAMB and fAMB.

Testing of LAMB-Ab in an in vivo mouse model was the topic of Article II. Also examined in this article was the effect which was due to the attachment of the antibody and that due to the specificy of this immunoglobulin. In this intraperitoneal-initiated model of candidiasis LAMB-Ab proved to be more effective at lower dosages than fAMB

or LAMB in both treatment and prophylaxis of murine candidiasis. Survival rates of mice treated with liposomal amphotericin B preparations containing unattached specific antibody or with attached nonspecific antibody in this therapy model supported the conclusion that the improved survival in LAMB-Ab treated mice was due to specific, attached immunoglobulin. Toxicity of the liposomal compound was tested grossly in vivo in this study. No toxicity, in the form of visual signs or death, was seen with the injection of 2.0 mg AMB/kg LAMB-Ab in uninfected mice.

A new mouse model was examined in Article III. In this study, a intravenous-initiated murine model of candidiasis was characterized by the counting of viable C. albicans in the spleen, liver, and kidneys of the infected mice. Preliminary results in this model showed at a treatment dosage of 0.6 mg AMB/kg, LAMB-Ab was more effective than fAMB and LAMB. All of these compounds had virtually identical counts of viable yeast in their organs at similar days of the infection. Though these numbers were substantially different than the control mice, they did not reflect the difference in survival afforded by the three compounds. At the larger dose of 1.2 mg AMB/kg, LAMB-Ab was still more effective than the other two preparations, but showed no significant increase over that which it produced at 0.6 mg AMB/kg. The fact that the

antibody preparation used in formulating these liposomes contained only 10% specific C. albicans antibody, is believed to be of some importance here. Assuming that the average antisera contains about 80% albumin, this protein could be causing an deleterious effect at higher dosages of LAMB-Ab.

To improve the effect of LAMB-Ab in future projects, and better ascertain this effect, the use of monoclonal antibody would seem to be the next logical step. Through this use, with proper controls, one could more fully understand the effect produced by binding antibody to liposomal amphotericin B. Labeling of this antibody could also prove useful in following the localization of LAMB-Ab in the murine model. Ultimately, LAMB-Ab produced with polyclonal antibody (perhaps a carefully selected mixture of monoclonal antibodies) should be thoroughly examined. The goal being to produce an anticandidal formulation with a wide spectrum of action against the many strains of C. albicans.

Using the monoclonal antibody-bearing liposomal amphotericin B, the first step should be to test this compound at previously used dosages, 0.6 and 1.2 mg AMB/kg, to compare this formulation to those cited in this dissertation. Along with this, more extensive organ counts of viable yeasts are needed to further explore the effect of LAMB-Ab on the mouse model. Increasingly higher

dosages also deem exploration to establish the toxicity and efficacy of this compound and the dose at which actual cure is effected. Multiple dosing therapies also merit study. Since clinically, free amphotericin B is given in multiple dosages over an extended period of time, this should also be examined in the murine model. Extending the murine model to more closely match the clinical reality should also include studies with neutropenic mice. In the clinical setting a vast number of the life-threatening mycoses which are treated with amphotericin B are in patients with hematologic malignancies and other compromised conditions.

Another possible future application of liposomal amphotericin B bearing antibody could easily be the preparation of this compound with specific antibodies to the other mycoses treated with the antifungal drug. The possibility of LAMB-Ab containing a mixture of differing antifungal immunoglobulins could also be explored. The final and most broad possibility is the application of this technology to encapsulate any compatible drug and bear any antibody which is specific to antigen which would improve the targeting of that drug.

The production and preliminary testing of LAMB-Ab has shown promise and success in the reduction of toxicity of amphotericin B and the increase in efficacy of the

antifungal compound. Though much is left that could or should be done, the groundwork of this research has been established and the path set.

BIBLIOGRAPHY

Adriano, S. M. and J. Schwarz. 1955. Experimental moniliasis in mice. Am. J. Pathol. 31: 859-873.

Ahrens, J., J. R. Graybill, P. C. Craven and R. L. Taylor. 1984. Treatment of experimental murine candidiasis with liposome-associated amphotericin B. Sabouraudia 22: 163-166.

Alving, C. R. 1987. Liposomes as carriers for vaccines. In M. J. Ostro [ed]. Liposomes. From Biophysics to Therapeutics. Marcel Dekker,Inc., New York, pp. 195-218.

Bachhawat, B. K. and P. Dasgupta. 1986. Approach to tissue targeting of drugs and proteins using glycolipid liposomes. In K. Yagi [ed]. Medical Application of Liposomes. Japan Scientific Societies Press, Tokyo, pp. 81-90.

Bangham, A. D. 1980. Development of the liposome concept. In G. Gregoriadis and A. C. Allison [eds]. Liposomes in Biological Systems. John Wiley & Sons, New York, pp. 1-24.

Bangham, A. D., M. M. Standish and J. C. Watkins. 1965. Diffusion of univalent ions across the lamellae of swollen phospholipids. J. Mol. Biol. 13: 238-252.

Becker, J. M., S. Marcus, J. Tullock, D. Miller, E. Krainer, R. K. Kahare and F. Naider. 1988. Use of the chitin-synthesis inhibitor nikkomycin to treat disseminated candidiasis in mice. J. Infect. Dis. 157: 212-214.

Bendayan, M. 1984. Protein A-gold electron microscopic immunocytochemistry: Methods, applications, and limitations. J. Electron Microscopy Technique 1: 243-270.

Bannatyne, R. M. and R. Cheung. 1977. Susceptibility of Candida albicans to amphotericin B and amphotericin B methyl ester. Antimicrob. Agents Chemother. 12: 449-450.

Beneke, E. S., J. W. Rippon and A. L. Rogers. 1984. Human Mycoses. 8th Ed. The Upjohn Co., Kalamazoo, Michigan, 88pp.

Bodey, G. P. 1984. Candidiasis in cancer patients. Am. J. Med. 77: 13-19.

Bolard, J., M. Cheron and J. Mazerski. 1984. Effect of surface curvature on the interation of single lamellar phospholipid vesicles with arommatic and nonaromatic heptaene antibiotics (vacidin A and amphotericin B). Biochem. Pharmacol. 33: 3675-3680.

Brajtburg, J., G. Medoff, G. S. Kobayashi and S. Elberg. 1980. Influence of extracellular K^+ or Mg^{2+} on the stages of the antifungal effects of amphotericin B and filipin. In J. D. Nelson and C. Grassi [eds]. Current Chemotherapy and Infectious Disease. Vol. II. Amer. Soc. Microbiol., Washington, DC, pp. 970-972.

Brandriss, M. W., S. M. Wolff, R. Moores and F. Stohlman. 1964. Anemia induced by amphotericin B. J. Am. Med. Assoc. 189: 663-665.

Brass, C., J. N. Galgiani, T. F. Blaschke, R. deFelice, R. A. O'Reilly and D. A. Stevens. 1982. Disposition of ketoconazole, an oral antifungal, in humans. Antimicrob. Agents Chemother. 21: 151-158.

Bullock, W. E. and D. Bathena. 1976. Can mannitol reduce amphotericin B nephrotoxicity? Double-blind study and description of a new vascular lesion in the kidneys. Antimicrob. Agents Chemother. 10: 555-563.

Burford-Mason, A. P., J. C. P. Weber and J. M. T. Willoughby. 1988. Oral carriage of Candida albicans, ABO blood group and secretor status in healthy subjects. J. Med. Vet. Mycol. 26: 49-56.

Butler, W. T., J. E. Bennett, D. W. Alling, P. T. Wertlake, J. P. Utz and G. J. Hill. 1964. Nephrotoxicity of amphotericin B; Early and late effects in 81 patients. Ann. Int. Med. 61: 175-179.

Carmo-Sousa, L. do. 1969. Distribution of yeasts in nature. In A. H. Rose and J. S. Harrison [eds]. The Yeasts. Vol. 1. Academic Press, London, pp. 79-105.

Chalkley, L. J., A. P. J. Trinci and A. M. S. Pope. 1985. Effect of mycolase and amphotericin B on Candida albicans and Candida pseudotropicalis in vitro and in vivo. Sabouraudia 23: 147-164.

Chapman, H. A. and J. B. Hibbs. 1978. Modulation of macrophage tumorcidal capability by polyene antibiotics: Support for membrane lipid as a regulatory determinant of macrophage function. Proc. Natl. Acad. USA 75: 4349-4353.

Clejan, S. and R. Bittman. 1985. Rates of amphotericin B and filipin association with sterols. J. Biol. Chem. 260: 2884-2889.

Cohen, B. E. 1983. The nature of the channels induced by amphotericin B in liposomes. In A. D. Bangham [ed]. Liposome Letters. Academic Press, London, pp. 127-135.

Cybulska, B., M. Herve, E. Borowski and C. M. Gary-Bobo. 1986. Effect of the polar head structure of polyene macrolide antifungal antibiotics on the mode of permeabilization of ergosterol- and cholesterol-containing lipidic vesicles studied by 31P-NMR. Mol. Pharmacol. 29: 293-298.

Dennis, D. L., C. G. Peterson and W. S. Fletcher. 1968. Candida septicaemia in the severely traumatized patient. J. Trama 8: 177-185.

Difco Laboratories. 1984. Bacto Candida albicans antisera. In Difco Manual. 10th Edition. Difco Laboratories Inc., Detroit, MI, pp. 196-198.

Drouhet, E. 1970. Basic Mechanisms of antifungal chemotherapy. In J. P. Utz [ed]. Modern Therapy. Vol. 7: Treatment of the Systemic Mycoses. Harper & Row, New York, pp.539-564.

Edwards, J. E., Jr., R. I. Lehrer, E. R. Stiehm, T. J. Fischer and L. S. Young. 1978. Severe candidal infections: Clinical perspective, immune defense mechanisms, and current concepts of therapy. Ann. Intern. Med. 89: 91-106.

Ellis, W. G., R. A. Sobel and S. L. Nielsen. 1982. Leukoencephalopathy in patients treated with amphotericin B methyl ester. J. Infect. Dis. 146: 125-137.

Emmons, C. W., C. H. Binford, J. P. Utz and K. J. Kwon-Chung. 1977. Medical Mycology. 3rd Edition. Lea & Febiger, Philadelphia, 592 pp.

Feld, R., G. P. Bodey, V. Rodriguez and M. Luna. 1974. Cause of death in patients with malignant lymphoma. Am. J. Med. Sci. 268: 97-106.

Fraser-Smith, E. B., D. A. Eppstein, M. A. Larsen and T. R. Matthews. 1983. Protective effect of a muramyl dipeptide analog encapsulated in or mixed with liposomes against Candida albicans infection. Infect. Immun. 39: 172-178.

Gehan, E. A. 1965. A generalized Wilcoxon test for comparing arbitrarily single-censored samples. Biometrika 52: 203-233.

Gold, W., H. A. Stout, J. F. Pagano and R. Donovick. 1956. Amphotericins A and B, antifungal antibiotics produced by a streptomycete. Antibiotics A. 1956: 579-586.

Gouge, T. H. and V. T. Andriole. 1971. An experimental model of amphotericin B nephrotoxicity with renal acidosis. J. Lab. Clin. Med. 78: 713-726.

Graybill, J. R. and J. Ahrens. 1984. R 51211 (itraconazole) therapy of murine cryptococcosis. Sabouraudia 22: 445-453.

Graybill, J. R. and P. C. Craven. 1983. Antifungal agents used in systemic mycoses: Activity and therapeutic use. Drugs 25: 41-62.

Graybill, J. R., P. C. Craven, R. L. Taylor, D. M. Williams and W. E. Magee. 1982. Treatment of murine cryptococcosis with liposome-associated amphotericin B. J. Infect. Dis. 145:748-752.

Graybill, J. R., S. H. Sun and J. Ahrens. 1986. Treatment of murine coccidioidal meningitis with fluconazole (UK 49,858). J. Med. Vet. Mycol. 24:113-119.

Gregoriadis, G. 1977. Targeting of drugs. Nature (London) 265: 407-411.

Gregoriadis, G. and E. D. Neerunjun. 1975. Homing of liposomes to target cells. Biochem. Biophys. Res. Commun. 65: 537-544.

Gregoriadis, G., D. E. Neerunjun and R. Hunt. 1977. Fate of a liposome-associated agent injected into normal and tumor-bearing rodents. Attempts to improve localization in tumor tissues. Life Sci. 21: 357-370.

Gregoriadis, G. and B. E. Ryman. 1972. Fate of protein-containing liposomes injected into rats. An approach to the treatment of storage diseases. Eur. J. Biochem. 24: 485-591.

Gregoriadis, G., J. Senior, B. Wolff and C. Kirby. 1985. Targeting of liposomes to accessible cells in vivo. Ann. NY Acad. Sci. 446: 319-340.

Hamilton-Miller, J. M. T. 1973. Chemistry and biology of the polyene macrolide antibiotics. Bacteriol. Rev. 37: 166-196.

Hashimoto, Y., H. Endoh and S. Minora. 1984. Chemical methods for the modification of liposomes with proteins or antibodies. In G. Gregoriadis [ed]. Liposome Technology. Vol. III. Targeted Drug Delivery and Biological Interaction. CRC Press, Boca Raton, Florida, pp. 41-50.

Hauser, W. E. and J. S. Remington. 1982. Effect of antibiotics on the immune response. Am. J. Med. 72: 711-716.

Hazen, E. L. and R. Brown. 1951. Fungicidin, an antibiotic produced by a soil actinomycete. Proc. Soc. Exp. Biol. (NY) 76: 93.

Heath, T. D., R. T. Fraley, J. Bentz, E. W. Voss, J. N. Herron and D. Papahadjopoulos. 1984. Antibody directed liposomes: Determination of affinity constants for soluble and liposome-bound antifluorescein. Biochim. Biophys. Acta 770: 148-158.

Heath, T. D., R. T. Fraley and D. Papahadjopoulos. 1980. Antibody targeting of liposomes: Cell specificity obtained by conjugation of $F(ab')_2$ to vesicle surface. Science 210: 539-541.

Heath, T. D., J. A. Montgomery, J. R. Piper and D. Papahadjoponlos. 1983. Antibody-targeted liposomes: Increase in specific toxicity of methotrexate-gamma-aspartate. Proc. Natl. Acad. Sci. USA 80: 1377-1381.

Heel, R. C., R. N. Brogden, G. E. Pakes, T. M. Speight and G. S. Avery. 1980. Miconazole: a preliminary review of its therapeutic efficacy in systemic fungal infections. Drugs 19: 7-30.

Hellebusch, A. A., F. Salama and E. Eadie. 1972. The use of mannitol to reduce the nephrotoxicity of amphotericin B. Surg. Gynecol. Obstet. 134: 241-252.

Hildick, G., H. Blank and I Sarkany. 1964. Fungus Diseases and their Treatment. Little, Brown and Co., Boston, 494 pp.

Hitchcock, C. A., K. J. Barrett-Bee and N. J. Russell. 1987. Inhibition of 14-alpha-sterol demethylase activity in Candida albicans Darlington does not correlate with resistance to azole. J. Med. Vet. Mycol. 25: 329-333.

Hopfer, R. L., K. Mills, R. Mehta, G. Lopez-Berestein, V. Fainstein and R. L. Juliano. 1984. In vitro antifungal activities of amphotericin B and liposome-encapsulated amphotericin B. Antimicrob. Agents Chemother. 25: 387-389.

Hospenthal, D. R., A. L. Rogers, E. S. Beneke. 1989. Effect of attachment of anticandidal antibody to the surfaces of liposomes encapsulating amphotericin B in the treatment of murine candidiasis. Antimicrob. Agents Chemother. 33: 16-18.

Hospenthal, D. R., A. L. Rogers and G. L. Mills. 1988. Development of amphotericin B liposomes bearing antibody specific to Candida albicans. Mycopathologia 101: 37-45.

Hsu, M. J. and R. L. Juliano. 1982. Interaction of liposomes with the reticuloendothelial system. II: Nonspecific and receptor-mediated uptake of liposomes by mouse peritoneal macrophges. Biochim. Biophys. Acta 720: 411-419.

Huang, A., L. Huang and S. J. Kennel. 1980. Monoclonal antibody covalently coupled with fatty acid. J. Biol. Chem. 255: 8015-8018.

Huang, A., S. J. Kennel and L. Huang. 1981. Immunoliposome labeling: A sensitive and specific method for cell surface labeling. J. Immunol. Methods 46: 141-151.

Huang, A., Y. S. Tsao, S. J. Kennel and L. Huang. 1982. Characterization of antibody covalently coupled to liposomes. Biochim. Biophys. Acta 716: 140-150.

Huang, L., A. Huang and S. J. Kennel. 1984. Coupling of antibodies with liposomes. In G. Gregoriadis [ed]. Liposome Technology. Vol. III. Targeted Drug Delivery and Biological Interaction. CRC Press, Boca Raton, Florida, pp. 51-62.

Huang, L. and S. J. Kennel. 1979. Binding of immunoglobulin G to phospholipid vesicles by sonication. Biochemistry 18: 1702-1707.

Hunt, C. A., Y. M. Rustum, E. Mayhew and D. Papahadjopoulos. 1979. Retention of cytosine arabinoside in mouse lung following intravenous administration in liposomes of different sizes. Drug Metab. Dispos. 7: 124-131.

Hurley, R. 1980. The pathogenic Candida species and disease caused by Candidas in man. In F. A. Skinner, S. M. Passmore and R. R. Davenport [eds]. Biology and Activity of Yeasts. Academic Press, New York, pp. 231-248.

Hurley, R. and H. I. Winner. 1963. Experimental renal moniliasis in the mouse. J. Pathol. Bacteriol. 86: 75-82.

Inagaki, J., V. Rodriguez and G. P. Bodey. 1974. Causes of death in cancer patients. Cancer 33: 568-573.

Isono, K., J. Nagatsu, Y. Kawashima and S. Suzuki. 1965. Studies on polyoxins, antifungal antibiotics. I. Isolation and characterization of polyoxins A and B. Agri. Biol. Chem. 29: 848-854.

Juliano, R. L., C. W. M. Grant, K. R. Barber and M. A. Kalp. 1987. Mechanism of the selective toxicity of amphotericin B incorporated into liposomes. Mol. Pharmacol. 31: 1-11.

Juliano, R. L., G. Lopez-Berestein, R. Hopfer, R. Mehta, K. Mehta and K. Mills. 1985. Selective toxicity and enhanced therapeutic index of liposomal polyene antibiotics in systemic fungal infections. Ann. NY Acad. Sci. 446: 390-402.

Juliano, R., G. Lopez-Berestein, R. Mehta, R. Hopfer, K. Mehta and L. Kasi. 1983. Pharmacokinetic and therapeutic consequences of liposomal drug delivery: fluorideoxyuridine and amphotericin B as examples. Biol. Cell 47: 39-46.

Juliano, R. L. and D. Stamp. 1979. Interactions of drugs with lipid membranes: Characteristics of liposomes containing polar or non-polar antitumor drugs. Biocim. Biophys. Acta 586: 137-145.

Juliano, R. L. and D. Stamp. 1978. Pharmacokinetics of liposome-encapsulated anti-tumor drugs. Studies with vinblastine, actinomycin D, cytosine arabinoside, and daunomycin. Biochem. Pharmacol. 27: 21-27.

Juliano, R. L. and D. Stamp. 1975. The effect of particle size and charge on the clearance of liposomes and liposome encapsulated drugs. Biochem. Biophys. Res. Commun. 63: 651-658.

Kauffman, C. A. and P. G. Jones. 1986. Candidiasis: A diagnostic and therapeutic challenge. 1986. Postgrad. Med. 80: 129-134.

Kirsh, R., R. Goldstein, J. Tarloff, D. Parris, J. Hook, N. Hanna, P. Bugelski and G. Poste. 1988. An emulsion of amphotericin B improves the therapeutic index when treating sysytemic murine candidiasis. J. Infect. Dis. 158: 1065-1070.

Kimelberg, H. K. and E. G. Mayhew. 1978. Properties and biological effects of liposomes and their uses in pharmacology and toxicology. Crit. Rev. Toxicol. 6: 25-79.

Kotler-Brajtburg, J., H. D. Price, G. Medoff, D. Schlessinger and G. S. Kobayashi. 1974. Molecular basis for selective toxicity of amphotericin B for yeast and filipin for animal cells. Antimicrob. Agents Chemother. 5: 377-382.

Lapidot, Y., S. Rappoport and Y. Wolman. 1967. Use of esters of N-hydrosuccinimide in the synthesis of N-acylamino acids. J. Lipid Res. 8: 142-145.

Leserman, L. D. 1981. Immunologic targeting of liposomes. In C. Nicolau and A. Paraf [eds]. Liposomes, Drugs andImmunocompetent Cell Functions. Academic Press, London, pp. 109-122.

Leserman, L. D., P. Machy, C. Devaux and J. Barbet. 1983. Antibody-bearing liposomes: Targeting in vivo. Biol. Cell 47: 111-116.

Lin, H. S., G. Medoff and G. S. Kobayashi. 1977. Effects of amphotericin B on macrophages and their precursor cells. Antimicrob. Agents Chemother. 11: 154-160.

Lopez-Berestein, G. 1986. Liposomal amphotericin B in the treatment of fungal infections. Ann. Intern. Med. 105: 130-131.

Lopez-Berestein, G., G. P. Bodey, L. S. Frankel and K. Mehta. 1987. Treatment of hepatosplenic candidiasis with liposomal-amphotericin B. J. Clin. Oncol. 5: 310-317.

Lopez-Berestein, G., V. Fainstein, R. Hopfer, K. Mehta, M. P. Sullivan, M. Keating, M. G. Rosenblum, R. Mehta, M. Luna, E. M. Hersh, J. Reuben, R. L. Juliano and G. P. Bodey. 1985. Liposomal amphotericin B for the treatment of systemic fungal infections in patients with cancer: A preliminary study. J. Infect. Dis. 151: 704-710.

Lopez-Berestein, G., R. L. Hopfer, R. Mehta, K. Mehta, E. M. Hersh and R. L. Juliano. 1984. Prophylaxis of Candida albicans infection in neutropenic mice with liposome-encapsulated amphotericin B Antimicrob. Agents Chemother. 25: 366-367.

Lopez-Berestein, G., R. L. Hopfer, R. Mehta, K. Mehta, E. M. Hersh and R. L. Juliano. 1984a. Liposome-encapsulated amphotericin B for treatment of disseminated candidiasis in neutropenic mice. J. Infect. Dis. 150: 278-283.

Lopez-Berestein, G. and R. L. Juliano. 1987a. Application of liposomes to the delivery of antifungal agents. In M. J. Ostro [ed]. Liposomes. From Biophysics to Therapeutics. Marcel Dekker, Inc., New York, pp.253-276.

Lopez-Berestein, G., L. Kasi, M. G. Rosenblum, T. Haynie, M. Jahns, H. Glenn, R. Mehta, G. M. Mavligit and E. M. Hersh. 1984b. Clinical Pharmacology of 99m Tc-labeled liposomes in patients with cancer. Cancer Res. 44: 375-378.

Lopez-Berestein, G., R. Mehta, R. L. Hopfer, K. Mills, L. Kasi, K. Mehta, V. Fainstein, M. Luna, E. M. Hersh and R. Juliano. 1983. Treatment and prophylaxis of disseminated infection due to Candida albicans in mice with liposome-encapsulated amphotericin B. J. Infect. Dis. 147: 939-945.

Lopez-Berestein, G., K. Mehta, R. Mehta, R. L. Juliano and E. M.Hersh. 1983a. The activation of human monocytes by liposome-encapsulated muramyl dipeptide analogues. J. Immunol. 130: 1500-1502.

Lopez-Berestein, G., M. G. Rosenblum and R. Mehta. 1984c. Altered tissue distribution of amphotericin B by liposome encapsulation: Comparison of normal mice to mice infected with Candida albicans. Cancer Drug Delivery 1: 199-205.

Louria, D. B., R. G. Brayton and G. Finkel. 1963. Studies of the pathogenesis of experimental Candida albicans infection in mice. Sabouraudia 2: 271-283.

Louria, D. B., D. P. Stiff and B. Bennett. 1962. Disseminated moniliasis in the adult. Medicine 41: 307-337.

Lowry, O. H., N. J. Rosenbrough, A. L. Farr and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265-275.

MacMillan, B. G., E. J. Law and I. A. Holder. 1972. Experience with Candida infections in the burn patient. Archives of Surgery 104: 509-514.

Marichal, P., J. Gorrens and H. V. Bossche. 1985. The action of itraconazole and ketoconazole on growth and sterol synthesis in Aspergillus fumigatus and Aspergillus niger. Sabouraudia 23: 13-21.

Massa, T., D. P. Sinha, J. D. Frantz, M. E. Filipek, R. C. Weglein, S. A. Steinberg, J. T. McGrath, B. F. Murphy, R. J. Szot, H. E. Black and E. Schwartz. 1985. Subchronic toxicity studies of N-D-ornithyl amphotericin B methyl ester in dogs and rats. Fundam. Appl. Toxicol. 5: 737-753.

McCurdy, D. K., M. Frederic and J.R. Elkinton. 1968. Renal tubular acidosis due to amphotericin B. New Eng. J. Med. 278: 124-125.

Medoff, G., J. Brajtburg, G. S. Kobayashi and J. Bolard. 1983. Antifungal agents useful in therapy of systemic fungal infections. Ann. Rev. Pharmacol. Toxicol. 23: 303-330.

Medoff, G. and G. S. Kobayashi. 1980. Strategies in the treatment of systemic fungal infections. New Eng. J. Med. 302: 145-155.

Mehta, K., G. Lopez-Berestein, E. M. Hersh and R. L. Juliano. 1982. Uptake of liposomes and liposome-encapsulated muramyl dipeptide by human peripheral blood monocytes. J. Reticuloendothel. Soc. 32: 155-164.

Mehta, R. T., R. L. Hopfer, L. A. Gunner, R. L. Juliano and G. Lopez-Berestein. 1987. Formulation, toxicity, and antifungal activity in vitro of liposome-encapsulated nyastatin as therapeutic agent for systemic candidiasis. Antimicrob. Agents Chemother. 31: 1897-1900.

Mehta, R. T., R. L. Hopfer, T. McQueen, R. L. Juliano and G. Lopez-Berestein. 1987a. Toxicity and therapeutic effects in mice of liposome-encapsulated nystatin for systemic fungal infections. Antimicrob. Agents Chemother. 31: 1901-1903.

Mehta, R., M. J. Hsu, R. L. Juliano, H. J. Krause and S. L. Regen. 1986. Polymerized phospholipid vesicles containing amphotericin B: Evaluation of toxic and antifungal activities in vitro. J. Pharm. Sci. 75: 579-581.

Mehta, R. T., G. Lopez-Berestein, R. L. Hopfer, K. Mehta, R. A. White and R. L. Juliano. 1985. Prophylaxis of murine candidiasis via application of liposome-encapsulted amphotericin B and a muramyl dipeptide analog, alone and in combination. Antimicrob. Agents Chemother. 28: 511-513.

Mehta, R., G. Lopez-Berestein, R. Hopfer, K. Mills and R. L. Juliano. 1984. Liposomal amphotericin B is toxic to fungal cells but not to mammalian cells. Biochim. Biophys. Acta 770: 230-234.

Mehta, R. T., K. Mehta, G. Lopez-Berestein and R. L. Juliano. 1985a. Effect of liposomal amphotericin B on murine macrophages and lymphocytes. Infect. Immun. 47: 429-433.

Merck & Co., Inc. 1983. The Merck Index. 10th Ed. Merck & Co., Inc., Rathway, New Jersey, pg. 85.

Meunier-Carpentier, F. 1984. Chemoprophylaxis for fungal infections. Am. J. Med. 76: 652-656.

Mourad, S. and L. Friedman. 1961. Pathogenicity of Candida. J.Bacteriol. 81: 550-556.

New, R. R. C., M. L. Chance and S. Heath. 1981. Antileishmanial activity of amphotericin and other antifungal agents entrapped in liposomes. J. Antimicrob. Chemother. 8: 371-381.

Nozawa, Y., Y. Banno, K. Ohki, T. Morita, S. Yoshioka and T. Sekiya. 1986. Interaction of targeted liposomes with hepatocytes. In K. Yagi [ed]. Medical Application of Liposomes. Japan Scientific Societies Press, Tokyo, pp. 55-65.

Odds, F. C. 1979. Candida and Candidosis. Leicester University Press, Great Britain, 382 pp.

Olds, G. R., S. J. Stewart and J. J. Ellner. 1981. Amphotericin B-induced resistance to Schistosoma mansoni. J. Immunol. 126: 1667-1670.

Olson, F., C. A. Hunt, F. C. Szoka, W. J. Vail and D. Papahadjopoulos. 1979. Preparation of liposomes of defined size distribution by extrusion through polycarbonate membranes. Biochim. Biophys. Acta 557: 9-23.

Ostro, M. J. 1987. Introduction. In M. J. Ostro [ed]. Liposomes. From Biophysics to Therapeutics. Marcel Dekker, Inc., New York, pp. vii-x.

Ostro, M. J. 1987a. Liposomes. Scientific American 256: 102-111.

Pagano, R. E. and J. N. Weinstein. 1978. Interactions of liposomes with mammalian cells. Ann. Rev. Biophys. Bioeng. 7: 435-468.

Panosian, C. B., M. Barza, F. Szoka and D. J. Wyler. 1984. Treatment of experimental cutaneous leishmaniasis with liposome-intercalated amphotericin B. Antimicrob. Agents Chemother. 25: 655-656.

Papahadjopoulos, D. 1986. A personal view of liposomes: From membrane modeling to drug delivery. In K. Yagi [ed]. Medical Application of liposomes. Japan Scientific Societies Press, Tokyo, pp. 1-9.

Papahadjopoulos, D., T. Heath, K. Bragman and K. Matthay. 1985. New methodology for liposome targeting to specific cells. Ann. NY Acad. Sci. 446: 341-348.

Parmegiani, R. M., D. Loebenberg, B. Antonacci, T. Yarosh-Tomaine, R. Scupp, J. J. Wright, P. J. S. Chiu and G. H. Miller. 1987. Comparative in vitro and in vivo evaluation of N-D-ornithyl amphotericin B methyl ester, amphotericin B methyl ester, and amphotericin B. Antimicrob. Agents Chemother. 31: 1756-1760.

Philippidis, P., J. L. Naiman, M. S. Sibinga and M. A. Valdes-Dapnea. 1971. Disseminated intravascular coagulation in Candida albicans septicemia. J. Pediatrics 78: 683-686.

Poznansky, M. J. and R. L. Juliano. 1984. Biological approaches to the controlled delivery of drugs: A critical review. Pharmacol. Rev. 36: 277-336.

Pratt, W. B. 1977. Chemotherapy of Infection. Oxford University Press, New York.

Reingold, A. L., X. D. Lu, B. D. Plikaytis and L. Ajello. 1986. Systemic mycoses in the United States, 1980-1982. J. Med. Vet. Mycol. 24: 433-436.

Richardson, V. J. 1983. Liposomes in antimicrobial chemotherapy. J. Antimicrob. Chemother. 12: 532-534.

Rippon, J. W. 1988. Medical Mycology. The Pathogenic Fungi and the Pathogenic Actinomycetes. 3rd Ed. W. B. Saunders Co., Philadephia, 797 pp.

Roberts, S., R. J. Hay and d. Mackenzie. 1984. A Clinician's Guide to Fungal Disease. Marcel dekker, Inc., New York, 252 pp.

Rogers, T. and E. Balish. 1976. Experimental Candida albicans infection in conventional mice and germfree rats. Infect. Immun. 14: 33-38.

Ryley, J. F., S. McGregor, S. C. Lister and K. P. Jackson. 1988. Kidney function in experimental systemic candidosis of mice. Mycoses 31: 203-207.

Schach, M. C. 1987. "First" liposome immunoassay streptest: Results in one minute. Clinical Lab Letter, November 13, pg. 163.

Schneider, M. 1985. Liposomes as drug carriers: 10 years of research. In P. Buri and A. Gumma [eds]. Drug Targeting. Elsevier Science Publishing, Inc., New York, pp. 119-134.

Seelig, M. S. and P. J. Kozinn. 1982. Clinical manifestations and management of candidosis in the immunocompromised patient. In D. W. Warnock and M. D. Richardson [ed]. Fungal Infection in the Compromised Patient. John Wiley & Sons Ltd., New York, pp. 49-92.

Shen, D., A. Haung and L. Haung. 1982. An improved method for covalent attachment of antibodies to liposomes. Biochim. Biophys. Acta 689: 31-37.

Shirkhoda, A., G. Lopez-Berestein, J. M. Holbert and M. A. Luna. 1986. Hepatosplenic fungal infections: CT and pathological evaluation after treatment with liposomal amphotericin B. Radiology 159: 349-353.

Skipski, V. P. and M. Barclay. 1969. Thin-layer chromatography of lipids. IN J. M. Lowenstein [ed]. Methods in Enzymology. Vol. XIV. Lipids. Academic Press, New York, pp. 530-597.

Smith, K. J., D. W. Warnock, C. T. C. Kennedy, E. M. Johnson, V. Hopwood, J. van Cutsem and H. V. Bossche. 1986. Azole resistance in Candida albicans. J. Med. Vet. Mycol. 24: 133-144.

Sokol-Anderson, M. L., J. Brajtburg and G. Medoff. 1986. Amphotericin B-induced oxidative damage and killing of Candida albicans. J. Infect. Dis. 154: 76-83.

Speller, D. C. E. 1980. Antifungal Chemotherapy. John Wiley & Sons Ltd., Chichester, England.

Stamp, D. and R. L. Juliano. 1979. Factors affecting the encapsulation of drugs within liposomes. Can. J. Physiol. Pharmacol. 57: 535-539.

Stevens, D. A. 1977. Miconazole in the treatment of systemic fungal infections. Amer. Rev. Respir. Dis. 116: 801-806.

Stone, H. H., L. D. Kolb, C. A. Currie, C. E. Geheber and J. Z. Cuzzell. 1974. Candida sepsis: Pathogenesis and principles of treatment. Annals of Surgery 179: 697-711.

Straub, S. X., R. F. Garry and W. E. Magee. 1974. Interferon induction by poly(I):poly(C) enclosed in phospholipid particles. Infect. Immun. 10: 783-792.

Sunamoto, J. 1986. Application of polysaccharide-coated liposomes in chemotherapy and immunotherapy. In K. Yagi [ed]. Medical Application of Liposomes. Japan Scientific Societies Press, Tokyo, pp. 121-129.

Sundstrom, P. M. and G. E. Kenny. 1984. Characterization of antigens specific to the germ tubes of Candida albicans by immunofluorescence. Infect. Immun. 43: 850-855.

Surarit, R. and M. G. Shepherd. 1987. The effects of azole and polyene antifungals on the plasma membrane enzymes of Candida albicans. J. Med. Vet. Mycol. 25: 403-413.

Szoka, F. C., D. Milholland and M. Barza. 1987. Effect of lipid composition and liposome size on toxicity and in vitro fungicidal activity of liposome-intercalated Amphotericin B. Antimicrob. Agents Chemother. 31: 421-429.

Szoka, F. and D. Papahadjopoulos. 1978. Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. Proc. Natl. Acad. Sci. USA 75: 4194-4198.

Taschdjian, C. L., M. S. Seelig and P. J. Kozinn. 1973. Serological diagnosis of candidal infections. CRC Critical Reviews in Clinical Laboratory Sciences 4: 19-59.

Taylor, R. L., D. M. Williams, P. C. Craven, J. R. Graybill, D. J. Drutz and W. E. Magee. 1982. Amphotericin B in liposomes: A novel therapy for histoplasmosis. Am. Rev. Respir. Dis. 125: 610-611.

Teerkink, T., B. D. Kruijff and R. A. Demel. 1980. The action of pimaricin, etruscomycin and amphotericin B on liposomes with varying sterol content. Biochim. Biophys. Acta 599: 484-492.

Torchilin, V. P., B. A. Khaw, V. N. Smirnov and E. Haber. 1979. Preservation of antimyosin antibody activity after covalent coupling to liposomes. Biochem. Biophys. Res. Commun. 89: 1114-1119.

Tremblay, C., M. Barza, C. Fiore and F. Szoka. 1984. Efficacy of liposome-intercalated amphotericin B in treatment of systemic candidiasis in mice. Antimicrob. Agents Chemother. 26: 170-173.

Tremblay, C., M. Barza, F. Szoka, M. Lahav and J. Baum. 1985. Reduced toxicity of liposome-associated amphotericin B injected intravitreally in rabbits. Ophthalmol. Vis. Sci. 26: 711-718.

Troke, P. F., R. J. Andrews, K. W. Brammer, M. S. Marriott and K. Richardson. 1985. Efficacy of UK-98,858 (fluconazole) against Candida albicans experimental infections in mice. Antimicrob. Agents Chemother. 28: 815-818.

Tucker, R. M., P. L. Williams, E. G. Arathoon, B. E. Levine, A. I. Hartstein, L. H. Hanson and D. A. Stevens. 1988. Pharmacokinetics of fluconazole in cerebrospinal fluid and serum in human coccidioidal meningitis. Antimicrob. Agents Chemother. 32: 369-373.

Vecchiarelli, A., G. Verducci, S. Perito, P. Puccetti, P. Marconi and F. Bistoni. 1986. Involvement of host macrophages in the immunoadjuvant activity of amphotericin B in a mouse fungal infection model. J. Antibiotics 39: 846-855.

Wertlake, P. T., W. T. Butler, G. J. Hill and J. P. Utz. 1963. Nephrotoxic tubular damage and calcium deposition following amphotericin B therapy. Am. J. Path. 43: 449-453.

Whittle, C. H. and G. A. Gresham. 1960. Candida in vitro and in vivo. Mycopathol. Mycol. Appl. 12: 207-215.

Yagi, K. and M. Naoi. 1986. Glycolipid insertion into liposomes for their targeting to specific organs. In K. Yagi [ed]. Medical Application of Liposomes. Japan Scientific Societies Press, Tokyo, pp.91-97.

Young, G. 1958. The process of invasion and the persistence of Candida albicans injected intraperitoneally into mice. J. Infect. Dis. 102: 114-120.

Young, L. S. 1982. The outlook for antifungal prophylaxis in the compromised host. J. Antimicrob. Chemother. 9: 338-340.