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TREATMENT OF CANDIDIASIS IN A NEUTROPENIC MURINE MODEL USING ANTIBODY-BEARING LIPOSOMAL AMPHOTERICIN B

By

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ABSTRACT

TREATMENT OF CANDIDIASIS IN A NEUTROPENIC MURINE MODEL USING ANTIBODY-BEARING LIPOSOMAL AMPHOTERICIN B

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The goal of this study was to investigate the efficacy of liposomal amphotericin B targeted with anti candidal antibodies in the treatment of disseminated candidiasis in a murine model made neutropenic using cyclophosphamide. To achieve the goal, crude rabbit antiserum or specific immunoglobulin G to Candida albicans was derivatized with palmitic acid and was incorporated into liposomes containing amphotericin B.

To develop a murine model, the effect of cyclophosphamide (CY) on the number of total and differential cells was first examined by administration of various dosages of the drug intraperitoneally. A dose dependent transient reduction in the number of granulocytes, lymphocytes, and monocytes of mice was observed. The susceptibility of mice to C. albicans infection after different doses of CY administration was examined, a dose of 150 mg/kg of CY was selected for induction of neutropenia because it lead to 100% mortality of C. albicans infected mice within 4 to 7 days.

Treatment with a single dose (0.6 mg amphotericin B /kg)

of liposomal amphotericin B complexed with rabbit antiserum (LAMB-Ab) to C. albicans resulted in an increase in the survival of neutropenic mice infected with 3 X 10^5 cfu of C. albicans compared to mice treated with identical doses of liposomal amphotericin B (LAMB) or free amphotericin B (fAMB/Fungizone).

In a second study, immunoglobulin G (IgG) was separated from the same antiserum and bound to liposomal amphotericin B (LAMB-IgG). This complex, LAMB-IgG significantly improved the survival (65%) of neutropenic mice with candidiasis compared to LAMB-Ab, LAMB or Fungizone. LAMB-IgG at a dose of 0.6 or 0.9 mg AMB/kg was more effective in the therapy of disseminated candidiasis compared to the same doses of Fungizone. However, treatment with lower and higher doses of LAMB-IgG or Fungizone equally cleared C. albicans from the spleen, liver, and lungs but not from the kidneys.

Results of this study suggest that LAMB-IgG is more effective than LAMB-Ab, LAMB, or fAMB in the therapy of disseminated candidiasis in neutropenic mice.

DEDICATED TO MY PARENTS

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INTRODUCTION

Disseminated candidiasis produced by Candida albicans is one of the leading causes of death in cancer patients undergoing chemotherapy, transplant recipients on immunosuppressive therapy, and in patients with hematologic malignancies. The incidence of the disease especially in neutropenic patients has been increasing steadily in recent years.

Despite its adverse effects to the host, the commercial form of amphotericin B, Fungizone, remains the drug of choice for many life-threatening mycotic infections including systemic candidiasis. To overcome the toxicity of amphotericin B and increase its therapeutic index by allowing much higher dosages, researchers have been encapsulating the drug in liposomes. Amphotericin B delivery by liposomes has shown potential advantages over the standard Fungizone therapy in various animal models and in a few patients with sytemic mycotic infections. Although promising results have been obtained, liposomal amphotericin B formulation has not been standardized for the therapy of mycotic infections. The optimum formulation of liposomal amphotericin B remains to be

determined for use in clinical therapy. As a result, different formulations of liposomal amphotericin B including liposomal amphotericin B-cholesterol sulphate complex, emulsion of amphotericin B with Intralipid or antibody-bearing liposomal amphotericin B have been under investigation to treat a variety of systemic fungal infections in animal models.

The incorporation of antibodies into the surface of liposomes in an attempt to target amphotericin B to fungal cells has shown encouraging results. In recent studies, researchers have used amphotericin B in immunoliposomes to treat both Cryptococcus neoformans and Candida albicans infections in mice. Based on the principle of new modalities for amphotericin B administration, it has been shown that commercial rabbit antiserum against C. albicans attached to liposomal amphotericin B significantly improves survival of normal mice compared to untreated controls or mice treated with Fungizone. Because of the increasing number of morbidity and mortality reports in neutropenic patients due to disseminated C. albicans infections, there is a need for a reproducible neutropenic animal model to develop and further evaluate the therapeutic effect of antibody-bearing liposomal amphotericin B.

The objectives of this research are to a) develop a neutropenic murine model using cyclophosphamide, b) assess the effect of cyclophosphamide on total and differential leukocyte

cell count, and c) utilize this murine model in the treatment studies of experimentally induced systemic candidiasis using antibody bearing liposomal amphotericin B. This study also investigates whether the increase in therapeutic efficacy of liposomal amphotericin B coupled with crude rabbit antiserum in the therapy of candidiasis observed in previous studies is truly due to antibodies (immunoglobulin G) or some other serum components.

Antibody molecules from rabbit antiserum were fractionated using the combination of caprylic acid-ammonium sulphate precipitation method. Different formulations of the liposomal amphotericin B including liposomal amphotericin B without antibodies, liposomal amphotericin B coupled with crude rabbit antiserum, liposomal amphoterin B coupled with purified immunoglobulin G and Fungizone were compared in the therapy of systemic candidiasis in neutropenic and non-neutropenic mice. Dose responses of the various liposomal amphotericin B formulations were also examined.

LITERATURE REVIEW

LITERATURE REVIEW

Candida albicans and Candidiasis

Candidiasis (candidosis) is caused by several members of the genus *Candida* and is the most prevalent mycotic disease in humans or other mammals occurring as a superficial or systemic forms. The clinical manifestation of candidiasis can be an acute, subacute or a chronic form (98). The incidence of disseminated candidiasis, in particular has increased markedly in the last two decades and remains as a major cause of morbidity and mortality in immunocompromised patients. Furthermore, it is becoming more serious and frequent as the number of AIDS (HIV infection), cancer and transplant patients increases. Death rate due to candidiasis have been steadily increasing in the last two decades. For instance, in the USA, the number of deaths due to candidiasis has increased roughly 3-fold between the late 1960s and the late 1970s and in France there has been a roughly 2-fold increase between 1973 and 1982 (90). It was reported that mortality associated with systemic candidiasis has reached up to 35% (90).

The genus *Candida* consists of more than 150 species, most with no sexual cycle. All *Candida* species isolated from clinical material are in their imperfect (asexual) states.

However, some sexual forms have been described while the organisms grow in culture. Candida species are yeast cells that reproduce predominantly by budding or fission. According to Odds (90), the following eight species of the genus Candida have been found to be pathogenic: C. albicans, C. stellatoidea, C.tropicalis, C. parapsilosis, C. krusei, C. pseudotropicalis, C. guilliermondii, and C. glabrata (or Torulopsis glabrata). However, recently 15 species of the genus Candida have been considered pathogenic (36).

The species of the genus Candida have been mainly separated on the basis of morphological and physiological properties, although both of these factors are by no means the only criteria for the identification of the species. Differences in antigenic structure (46a), DNA composition (91), DNA restriction fragment length polymorphism and electrophoretic karyotypes of the species (79), are currently providing more reliable interrelationships between the Candida species. Antigenic structures of C. albicans and C. tropicalis are very similar, and C. parapsilosis is closer to both of these species than other members of the genus (46a). The remaining species such as C. guilliermondii, C. keifyr, and C. krusei have relatively distinct components of antigens but several species still show some cross-reactivity.

Candidiasis accounts for 70-80% of all systemic fungal infections of which 60-75% are caused by C. albicans and 20-25% by C. tropicalis as reported by Wingard et al. (132).

Candida albicans is a dimorphic fungus belonging to the form-phylum Deuteromycota, the form-class Blastomycetes, and the form-family Cryptococcaeae (98). The dimorphic property of C. albicans depends on environmental conditions: temperature, pH, and growth media (90). Factors enhancing filamentous formation of the organism in vitro are: pH > 6.5-7.0, temperature > 35°C, non-fermentable carbon source, or CO_2:O_2 2:1. Yeast cell formation in vitro is favored by glucose as carbon source, pH < 6.5-7.0, and temperature < 35°C.

Based on serum agglutination tests, C. albicans isolates have been divided into 2 serotypes A and B with type A being recovered more commonly than type B in clinical materials (46b). In a recent review, Merz et al. (82) stated that there are many typing methods based on variable phenotyping characteristics within C. albicans including morphotyping, serotyping, antibiogram, resistogram typing, biotyping on commercial carbon assimilation patterns, enzyme profiles, sensitivity to yeast killer toxins, and typing based on protein viability.

Wide range of carrier rates for C. albicans in humans has been reported. C. albicans may exist in the yeast phase (blastospore) as a normal flora on the mucous membranes of various parts of the alimentary tract and vagina in 10 to 50% of healthy individuals (90,98). The incidence of C. albicans in normal nonpregnant women is about 5 percent and can be as

high as 30 percent in pregnant women or in women taking oral contraceptics (90). It exists in balance with other normal flora of the body. But various prediposing factors may upset the this balance and lead to infection. As Rippon (98) described it, C. albicans can become pathogenic due to premature birth, physiologic change, or prolonged administration of antibiotics by the host. Rippon (98) has defined 3 types of infections caused by C. albicans: mucocutaneous, cutaneous and systemic candidiasis. Infection in the nails (onychomycosis), oral candidiasis (thrush) or other chronic mucocutaneous candidiasis (CMC) are classified under mucocutaneous or superficial candidiasis. Cutaneous infection includes intertriginous, epiglottitis, esophagitis and other generalized candidiasis. Systemic candidiasis appears as gastrointestinal infections, genitourinary infection, esophagitis, meningitis, endocarditis, and septicemia.

Disseminated infection due to C. albicans is the most life-threatening candidiasis and occurs commonly in debilitated or immunocompromised hosts. According to Odds (90) definition, disseminated candidiasis is an infectious process in which Candida lesions occur in a minimum of 2 body sites.

The prevalence of this opportunistic infection is steadily increasing in the era of modern medicine and chemotherapy. For example, in the United States this disease

affects more than 120,000 patients annually, accounts for 15% of hospital acquired infections and has a high mortality rate (131). The major predisposing factors for disseminated candidiasis includes the wider use of immunosuppressive drugs, severe burns, malignancies, post-surgery, and intravenous hyperalimentation (90,98,131).

Animal models of candidiasis

Several experimental infections in rodents have been shown to resemble disseminated candidiasis in humans (96). Researchers in the pharmaceutical industries have made a substantial contribution to developing animal models of candidiasis which can be useful for preclinical evaluation of antifungal regimens (122).

Many animal models for use in the examination of cutaneous candidiasis have been described. Ray et al.(96) used the epicutaneous route of infection in infant rats and mice along with an occlusive dressing that resulted in subcorneal microabcesses detectable histologically after 21 hours. Vaginal candidiasis has also been studied in many animals models. For instance, experimental vaginal candidiasis in a mouse model was produced by the introduction of a knobbed glass rod heavily coverd by C. albicans into the genital organ (120). The use of various animal models to study oropharyngeal candidiasis has also been reported. Russel et al. (102) used tetracycline treated rats to examine the mouth area for increased colonization of C. albicans.

Experimental models of systemic candidiasis have also been produced in animals such as rabbit, rat, guinea pig, and mouse. The most common means for establishing systemic candidiasis has been by the intravenous inoculation. Various aspects of the disease have been studied mainly in the mouse including the spread and invasion of the etiologic agent in the animal body and interaction of yeast cells with innate host resistance.

The gastrointestinal tract in animal models as the main route of establishment of disseminated infection with C. albicans has been investigated in many experiments (18,29,39). A combination of antibiotics such as polymyxin B, gentamicin, and the antineoplastic drug, cytarabine have been used to make a mouse susceptible to C. albicans or C. tropicalis infection (132). In that study, both organisms were recovered from the visceral organs of the mice after antibiotic administration but not in untreated animals. Other researchers reported that oral-intragastric inoculation of infant mice compromised by a short fast had systemic spread of C. albicans and lethality after 30 minutes of inoculation (90). Intraperitonial inoculation of mice with Candida may lead to disseminated infection, but the inoculum given by this route needs to be about 10 times higher than intravenous inoculum. Many investigators agree that for most C. albicans isolates the intravenous LD_{50} in normal mice is 10^4 to 10^6 yeasts (90). In the literature it is reported that 10^6 C. albicans yeasts

is rapidly lethal whereas less than 10^4 yeast cells produce a chronic infection in mice (90,98).

Animal models injected intravenously with viable radiolabeled yeasts have been used to follow the course of experimental disseminated candidiasis. It has been found that lungs and livers are loaded with yeasts after a few hours of inoculation intravenously. This conclusion is based on the highest percentages of colony forming units (CFU) or radioactive counts recovered in the liver, lungs, and spleen (77). Time dependent distribution of C. albicans from blood to lungs, liver then to kidneys was revealed (124). In this study, a gradual decline in numbers of organisms in all organs is observed, except in the kidneys. It is believed that the kidneys are the most severely infected organs by C. albicans.

In general, irrespective of the route of inoculation, candidiasis is established and the course of the infection is basically similar in many different animal models so far developed.

Different murine strains have shown a variation in susceptibility to C. albicans infection depending on the immunological and genetic factors of the animals. For instance, C3H, BALB/c and hybrid CD2F1 mice are susceptible to C. albicans infection whereas C57B1/6 strain is resistant to the same infection (11). The congenically athymic (nude) mice and New Zealand Black (NZB) mice are commonly utilized to study the role of cell-mediated immunity in disseminated

candidiasis (19,20). It has been shown that nude mice have higher resistance to C. albicans infection compared to the phenotypically normal littermates. It is believed that activated macrophages are responsible for rapid clearance of the etiologic agent from the liver of the nude mice. Similarily, the NZB mice have shown a greater susceptibility to lethal infection with opportunistic fungal pathogens such as C. albicans, Cryptococcus neoformans, and Aspergillus fumigatus (19). The enhanced susceptibility of NZB is associated with a deficiency of cell-mediated immunity. Also, mouse strains such as DBA/2J, and A/J that have shown deficiency in the C5 complement have an increased susceptibility to C. albicans infection (35).

Virulence factors of C. albicans

The ability of Candida species to cause disease in humans is highly variable. Candida albicans is the most pathogenic species among the few pathogenic members of the genus. The variation in pathogenicity may be associated with the difference in virulence factors. The determinants of virulence of C. albicans, have not been completely defined. However, a number of virulence factors including production of inducible proteases, phospholipases, Candida endotoxin, dimorphism, cell surface composition, phenotype switching, adherence and complement receptors have been proposed (36,90).

There is evidence that adherence is involved in the colonization of C. albicans as shown by its attachment to rat

tongue and cheek cells, although adherence of C. albicans was suppressed by mixed human salivary bacteria (72). In another study, C. albicans showed a greater degree of adherence to vaginal and buccal epithelial cells than other Candida species tested (61). The optimum pH and temperature for adherence are, respectively, pH 6-8 and 37°C. Recently it has been shown that the complement receptor of C. albicans, CR3, and the plastic receptor, CR2, both considered mannoproteins and expressed in germ tubes, play a role in adherence (14).

Although many comparative studies have been conducted, it is not yet clear whether the yeast form or the filamentous form is more important in virulence activity. Some investigators believe that both are equally important as a cause of infection, whereas others claim that the mycelial form is associated with infection, with easier penetration of the tissue than yeast cells (44). On the other hand, in vitro studies suggest that the yeasts are capable of penetrating the epithelial cells or resist killing from polymorphonuclear phagocytes (63). In their study, yeast cells were able to adhere and penetrate the epithelial cells before germ tube formation. Regarding adherence, it has been demonstrated that germinated C. albicans (mycelial form) has significantly greater adherence to buccal epithelial cells than yeast cells. This was evidenced by mannose-mediated adherence of C. albicans to human buccal cells in vitro (104).

Haemolysin and endotoxin production has been confirmed

from C. albicans strains (36). High-molecular weight glycoproteins and canditoxin from C. albicans were found to be toxic to mice. In addition, the glycoproteins of C. albicans were involved in adherence and inhibition of neutrophil attachment to hyphae. In the same study, low-molecular weight toxins from C. albicans strains were also found to be shock-producing and lethal to experimental animals. Other toxic substances from surface cell components of C. albicans that are pyrogenic in rabbits and lethal to mice and chicken embryos have also been discovered.

Enzymes produced by C. albicans that may be involved in pathogenicity include proteinases, phospho-lipases and lysophospholipases (36).

Immunology of Candida albicans

Candidiasis in healthy individuals is rarely encountered because the nonspecific host defense mechanisms greatly contribute to resistance against Candida infection. Innate and nonimmune factors such as skin and mucous membranes, microbial flora, hormones, phagocytic cells, the complement system and other serum factors contribute to resistance against Candida infections (22,104,105).

Phagocytosis and destruction by tissue macrophages or circulating polymorphonuclear (PMN) and monocytes appears to be the ultimate fate of C. albicans. The action of PMN and macrophages in killing C. albicans has been extensively studied in vitro (28,69). Candidacidal activity of PMN cells

and monocytes is due to the participation of lysosomal myeloperoxidase and its oxidant substrate hydrogen peroxide (68). Evans et al.(28) reported the initial appearance of PMN leukocytes followed by macrophages in the liver and lungs of mice challenged with C. albicans. In the same study, lungs were more resistant to infection than other organs although macrophages were effective in the the liver. In another similar study, Leijh et al. (69) reported that up to 96% of the cells in a C. albicans inoculum were ingested by human granulocytes and monocytes in 1 h. The granulocytes and monocytes killed 58% and 50%, respectively of the organism during the hour.

As evidenced by the detection of several broken substances of C. albicans inside human neutrophil cells (68), it is accepted that neutrophils are the most effective candidacidal phagocytic cells. Djeeu et al. (23) showed that the growth of C. albicans was inhibited by human PMN neutrophils which had been activated by interferon-gamma and tumor necrosis factor (TNF). On the other hand Kolotila et al. (64) using in vitro experiment demonstrated that neutrophils and their oxidants are capable of killing yeast cells or inhibiting phenotypic switching of C. albicans strain WO-1. Moreover, Bostoni et al.(10) reported evidence for macrophage-mediated protection against lethal C. albicans infection. In that study, mice exposed to a non-virulent strain of C. albicans, PCA-2, were protected against a

subsequent intravenous challenge with a pathogenic parent strain of PCA-2. It is suggested that the protection was due to macrophage activation by preexposure to C. albicans antigens that increase macrophage candidacidal activity. In addition, a heat-stable substance with a molecular weight of 10,000 to 20, 000 dalton that inhibits in vitro growth of C. albicans has been identified (78). Copper and iron in the human serum are also considered as factors involved in resistance against Candida infection (104).

Although the nonspecific immune mechanism is a major factor contributing to the increase in resistance to Candida infection, both humoral and cellular arms of immune system are believed to play a role in resistance.

Antibodies elicited against mannonproteins, cytoplasmic proteins and ribosomes of C. albicans have shown less effective in protection against infection (115). However, antibodies neutralizing proteinase released from C. albicans or inhibiting the adherence of C. albicans to epithelial cells by blocking adhesins have been reported (32). Many experimental animals have been used in investigations to demonstrate that antibodies produced against C. albicans are protective to subsequent challenge with the same yeast (99,109). It was shown that cyclophosphamide-pretreated mice vaccinated with C. albicans ribosomes, with or without addition of incomplete Freud's adjuvant, led to a partial protection against a lethal intraperitoneal or intravenous

challenge with live yeasts (109).

Cell-mediated immunity (CMI) has a major role in defense against fungi including C. albicans by the generation of antigen specific T cells, followed by the release of lymphokines in response to the antigen. Cell-mediated immunity has important anticandidal effect by elicitation of an inhibitory factor from sensitized lymphocytes (8). Several investigators have demonstrated that deficiency in CMI is the major cause of chronic mucocutaneous candidiasis. Nelson et al.(88) stated that mannan and oligosaccharide fragments of mannan are major inhibitors of CMI resulting in chronic mucocutaneous candidiasis (CMC). The mechanism of mannan suppression has not yet been clearly defined, but it is suggested that mannan may act on monocytes or suppressor T lymphocytes. Another mechanism of mannan suppression suggested is that it interfers with cytotoxic activities or lymphocytic-monocyte interaction. A low molecular weight, (10,000 dalton) plasma inhibitor of T-lymphocyte function, suppressing both rosette formation and mitogen responsiveness of T-cells was also isolated from a patient with esophageal candidiasis (67).

Genetics of Candida albicans

It has been difficult to do genetic analysis on C. albicans because it lacks a sexual cycle. However, the analysis of parasexual events and the use of molecular approaches have lead to a better understanding of the genetic

system in C. albicans (79,101,107). Candida albicans and Saccharomyces cervisiae are closely related organisms and as a result, S. cervisiae has been greatly utilized in the isolation of C. albicans genes and development of its DNA transformations. Several C. albicans genes have been successfully cloned into S. cervisiae including genes for actin, tubulin, and genes involved in amino acid biosynthesis or sugar utilization. A C. albicans gene library that represents the whole genome of the organism had been constructed in S. ceverisiae (24).

The analysis of genetics in C. albicans has already been applied to investigate its drug resistance, virulence determinants, and the genetic variation of strains by means of recombinant DNA techniques (34,62). Kirsch et al.(62) recently showed that auxotrophic mutants of C. albicans for adenine, uracil or heme produced by molecular biology techniques had a decreased pathogenicity to mice compared to the wild type strain. Furthermore, by applying recombinant DNA techniques, a species-specific probe for the secretory acid-proteinase gene from C. albicans has been constructed for use in distingushing the organism from other yeasts (34).

Laboratory diagnosis of candidiasis

Laboratory diagnosis of systemic candidiasis is very complex because of difficulties in distinguishing C. albicans normal colonization of the body from invasive candidiasis. Because of this problem, definition of invasive candidiasis

was given to be applied in accurate diagnosis and chemotherapy. In this regard, it is believed that invasive candidiasis is present when Candida organisms are recovered from multiple blood cultures drawn over a period of days, or the organism is demonstrated histologically in tissues (58). This is based on the fact that recovery of an organism from a normally sterile site or from several different body sites is an indicator of disseminated infection.

Direct microscopic examination for fungal elements such as budding yeast, germ tubes, pseudohyphae or true hyphae, is the most rapid and useful of all diagnostic methods available (6,58). However, C. albicans may be identified by its ability to produce germ tubes or chlamydospores (6,90,98). Recovery of the organism from repeated blood cultures and confirmed by carbohydrate utilization tests such as API-20C is a method used to make a final identification of the species (58,87).

As an alternative method, standard serological tests are utilized for the detection of anti Candida antibodies in patients sera. However, the serological diagnosis of disseminated candidiasis is not the best choice because most people have anti Candida antibody due to the fact that C. albicans may be is a part of their normal flora. Furthermore, many patients with C. albicans infections have very low levels of antibody response that result in lower sensitivity of antibody detection. Lack of specificity in distinguishing

between colonization and deep seated infection of C. albicans is another problem encountered in the diagnosis of systemic candidiasis. Because of the insensitivity, tests for the detection of circulating Candida antigens and metabolites have been developed. The Candida Detection System, CAND-TEC from Ramco Laboratories, Inc, Houston, Tex., is a latex agglutination for the detection of circulating Candida antigens (87). Recently, however, the use of selective cytoplasmic antigens of C. albicans, 47K, 46k, or 29k have shown a promising results in detecting antibodies in patients with invasive candidiasis (133).

Antifungal agents

The major classes of antifungal agents commonly used for treatment of mycotic infections including candidiasis are macrolide antibiotics, flucytosine, imidazole and triazole compounds (90,98). Each group of the antifungal agents has shown limitations on the clinical use. Thus, a careful preclinical safety evaluation of a drug must be performed before the drug is recommended for use in patients.

The inhibitory activity of any antifungal agent against test organisms is evaluated in vitro and its value and safety is examined in animal models before it is used for therapy clinically. Measurement of the inhibitory potency of an antifungal agent includes determination of minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) in vitro. In several reports, however, MIC and MFC values for

many antifungal agents have shown significant interlaboratory variations using the same test procedures (15,90,98). The pharmacokinetic properties of an antifungal agent in animal models or *in vitro* inhibitory potency are not always consistent with results shown in clinical cases. In general, most fungal MICs do not correlate with the efficacy of the drug *in vivo*. For example, it was demonstrated that the concentration of amphotericin B which was effective as fungistatic or fungicidal in an animal model was not an effective dose in the treatment of candidiasis in patients (94). The major reasons that may account for the irreproducible results of antifungal agents *in vivo* and *in vitro* could be due to variation in dose of drug, testing method, inoculum size, route of inoculation, timing, or medium composition. Difference in inoculum size, for instance is one of the chief reasons for obtaining inconsistent results. If a high dose of the test organism is inoculated, the host may die before the action of the drug is evaluated. On the other hand, low dose inoculation of the test organism may lead to high survival rate of the host, so that the action of the drug may not be known. To improve the precision and reproducibility of antifungal tests *in vitro*, microtitration well broth assays have been recommended for use rather than tube or agar dilution tests (97).

Polyene macrolide antibiotics are active against a variety of fungi although they show some levels of toxicity

which limits their use in clinical practice. Nystatin, amphotericin B, candicidin, and natamycin are members of the polyene family commonly used in treatment of fungal infections (90,98). Although most Candida species are sensitive to polyene, very few polyene resistant isolates of Candida have been reported, and all of these have low membrane ergosterol content (21). These polyene-resistant Candida isolates were produced either by several passages on media containing increasing concentrations of a polyene or by mutagenic treatments.

Amphotericin B

Amphotericin B (AMB) has been the drug of choice for the treatment of most life threatening systemic fungal infections for more than 30 years, despite the emergence of newer antifungal agents and its toxicity (33,40,90,98).

Amphotericin B has both fungistatic and fungicidal activities against most members of fungal pathogens including candidiasis, cryptococcosis, histoplasmosis, coccidioidomycosis, blastomycosis, paracoccidioidomycosis, sporotrichosis (33,90,98). Amphotericin B inhibits most Candida isolates at a concentration of 3ug/ml in vitro. Although it is a broad-spectrum antibiotic, treatments of many opportunistic infections such as aspergillosis and mucormycoses have shown variable results of resistance and/or sensitivity. Furthermore, members of the organisms that cause chromoblastomycosis, and Pseudoallescheria boydii are often

resistant to AMB (33,90,98). Other forms of fungal infections are also efffectively treated with AMB. Recently, the use of AMB for therapy of fungal cystitis, peritonitis, dermatomycoses and fungal eye infections have been reported (40,90,98).

Amphotericin B is a secondary metabolite produced by Streptomyces nodosus, an actinomycete commonly living in the soil (38). It has a molecular weight of 924.11 that has an amphipathic (hydrophilic and hydrophobic) property. It contains 7 double bonded carbon atoms along the hydrophobic side of the ring, multiple hydroxyl groups along the hydrophilic side, and a mycoamine side chain with a chemical formula $C_{47}H_{73}NO_{17}$ (33,40). Since AMB is insoluble in water, its commercial form, Fungizone, is prepared by mixing deoxycholate, AMB and a buffer. Fungizone, as a deoxycholate micelle, is currently in use for clinical therapy of the life-threatening fungal infections even though it has many serious adverse side effects, including severe nephrotoxicity.

There is no standardized dosage schedule for intravenous AMB administration, although 0.25 mg/kg/day to 1 mg/kg/day of AMB is currently recommended for routine use in therapy. AMB is very poorly adsorbed when given orally. Following intravenous adminstration, however, AMB is initially detected in the liver, spleen, lungs, kidneys, muscle and skin. It has been revealed that peak serum concentration of AMB could reach up to 2 to 3 ug/ml at the end of an infusion and usually

remains above 0.5 ug/ml for 24 hr. Amphotericin B does not penetrate into the CNS effectively, probably due to a combination of its high protein binding activity and high molecular weight. Due to low distribution of AMB in the body, treatment of soft tissue infections are not recommended, but this feature has the advantage of treating infections in well-perfused organs such as the heart and lungs (33,40,81). Amphotericin B exhibits very low systemic clearance, reflecting a degree of stability and the drug persists in the body for several days. For example, its clearance from rabbit lung, skin and muscle occurred in 3 days after a single dose of 1 mg/kg, but persisted for a longer period in other organs (81). Renal levels have persisted for 30 to 60 days in dogs and even 1 year in humans (81).

The mechanism by which AMB causes toxicity to fungal cells has been extensively investigated in many laboratories (9, 12a,33,40, 81,116). The drug binds more strongly to ergosterol of fungal membrane than to cholesterol, present in mammalian membranes. It does not bind either to bacterial cell wall or viral particles. Two kinds of binding forces of AMB to ergosterol or cholesterol have been proposed (12a). The first is the formation of hydrogen bonding (specific forces) between the hydroxyl groups of sterols and the carboxyl group of AMB. The second type is the binding forces of double bonds of AMB and the sterol molecule, which are governed by Van der Waals forces (nonspecific forces). The

function of sterols is to maintain the fluidity of the lipid bilayer of the cell membrane. When AMB binds to ergosterol, the composition of ergosterol is disturbed inducing local changes and as a consequence the membrane is rendered permeable to various compounds such as potassium ions, hemoglobin and small enzymes. The permeability of the plasma membrane to potassium ions leads to the uptake of protons causing acidification of the cell, resulting in its death. In contrast, other researchers suggest that lethality is not a simple consequence of changes in permeability of the cell membranes (12a). For example one hypothesis suggests three events that may happen sequenctially during the interaction of fungal cell and AMB: a) stimulation of fungal cells, b) permeabilization, and c) lethality. The hydrophobic part of AMB would seem to interact with a sterol molecule or with the acyl side chain of a phospholipid present in the membrane. Altered cell membrane lipid composition has been observed in a AMB-resistant mutants of C. albicans with reduced ergosterol content, whereas others indicated that resistant mutants of C. albicans have an increased ergosterol content (9). In addition to the selective toxicity of AMB on ergosterol, it inactivates some membrane enzymes of C. albicans. Surarit and Sheperd (116) have reported the inhibition of ATPase, glucan synthetase, adenyl cyclase, and 5′ nucleotidase enzymes.

There are reports that support the action of AMB as an immunoadjuvant, such as activation of macrophages to kill

bacteria *in vitro* (73), other parasites (113), or tumor cell lines (16) following AMB treatment. Vacchiarelli et al.(128) also reported the correlation of the *in vivo* augmentation of resistance to *C. albicans* infection and the *in vitro* increased killing of *C. albicans* by murine macrophages after AMB administration. There are other reports that indicated an augmentation of the polymorphonuclear leukocyte immunoglobulin IgG-mediated ingestion of opsonized sheep erythrocytes (9). However, this action was inhibited by superoxide dismutase and furthermore, it is evidenced that the active oxygen species and catalase can inhibit the lytic or lethal action of AMB (9).

Although the antifungal property of amphotericin B appears very promising for the development of a chemotherapeutic agent, its high toxicity, insoluble in water and other physical and chemical properties does not encourage direct use. Problems associated with the use of AMB include renal toxicity, sodium depletion, decrease in haemoglobin concentration, and adverse drug reaction. Clinical and laboratory tests have manifested renal tubular acidosis, magnesium and potassium wasting due to membrane permeability (33). Due to the serious parenteral toxicity of AMB, many attempts have been made to overcome these problems by chemical modification of amphotericin B molecular structure. Accordingly, a variety of semisynthetic antifungal AMB derivatives have been produced by N-acylation or

esterification. Amphotericin B ester chloride and N-D-Ornithyl AMB methyl ester significantly reduced toxicity and are more efficacious than Fungizone as reported by Permegiani et.al (93). However, severe neurotoxicity resulted when high doses and prolonged treatment of the derivatives were employed in laboratory animals.

Flucytosine (5-fluorocytosine) is a synthetic antifungal agent which is effective in the treatment of candidiasis and cryptococcosis (15,40). Its mechanism of action is the conversion of the compound to 5-flurouracil by a deaminase enzyme so that 5-fluorouracil substitutes for uracil as a substrate and this interfers with the synthesis of RNA (40). Taking 150 mg/kg/day of flucytosine is recommended for clinical use. Unlike the polyene antifungal agents, flucytosine is not a reproducibly effective inhibitor of Candida species in vitro. Flucytosine has shown different interlaboratory MIC values (94). Furthermore, significant numbers of flucytosine resistant mutants of fungi have been recovered, and as a result flucytosine is most commonly used in combination therapy with AMB. High serum levels of flucytosine have caused depression of bone marrow function and abnormal liver function (15).

Imidazoles and triazoles are synthetic compounds which were used in clinical practice in the early 1970s (5). The mechanism of these compounds is believed to be inhibition of ergosterol synthesis.

Miconazole is one of the first generations of imidazoles (clotrimazole and econazole) more abundantly utilized than any other azoles in the treatment of superficial and systemic forms of candidiasis. Miconazole MICs for Candida isolates have been reported in the range of 0.1 ug/ml to 25 ug/ml in vitro. The recommended dosage schedule for intravenous miconazole is 600 mg every 8 hr for adults and 15-40 mg/kg every 8 hr for children. Adverse reactions of miconazole include haematological disturbances and skin rashes (5).

Ketoconazole is an orally active and a wide-spectrum azole with a longer half-life in serum and is less toxic than micronazole (48). Its various formulations include creams, tablets and suspensions. Wide ranges of C. albicans infections have been successfully treated using ketokonazole. MICs ranging from 1ug/ml to 20 ug/ml of ketoconazole have been suggested (48,90).

Itraconazole is one of the new azoles with the largest and most complex azole that has reached the stage of clinical evaluation. The drug is an orally active triazole with a high tissue affinity and it has a longer pharmacokinetic half-life than ketoconazole as demonstrated in animal models and several clinical trials (127). Most anti Candida MICs reported are less than 100 ug/ml in vitro.

Fluconazole is a new bis-triazole which is active against several mycotic infections including candidiasis. It can be given orally or systemically as evidenced in animal models or

in a few clinical trials. Recently, it was demonstrated that efficacious concentrations of fluconazole in vaginal secretion of patients is achieved by a 150-mg single oral dose of the drug (54). The mean peak concentrations of fluconazole in plasma and vaginal secretions of females after taking the drug were shown to be 2.82 ug/ml and 2.43 ug/g, respectively. Other studies of fluconazole have indicated its good pharmacokinetics, especially in the cerebrospinal fluid with minimal toxicity, and it has a broad-spectrum of antifungal activity (103). A long plasma half-life (25 h) and low protein binding (12%) have been reported (103). At present, the suggested MIC for Candida isolates is less than 100 ug/ml. Several clinical trials are currently undergoing for the use of fluconazole in the treatment of disseminated candidiasis.

Several new antifungal agents such as cilofungin and SCH39304 that may have potent antifungal activity are currently under investigation both in vitro and in animal models (17,116).

Liposomes as drug carriers

Many strategies have been developed to overcome the toxicity of AMB, including the discovery of alternative antifungal agents such as the new triazoles, synthesis of AMB derivatives, synergstic action of AMB and Flucytosine combination, and loading of AMB in liposomes (12a,33). Use of liposomes as drug carriers has been implemented in the treatment of fungal infections and this approach has

apparently led to the decrease of AMB toxicity in animals and even some patients.

The liposome technology was initially used to study ion transports across cell membranes in 1961. Since then it has been evaluated for drug or other delivery systems (92,106). Liposomes are microscopic (250 A to 20 um in diameter) synthetic phospholipid vesicles composed of one or more membranes (92). The amphipathic feature of phospholipids enables the formation of enclosed vesicles due to the association of hydrophobic tails which exclude water and the grouping of hydrophilic heads towards water. Liposomes have been widely used for drug, vaccine, various antigens, and hormones delivery or as emollients in cosmetics (92,106). Liposomes can encapsulate water-soluble drugs in the aqueous portion and lipid-soluble drugs within the membrane itself.

Interaction of liposomes and their contents with host cells is not completely understood. However, four mechanisms of releasing liposome contents into cells have been suggested. These are : a) adsorption, b) endocytosis, c) lipid exchange, or d) fusion (92).

Three classes of liposomes have been introduced depending on their sizes and lamellar nature. The three categories of liposomes are small unilamellar (SUV), large unilamellar (LUV) and multilamellar (MLU) vesicles. Large multilamellar liposomes are very sensitive to osmotic gradients and can swell or shrink very easily when exposure to pressure. Large

multilamellar vesicles are capable of holding up to 4 ul/mg of phospholipid (95). Small unilamellar vesicles (0.2 to 0.6 um in diameter) are usually produced by ultrasonic dispersion, French Press or high pressure homoginization of larger vesicles (95). Small unilamellar vesicles can also be produced by sonication of these large unilamellar vesicles. Small unilamellar vesicles are more stable than the MLUs and have a longer half-life in the blood. Large unilamellar (0.20 to 10 um in diameter), are commonly prepared by reverse-phase evaporation as described by (118) and have the capacity to hold up to 14 ul/mg of lipid (95).

Although intravenous injection is most common, liposomes can be introduced by other routes into to the body including oral or local application of liposomes (92,106). After injection, liposomes are rapidly cleared from the blood predominantly by cells of the reticuloendothelial system. In addition to organs rich in reticuloendothelial cells, liposome-encapsulated drugs have been used efficiently to treat diseases outside reticuloendothelial system. Researchers have shown that radioactively labeled liposomes are accumulated in inflammed or infected sites of patients. For instance, negatively charged liposomes labeled with [^{99m}Tc] pertechnatate injected into mice were detected in large amounts in the liver, spleen, lungs, and kidneys (60). Similar disposition of labeled liposomes in the above organs was observed in cancer patients as well (74).

Although the ultimate fate of liposomes in vivo is not clear, one of the following events may occur. It is proposed that liposomes may break down by exchange of lipids with various serum components, be degraded by phospholipases, taken up by circulating phagocytic cells, or filtered out of the blood through fenestrated capillaries (92).

Cholesterol plays an important role in maintaining the stability of liposomes in vivo. Liposomes tend to leak their contents if cholesterol is not included in the vesicle membrane. Other major factors that influence the features of liposomes include the half-life of liposomes, which increases as the lipid content increases. Furthermore, small liposomes are cleared more slowly than large liposomes and charged liposomal systems are cleared more rapidly than uncharged systems. Liposomes are cleared mainly by the macrophage-phagocytic system; but larger particles may be trapped in capillaries whereas smaller liposomes may even escape engulfment by Kupffers'cells. In the selection of liposomes as drug carriers, several major factors such as size, charge, membrane fluidity, biodegradability should be considered (92,106).

Liposomal encapsulation of drug represents a new drug delivery system that appears to offer important thearapeutic advantages over existing methods of drug delivery. It has been demonstrated that the incorporation of AMB into liposomes can significantly reduce the toxicity of the drug, without the

loss of antifungal activity (59). Gregoriadis et al.(42) first used liposomes to deliver albumin into the liver of a rat. Encapsulation of drugs and liposomal preparation was first described by Bangham et al. (4) as follows: Lipids were dissolved in an organic solvent then the solvents was removed by evaporation leaving the lipids as a film on wall of a glass container. The drug to be encapsulated was added to the flask in an aqueous solution. Large multilamellar liposomes produced by a such procedure have a concentric appearance of a sliced onion seen under electron microscope.

Incorporating a drug simultaneously with liposome formation is the second technique for liposomal drug preparation. In this procedure, the drug to be encapsulated is added to phospholipids with organic solvents (ethanol, ether, methanol) in the presence of an aqueous buffer. The drug trapping liposomes are formed subsequently by the evaporation of the solvent under reduced pressure.

Another technique of drug loading involves the uptake of charged, amphipathic drugs into preformed liposomes due to transmembrane pH gradients. When a cationic drug is involved, low intraliposomal pH results in an extremely high drug:lipid ratio and trapping efficiencies are independent of the lipid composition.

The loading of drugs into liposomes depends on properties of both the drug and the lipids. Hydrophobic drugs are captured in the lipid hydrocarbon, whereas hydrophilic drugs are

trapped into the interior aqueous compartment(s). Incorporating negatively charged lipids (e.g, phosphatidylserine, cardiolipin) in liposomes has improved the affinity of binding the drugs to liposomes and by increasing the drug:lipid ratio and the trapping efficiencies (4,42,89).

Based on the effective treatment of leishmaniasis in mice with liposomal antimonial drugs or liposomal AMB (89), several researchers have investigated the use of liposome-encapsulated antimicrobial agents to treat infected animals (41,75,83,117,121). Liposome-encapsulated drugs have been used in experimental animal models for the treatment of parasitic , bacterial, and fungal diseases. Because of the severe toxicity of AMB, the most advanced application of liposome-based therapy has been in the treatment of systemic fungal infections.

Several investigators have characterized and evaluated the therapeutic effect or the toxicity of liposomal amphotericin B *in vitro* as well as *in vivo* (83,117). They concluded that lipid composition or formulation, liposome size, or liposome storage and other factors can influence the therapeutic effects of liposomal AMB. For instance, SUV liposomes with AMB are less toxic to cells than are the MUV or LUV formulations (117). Juliano et al. (83) have demonstrated that liposomes made of saturated acyl chains loaded with amphotericin B were selectivly toxic to *C. albicans* but not to human erythrocytes. However, amphotericin B containing

liposomes made of unsaturated acyl chains were as toxic as free AMB to erythrocytes.

The use of liposomes as a drug delivery system for AMB significantly improved its therapeutic index in the treatment and prophylaxis of disseminated C. albicans infection in mice (75). The reason liposome-encapsulated AMB raises the therapeutic index is not very clear, but it may be due to increased delivery of the drug to the liver and kidneys (75). Another possible reason could be the agumenting effect of AMB on the immune response, because AMB has been shown to activate macrophages and to increase T and B cells function both of which play a role in resistance to fungal infection. Intercalation of AMB into liposomes for the treatment of murine cryptococcosis (41), histoplasmosis (121) and candidiasis (75) has shown that AMB is less toxic to host cells and provides a higher therapeutic index of liposomal AMB than Fungizone. In all of those studies, liposomal AMB was shown to be as potent as free AMB whereas its toxicity to the host was decreased.

Based on successful results of liposomal amphotericin B (LAMB) tested in animal models (76,84,108,129), preliminary studies on LAMB for the treatment of systemic fungal infections in patients with cancer have been conducted and satisfactory results were obtained. The preliminary results suggest that LAMB has a wider therapeutic index than free amphotericin B (fAMB) and it allows for the use of higher

doses. Lopez et al (76) initiated the implementation of liposomal AMB in patients with fungal infections. Significant clinical improvement was observed in some patients after the use of liposomal AMB in the treatment of cancer patients with fungal infections. In addition, the concentrations of AMB in serum given in LAMB form were greater than concentrations obtained with Fungizone (76,108). There are reports which suggest that the toxicity of AMB to host cells is decreased when the drug is given together with Intralipid formulation (commercially available), cholesterol, lipoproteins, detergents, and lauryl ester of sucrose (12b). Amphotericin B complexed with Intralipid or addition of cholesterol to dispersion of AMB has inhibited the drug toxicity to erythrocytes but not to fungal cells. Gruda et al.(45) showed that lauryl ester of sucrose complexed with AMB was able to bind to ergosterol but not to cholesterol indicating a possible explanation of its reduced toxicity to host cells. Furthermore, other investigators showed that AMB administered together with ester sucrose was much less toxic than given as Fungizone (12b). In a similar study, sonicated unilamellar liposomes made of egg-yolk lecithin, cholesterol and stearylamine with AMB were more effective than Fungizone in the treatment of fungal infections in cancer patients (108).

Although promising results have been obtained from liposomes, there is yet no standardized preparation of LAMB for the therapy of the life-threatening systemic fungal

infections. Despite the failures of therapy, toxicity, relapses, and adverse effects of Fungizone, the commercial form of AMB with deoxycholate and buffer, remains the drug of choice for many systemic fungal infections. In general, the drug delivery by liposomes has many potential advantages over the standard amphotericin B therapy. Therefore properties of both the liposomes and the entrapped drug including the chemical composition, the charge, structure, mode of preparation and *in vivo* effect should be further investigated before widely applied in medicine.

Coupling of antibody to liposomes

The problems encountered in the management of systemic fungal infections in immunocompromised patients and the wide use of liposomes in cancer therapy have both facilitated the development of various methods of targeting liposomes with their contents to specific sites or cells. Other advantages of liposome targeting include, use of liposomes to entrap vaccines for stimulation of the immune system or use of ligands coupled to liposomes in order to target various cells in the body. To enhance the specific delivery of drugs in liposomes to infection sites, research workers have been attaching various molecules including glycoproteins, glycolipids, or antibodies to the surface of liposomes (43). Among the molecules under investigation, antibodies have been commonly used for targeting liposomes because of their high specificity. Several investigators have attempted to increase

the extent of liposome interaction with cell specific antibodies (43,71,123,130). Although numerous ways were previously attempted to attach antibodies to liposomes, antibody-bearing liposomes for drug targeting was first successfully introduced in oncology (130). Using the same principle, Torchlin et al.(123) covalently coupled antimyosin antibodies to activated liposomes. This complex was then used to target the Indium-111 chloride to a dog's heart, but it was not stable. The use of glutaraldehyde or carodiimide for covalently coupling antibodies to liposomes lead to the production of homocoupling between antibodies or between liposomes. However, Leserman et al.(71) introduced a better way of associating of antibodies with liposomes by modifying both the amine end of the antibody and phospholipids used in liposome preparation with the addition of the cross-linking reagent, N-hydroxy succinimidyl -1-3-(2 pyridildithio) propionate (NHSP). The cross-linking reagent, N-hydroxysuccinimide esters of short or long fatty acids for use in the preparation of N-acylamino acids was first introduced by Lapidot et al.(66). In their experiment, N-hydroxy-succinimide (NHSP) esters of fatty acids reacted readily with the sodium salt of free aminoacids in aqueous solutions to form the corresponding N-acylamino acids. Huang et al.(55) developed a method with which monoclonal antibody to mouse histocompatibility antigen, H-^{2k} was derivatized with palmitic acid using an activated ester of N-hydroxysuccinimide.

Furthermore, Huang et al.(56) characterized features of antibody molecules coupled to liposomes in terms of the time course of reaction, the ratio of NHSP to immunoglobulin(IgG) ratio, stoichiometry of coupling, and distribution of palmitic acid in the body. In their study, the optimal molar ratio of NHSP to IgG was 10 and 20, yielding about 4-5 palmityl chains per IgG molecule. However, the coupling was found to decrease the antigen binding capacity to 3-4 fold. Incorporation of the coupled antibody into unilamellar liposomes (1000 A in diameter) was achieved with a deoxycholate-dialysis method with lipid to protein ratio (10:1 w/w). There were about 48 IgG molecules incorporated per liposome. The distribution of antibodies among the liposome was heterogeneous. Binding of antibody to target cells was accompanied by binding of liposomal lipids, where binding was inhibited by pretreatment of cells with unmodified antibodies. In a similar study, liposomes conjugated with protein resulted in the binding of up to 200 microgram of immunoglobulin G per micromole of of lipid (47). In their experiment, anti-human erythrocytes, F(ab) in liposomes (140 molecule/ liposome) enhanced the binding of liposomes to human erythrocytes resulting 80% association of liposome and its contents with cells.

The antigen binding capacity of IgG subunits covalently coupled to liposomes was investigated by Martin et al.(80). At the same time it was discovered that the $F(ab')_2$ portion of an immunoglobulin is responsible for the binding of IgG to

liposomes without losing its activity. Covalent attachment of Fab fragment and phospholipid was achieved via a disulfide interchange reaction between the thiol group exposed on each Fab' fragment and a pyridythio derivative of the phospholipid. In the same study, 6000 F(ab)' molecules were associated to a 0.2ug liposome. Also, the F(ab)' fragments of anti human erythrocytes showed a strong reactivity to human erythrocytes.

The fate of protein containing liposomes injected into rats was first investigated by Gregoriadis et al.(42). In that study, radio-labeled proteins or liposomes were abundantly recovered in the lysosomes and to lesser extent in the spleen within less than 10 minutes after injection. The fate of antibody-bearing liposomes injected intravenously into mice were also examined by Lesserman (130). Antibody-bearing liposomes in mice having affinity for cell surface determinant rapidly disappeared from the plasma, whereas antibodies bearing no cell surface determinants lasted longer in the animal circulation.

At present, it is accepted that antibodies are modified by reacting with palmitic acid, then allowed to react with liposomes prepared by reverse-phase evaporation and the product is stabilized by the removal and dialysis of the destabilizing emulsifier and residual organic solvents as described by Shen et al.(111). Encapsulation of drugs or other molecules in liposomes can as well be performed using freeze and thaw procedure (31). In this procedure, molecules

to be entrapped are added to liposomes, where the mixture is rapidly frozen in a dry ice/acetone bath followed by thawing slowly. Using the same application, investigators have been modifying antibodies for targeting drugs in liposomes to specific cells or cites. Recently, Dromer et al. (25) investigated whether specific targeting of AMB with liposomes bearing anti Cryptococcus neoformans serotype A monoclonal antibody (IgG1) was protective for cryptococcosis in mice. In their studies, mice treated 24 hours after infection with a single injection of AMB (.12 mg/kg of body weight) bearing IgG1 survived longer than mice treated with free AMB or AMB incorporated into nonspecific IgG or control immunoliposomes.

In a similar fashion, Hospenthal et al.(52) have developed amphotericin B liposomes bearing anti candidal antibodies in crude rabbit anserum for use in the treatment of systemic candidiasis in normal mice. Their experimental results showed that, mice treated with a single injection of antibody bearing liposomal amphotericin B (.6 mg AMB/kg) after 2 days of infection survived longer mice treated with the same dose of commercial amphotericin B (Fungizone) or liposomal amphotericin B (51,53).

Cyclophosphamide

Treatment of experimental animals using various immunosuppressive drugs increases susceptibility of the animal to infection. Cyclophosphamide is one of the predisposing factors for infection when administered prior to inoculation

of a host with a pathogen or an opportunistic organism.

Cyclophosphamide (CY), a member of the nitrogen mustard family is a drug toxic to lymphoid tissues or rapidly dividing cells (37). CY was synthesized for the first time in 1958 in an effort to modify the chemical structure of the nitrogen mustard compounds to achieve greater selectivity for neoplastic tissues in cancer therapy (2). It is prepared by treating N, H-bis (2-chloroethyl) phosphoramidicdichloride with an equal amount of 3-amino-1-propanol in the presence of two molar equivalents of triethylamine. It has different trade names in different countries and a correct chemical name,(2-[bis(2-chloroethyl)amino]tetrahydro-2H-1,3,2-oxaza-phosphorine 2-oxide.

The initial success of CY as an antitumor agent in experimental tumor studies led to its common use as an immunosuppressive agent in experimental animals for research purposes (2,37,49). Early experiments in animal models have shown that the lymphoid tissues of thymus and bone marrow (hematopoietic stem cells) are very sensitive to CY treatments. As an alkylating agent, CY has an effect on cell growth, DNA being the main target of action. Nucleic acid synthesis in mice as assessed by 5-iodo-2-deoxyuridine-^{125}I uptake by the thymus and bone marrow cells remained suppressed for 10 days, with recovery following 14 days after treatment (126). In a similar study, DNA synthesis in mice embryo, as measured by ^{14}C-thymidine incorporation was significantly

reduced at 12 h after treatment (112). RNA and protein products as well were reduced as a result of DNA synthesis inhibition. It is concluded that purines are removed from the nucleic acid (depurination) due to alkyation by CY and the chain of the double helix falls apart. Another effect of alkylation by CY is the formation of rigid links between the chains leading to blockage of DNA replication in dividing cells.

Cyclophosphamide does not have alkylating or cytotoxic action *in vitro*. According to Foley (30), CY requires an oxidase-mediated activation in the liver to generate the reactive cytotoxic species, phosphoamide mustard, a bifunctional alkylating agent capable of forming DNA-protein cross-links and DNA instrand cross-links. In another study it was found that CY is metabolized *in vivo* by liver microsomal enzymes into an active metabolite that has alkylating and cytotoxic action (13). Furthermore, the metabolic pathway of CY leading to the formation of alkylating metabolites aldophosphamide and its product carboxyphosphamide was proposed by Hill (50).

Cyclophosphamide injected into mice was rapidly metabolized with a rate based on the amount of dose injected versus the cytostatic activity demonstrable in the serum (2). Its half-life in mouse serum was about 20 minutes and only 5% of 100 mg/kg dose was extracted unchanged in a 24 hour period. Using whole-body autoradiography technique, cyclophosphamide

was found accumulated within 30 minutes of intraperitoneal injection in the liver, gall bladder, small intestine and kidneys of mice with later accumulation in the thymus, spleen and testes (2).

Several experiments have been reported on the efforts made to enhance susceptibility to bacterial, mycotic, viral, or parasitic infections by pretreatment of animal models with CY (1,20,26,27,57,65,86,119). Alder, et al.(1) made mice susceptible to infection with Leptospira interrogans serovar pomona by treatment with a single dose of 300 mg/kg Cy administered from 4 days before to 1 day after infection. Epstein et al.(27) developed a canine model using CY injection to facilitate pseudomonas septicemia in the host, where all the neutropenic dogs died after challenge with the bacterium. Multiplication of Histoplasma capsulatum in neutropenic mice was conducted by Cozad et al.(20) where the number of H. capsulatum cells in specific organs after CY treatment markedly increased as compared to control animals, however, the number greatly decreased when assessed 15 days after CY administration. Likewise, severe candidiasis was established in mice pretreated with CY before challenge with Candida albicans (86). Hurd et al.(57) were able to demonstrate the conversion of a relatively harmless Kunz infleunza virus into a fatal pneumonic illness in mice pretreated with a dose of 300 mg/kg CY. The growth of Rickettsia sennetsu was significantly enhanced in mice injected with CY (119). Dogs

made neutropenic by injection with cyclophosphamide developed disseminated candidiasis following Candida albicans challenge (26). In the neutropenic dogs, granulocyte counts were markedly reduced compared to normal control dogs. In the same investigation, gross pathology was demonstrated in the immune system organs of the drug treated animals. A model of baby guinea pigs with a significant lower number of polymorphonuclear cells and lymphocytes was produced by utilizing a 20 mg/kg of CY given intraperitonealy daily for 5 days (65).

From previous experimental studies, it has been concluded that the interval of time between CY administration and the amounts are important in obtaining maximum effectiveness to encourage infections.

CY is a potent immunosuppressive drug under certain conditions, but it can also potentiate immune response to a certain extent. It appears to be cytotoxic to rapidly dividing cells whether T or B lymphocytes, and thus results in the suppression of both cell-mediated and humoral immunity (3,7a,7b,70,85,100,114,125a,125b). Both delayed hypersensitivity and antibody production are defective immediately after treatment. In previous studies, pigs or mice given in a single dose of 250-300 mg CY per kilogram intraperitoneally 3 days before immunization caused a marked augmentation of certain types of delayed hypersensitivity to methylated human serum or sheep erythrocytes or 2,4

dinitroflourobenzene as antigens (125a,125b). However, delayed hypersensitivity response was depressed when CY was given 3 days after contact with 2,4 dinitrofluorobenzene (125b). It was also found that agumentation of delayed type hypersensitivity is not the same to all antigens. Berd, et al. (7a,7b) showed that administration of CY (1000 mg/sqm) to cancer patients 3 days before sensitiztion agumented development of delayed type hypersensitivity to primary antigens called keyhole limpet hemocyanin(KLH), or 1-chloro-2,4 dinitrobenzene (DNCB). However, there was no antibody response to either antigen in the sera of the patients. The same investigators pretreated other groups of cancer patients with low-dose of CY (300 mg/sqm) and found that there was significant agumentation of delayed type hypersensitivity to the same antigens (7b). Also pretreatment of patients with 300 mg/sqm indicated antibody titers in the patients sera against KLH and DNCB as the primary antigens . The investigators suggested that delayed hypersensitivity is enhanced because cyclophosphamide damages suppressive T cells in the immune regulatory system. Their conclusion was supported by Mitsuoka et al. (85) who observed the depletion of specifically suppressor T cells followed with enhanced delayed hypersensitivity after CY pretreatment of mice. In the same study, other T cell subpopulations were also damaged, however, recovered within a short period from CY-induced damage compared to T suppressor cells.

Rollinghoff et al.(100) suggested that suppressor T lymphocytes are cyclophosphamide-sensitive cells by observing the lack of the *in vivo* generation of antigen-specific cytotoxic T-lymphocytes in mice after CY treatment. The major role of suppressor cells is to enhance the differentiation of cytotoxic T cells which are effective in cell killing activity.

In a study of differential effect of cyclophosphamide on immune response (3), it was indicated that the action of CY on B cells was more severe and longer-lasting than the effect on the T cells of mice. Histologically, B cell areas were severely depleted than were to T cell areas after CY treatment. In a different study (70) CY severely suppressed antibody formation and immunoglobulin synthesis in adolescent chickens. A marked impairment of B lymphocytes ability to regenerate surface immunoglobulins occurred after CY treatment in mice (114). The observations from several and similar studies lead to the conclusion that CY indiscriminately interferes with enhancing antibody production. In contrast, it has been reported that there are at least 2 populations of B cells. The B cells that are susceptible to CY and the long-lived B cells that are not affected by CY treatment (110). In their studies high antibody titers in CY-treated guinea pig sera were recovered, thus suggesting the long-lived B cell precursors are the producers of the antibodies.

Initial reports indicated that CY selectively depleted non-thymus dependent areas of lymphoid tissues, suggesting depression of antibody production but not T cell-mediated immunity. However, more recent information by many researchers has shown that any rapidly dividing cell is affected by cyclophosphamide.

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ARTICLE I

EVALUATION OF ANTIBODY-BEARING LIPOSOMAL AMPHOTERICIN B IN THE TREATMENT OF SYSTEMIC CANDIDIASIS IN A NEUTROPENIC MURINE MODEL

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ABSTRACT

Efficacy of liposomal amphotericin B with anticandidal antibodies attached on its surface (LAMB-Ab) was investigated in the treatment of experimental systemic candidiasis in a neutropenic mouse model. Neutropenic condition in this mouse model was produced by an intraperitoneal injection of cyclophosphamide at a dose of 150 mg/kg 2 days prior to infection with Candida albicans. Neutropenic induction in the model was manifested by the transient reduction in number of leukocytes following cyclophosphamide administration. A marked decrease in the number of total and differential cell counts of mice occurred shortly after cyclophosphamide injection (1 to 2 days) followed by a gradual increase in a 15 days period. An increased susceptibility of the neutropenic mice to C. albicans infection was demonstrated to occur 2 days after cyclophosphamide administration.

Treatment with a single dose (0.6 mg amphotericin B per kg body weight) of LAMB-Ab resulted in a significant increase in the survival rate of neutropenic mice infected with 3×10^5 cfu of C. albicans compared to controls. In

addition, survival was better in neutropenic mice treated with LAMB-Ab than in neutropenic mice treated with the same dose of liposomal amphotericin B (LAMB) or free amphotericin B (fAMB). Results of this study suggest that LAMB-Ab is more effective than LAMB or fAMB in the therapy of disseminated candidiasis in neutropenic mice.

INTRODUCTION

Disseminated candidiasis produced by Candida albicans is one of the major causes of morbidity and mortality in the neutropenic patients (1-4,13,14). The incidence of this disease has increased due to the frequent use of intravascular catheters, immunosuppressive therapies, broad-spectrum antibiotics therapy, and due to effects of other predisposing factors (1,2,14).

Amphotericin B (AMB) has been shown to be the most effective drug for the treatment of disseminated fungal infections including candidiasis, but its use is limited by its toxicity to mammalian cells (2,12). Recently, researchers have explored the use of liposome-encapsulated AMB in the treatment of fungal infections in humans as well as in experimental animal models (12,15,16,19). As a result of this approach, AMB toxicity to the host has been minimized, and its therapeutic index improved without loss of activity against most systemic fungal infections. Incorporation of antibodies to the surfaces of liposomes in an attempt to enhance delivery of drugs to specific cells or antigens has been previously examined (11,17). Based on this application, AMB encapsulated in liposomes with

antibodies from rabbit antiserum against C. albicans attached to their surfaces have been developed in our laboratory (8). In recent studies (7,9), treatment of systemic candidiasis in a non-neutropenic mice he liposomal AMB bearing antibodies was performed. Non-neutropenic mice with systemic candidiasis that were treated with antibody bearing liposomal amphotericin B showed an increased survival rate compared to free amphotericin B (Fungizone) or other liposomal preparations containing amphotericin B.

Because neutropenia is a common predisposing factor leading to many opportunistic mycoses, development of a neutropenic murine model using cyclophosphamide as an immunosuppressive agent was undertaken. Induction of neutropenia was evaluated by enumeration of leukocytes. After establishing a fatal systemic C. albicans infection, the model was utilized in evaluating the efficacy of antibody bearing liposomal amphotericin B in the therapy of experimentally induced systemic candidiasis.

MATERIALS AND METHODS

Animals: White Swiss CD1 female mice (4 weeks old) each weighing 19 to 22 grams purchased from Charles River Laboratories Inc. Portage, MI. were used in the study. Mice were housed five animals per cage and allowed free access to food and water in accordance with animal care regulations of University Laboratory Animal Resources at Michigan State University.

Drugs, lipids, and antisera: Dimyristoyl phosphatidylcholine (DMPC), dimyristoyl phosphatidylglycerol (DMPG), and cyclophosphamide were purchased from Sigma Chemical Co. St. Louis, Mo. Amphotericin B, without emulsifier for use in liposomal amphotericin B preparation, and the commercial form, Fungizone was provided by Squibb Pharmaceutical, New Brunswick, N.J. Adsorbed antiserum to C. albicans produced in rabbits was obtained from Difco, Detroit, MI.

Cyclophosphamide stock solution: The stock solution of cyclophosphamide (CY) was prepared by injecting 25 ml of sterile saline (0.9%) into a vial containing 1 gram of CY. The solution was stored at 4°C for use within 3 months after reconstitution.

Neutropenia induction: Three treatment groups each consisting of 5 mice were injected intraperitoneally with

100, 150, or 200 mg CY/kg of body weight. Each dose was given in a 0.2 ml aliquot of CY solution. Mice injected with 0.2 ml sterile saline were used as controls. Five mice were bled for total and differential cell counts on days 0, 1, 2, 7, and 15 after CY injection. Blood samples were obtained from intracardial puncture of mice anesthesized with Metofane (Pitman-Moore, Inc. Washington Crossing, NJ). Blood was collected via tuberculin syringes with 30 G needles and then transferred into 5 ml Microtainer test tubes containing EDTA (Becton, Dickinson Company, Rutherford, NJ). Total and differential leukocyte counts were performed by the Clinical Pathology Laboratory, Michigan State University within 1 hour of sample collection using the Technicon H1 cytogram system (Technicon Instruments Corporation, Tarrytown, NY).

Inoculum preparation: A clinical isolate of Candida albicans, strain AK 785, initially recovered from a patient with evidence of mucocutaneous candidiasis was donated by Michael Kennedy (The Upjohn Co., Kalamazoo, MI.) for animal inoculation in the study. To prepare for animal inoculation, the stock culture maintained at -20°C was subcultured on Sabouraud glucose agar and incubated overnight at 37°C. An inoculum from the slant was transferred into 100 ml of tryptic soy broth containing 4% glucose and incubated for 12 to 16 hr at 37°C on a rotary shaker. Yeast cells were harvested by centrifugation

(2,000 x g for 10 minutes) and washed 3 times in phosphate-buffered saline (PBS). Yeast cells for mouse inoculations were counted in a hemocytometer and adjusted to the desired concentration with PBS. Viability and the number of cells was checked by plating the suspension for inoculation on Sabouraud glucose agar with 10 to 100-fold serial dilutions.

Experimental infection: In a preliminary experiment, three groups of mice, each consisting of 10 animals, received 100, 150, or 200 mg/kg of CY and were then challenged with 3 X 10^5 colony forming units (cfu) of C. albicans, strain AK 785. Mice that received no CY but inoculated with the same dose of the strain were used as controls. The survival rate of the animals was followed for a period of 21 days. This experiment was carried out to determine the optimal concentration to be used in establishing neutropenia and causing 100% mortality of the mice from infection, but allowing a therapeutic window for providing antifungal therapy.

Other groups of mice consisting of 10 animals in each group that received 150 mg/kg of CY were challenged in the same manner after 1, 2, 7, or 15 days of CY administration. Survival rate was followed for 21 days. This experiment was set up to examine the effect of CY through time on the susceptibility of the mice for C. albicans infection as well as to observe whether there was a correlation between

reduction in cell counts and susceptibility to infection.

Palmitoyl antibody preparation: Adsorbed rabbit antiserum to C. albicans was reacted with N-hydroxysuccinimide ester of palmitic acid (NHSP) to produce palmitoyl antibody and attached to liposomal amphotericin B for use in the treatment studies of systemic candidiasis (8). In this preparation, 0.5 ml of radio-labeled NHSP, 5 ml of the crude rabbit antiserum, and 5 ml PBS with 1% deoxycholate (DOC) was mixed and incubated overnight at 37°C. After centrifugation at 3400 X g for 15 minutes, the supernatant was carefully loaded into a Sephadex G-75 column (1.9 X 20 cm) equilibrated with PBS. Palmitoyl antibody was eluted in the void volume and the product was concentrated by using ultrafiltration (Amicon, XM-50). The radioactivity associated with the product and protein content were measured by using the Tracerlab 4 pie Scanner and the Lowry method, respectively. Antibody activity of both palmitoyl antibody and crude antiserum was checked by using the slide agglutination test against the C. albicans isolate used in the study. The final protein concentration was about 1.6 ug/ml. Modified antibody was stored in aliquots of 1 ml at -20°C for further use.

Antibody-bearing liposome preparation: Liposomes containing AMB were prepared by reverse-phase evaporation as previously described (8). Briefly, amphotericin B dissolved in methanol was mixed with chloroform-based

solutions of the phospholipids (DMPC/DMPG ratio, 7:3) and 1 ml PBS. Organic solvents were evaporated under nitrogen and heat (45°C) until the solution was reduced to approximately one ml. The suspension was then sonicated for 2 seconds at 1000 W and incubated for 1 hour at room temperature. One ml of modified antibody with 0.15% deoxycholate (DOC), prepared by covalent binding of palmitic residues to immunoglobulins was added to the reaction mixture and left at room temperature for 2 hours. The resulting solution was dialyzed (using spectapor 2 dialysis tubing) overnight at 4°C with 3 changes of 500 ml PBS. The product was recovered by centrifugation (30,000 x g, 15 minutes, 4°C). Liposomal AMB without modified antibodies was prepared using the same procedures. Dosages of liposomal compounds were determined by AMB content via spectrophotometry at 405 nm. Fresh liposomes were prepared in every treatment study.

Treatment studies: The survival of neutropenic mice with systemic candidiasis was examined after treatment with liposomal or free amphotericin B (fAMB/Fungizone). Neutropenic mice were produced by a single intraperitoneal injection of cyclophosphamide (150 mg CY/kg of body weight). Two days following administration of CY, mice were infected intravenously with 3 x 10^5 cfu of Candida albicans, strain AK 785. Each mouse then was treated with a single dosage of 0.6 mg AMB/kg in 0.2 ml aliquot of LAMB-Ab,

LAMB, or fAMB on day 2 following infection. For comparison, groups of normal mice (non-neutropenic) were also infected and treated with either LAMB or fAMB at identical dosages. Animals infected but not treated with any of the AMB preparations were used as controls. Each treatment group consisted of 8 to 10 mice. Survival rate was recorded every 24 hours. Animals were evaluated up to 21 days after infection.

Statistical analysis: The significance of differences in cell counts between the different treatment groups were analyzed by using ANOVA and a multiple comparison of variance, least significant difference (LSD) test. Survival distribution differences were evaluated by using a generalized Wilcoxon test (6).

RESULTS

Effect of Cyclophosphamide on cell counts of in mice

The total and differential cell counts of mice injected with different doses of CY are described. Before CY treatment, the mean leukocyte counts of all treatment groups ranged from 6.23 to 6.6 x 10^3/ul with no significant difference between groups. Twenty four hours after CY treatment (Figure 1), significant total cell count differences were obtained between the treatment groups and controls ($P < 0.05$). One day after the drug treatment leukocyte counts were reduced up to 32% in the 100 mg/kg of CY (low dose) receiving group and 60% in both 150 (medium dose) and 200 mg/kg of CY (high dose) receiving groups. The minimum levels of mean total leukocyte counts, 1.53 x 10^3/ul and 2.19 x 10^3/ul in the medium and high dose receiving groups respectively, were noted on day 2 after CY administration. The extent of recovery of total leukocyte counts in mice also appeared proportional to the dose of CY administered. After 15 days of CY treatment, total leukocyte counts in mice treated with 100 mg/kg of CY recovered to the levels of the baseline. Mice that received 150 mg/kg of CY exhibited less elevation in cell counts 15 days after the drug treatment compared to the group

receiving the low dose and controls. Moreover, the 200 mg/kg group remained significantly below the 100 mg/kg group or the control group on day 15 after CY administration ($P < 0.05$).

A decrease in neutrophil cell count was seen as early as 1 day after CY treatment (Figure 2). The mean neutrophil counts in all treatment groups were significantly lower than that of control animals by days 1 and 2 after the drug administration ($P < 0.05$). Treatment with 200 mg/kg or 150 mg/kg CY resulted in significant reduction in neutrophil counts compared to the treatment with 100 mg/kg of CY on days 1, 2, 7 ($P < 0.05$).

In all animals, an initial decrease followed by an increase in neutrophil count was observed. By day 7 after drug treatment, a slight elevation in neutrophil counts was seen followed by regaining the normal values on day 15 after CY treatment. Mice treated with sterile saline showed no significant change in cell counts throughout the study. Although there was a linear decrease in the first two days after CY treatment, it was accompanied by a pronounced rise, both in total leukocyte and neutrophil counts.

The effect of CY on a variety of cell types other than neutrophils was also examined. A similar pattern of reduction and increase in cell counts observed in total leukocyte and neutrophils was obtained for lymphocytes, monocytes, basophils, and eosinophils. However, no

significant change in red blood cells and platelets counts was observed.

In normal (nontreated animals), the lymphocyte counts remained approximately the same throughout the 15 days of the experiment. On the other hand, a linear decrease in cell counts was observed during the course of the first 2 days in all treatment groups (Figure 3). A statistically significant difference in the means of lymphocytes of the treatment groups and controls was indicated on days 1, 2, 7 and 15 after drug administration ($P < 0.05$).

An increase in lymphocyte count was observed in both 100 and 150-mg/kg CY animals on day 7, but not in the 200 mg/kg of CY animals. On day 15, a marked increase of lymphocyte counts was obtained, reaching pretreatment values in 100, 150, and 200 mg/kg of CY groups. Using analysis of variance, it was found that there was a significant difference in the means of lymphocytes of high dose animals compared to controls ($P < 0.05$). A slight difference in the means of lymphocyte between the other treatment groups at 15 days after drug injection was also observed ($P < 0.05$).

As shown in Figure 4, during the first 2 days after drug administration varible monocyte counts were recorded in animals receiving the drug ($p < 0.05$). On day 1 after the drug treatment, the highest decrease in monocyte counts was observed, especially in animals receiving 100 and 150 mg/kg of CY. Approximately 30% increase of monocyte counts was

recorded in 100 and 150 mg/kg of CY receiving animals 2 days after drug administration. On day 7, all animals receiving drugs showed a linear increase in monocyte counts and furthermore, animal receiving high and medium doses had values higher than the pretreatment counts on day 15. Unlike other cell types, a dose-response trend was not observed regarding monocyte counts unlike other cell types.

Basophil counts of untreated animals were variable during the course of first 2 days although no statistically significant difference was obtained. Treatment groups had a highly decreased basophil count on day 1 after CY administration and remained decreased for the next 24 hours except for animals receiving high dose (Figure 5). Even though there was an increase in animals receiving the medium dose on day 7 and other groups on day 15, basophil counts remained below 40% of the pretreatment values regardless of the dosages used in the study. A statistically significant difference in means of basophil counts between treatment groups and the controls was observed 15 days after drug treatment ($P < 0.05$). However, comparison of the mean basophil counts of the 3 treatment groups on day 15 showed no significant difference.

As illustrated in Figure 6, animals that received 100 mg/kg of CY showed a drop of 24% in eosinophils, whereas animals receiving 150 or 200 mg/kg of CY had more than 90% reduction in eosinophils on day 2 after drug administration.

The reduction was statistically significant between the treatment groups ($P < 0.05$). Further reduction was observed on day 2 after drug administration except in animals receiving 150 mg/kg of CY. Despite a marked reduction seen in eosinophil counts in the course of 7 days, pretreatment values were regained by day 15 after drug administration in aminals receiving low and medium dose, whereas the high dose treated groups had greater than the pretreatment values. Comparison of the means of eosinophil counts of the animals receiving different doses using analysis of variance showed that there was significant difference in counts between animal groups on 1, 2, and 7 days after drug administration ($P < 0.05$). Moreover, on day 15, the animals receiving the high dose showed significant difference from controls and other groups ($P < 0.05$).

Cyclophosphamide administration did not significantly affect red blood cells (RBC) counts in treatment groups and control animals during the course of study. A dose-response effect was not observed regarding the counts of RBC as shown in Figure 7. The mean of RBC of the groups that received the lowest dose had the highest decline on day 7 after drug treatment. Mice that received 150 mg/kg of CY also had a drop in counts that was followed by an increase in counts approaching the pretreatment values.

As shown in Figure 8, there was significant decrease in platelet counts for animals receiving the low dose on day 2.

After receiving the drug there was a rebound on day 7. A slight deviation in animals receiving 150 mg/kg of the drug was observed on day 1 and 2 that was follwed by a slight increase in counts. The animals receiving 200 mg/kg of CY had a linear decrease of platelet counts during the first 2 days followed by an increase on day 7 remaining above the pretreatment value there after. Similar to RBC counts, a dose response effect was not observed in the case of platelets.

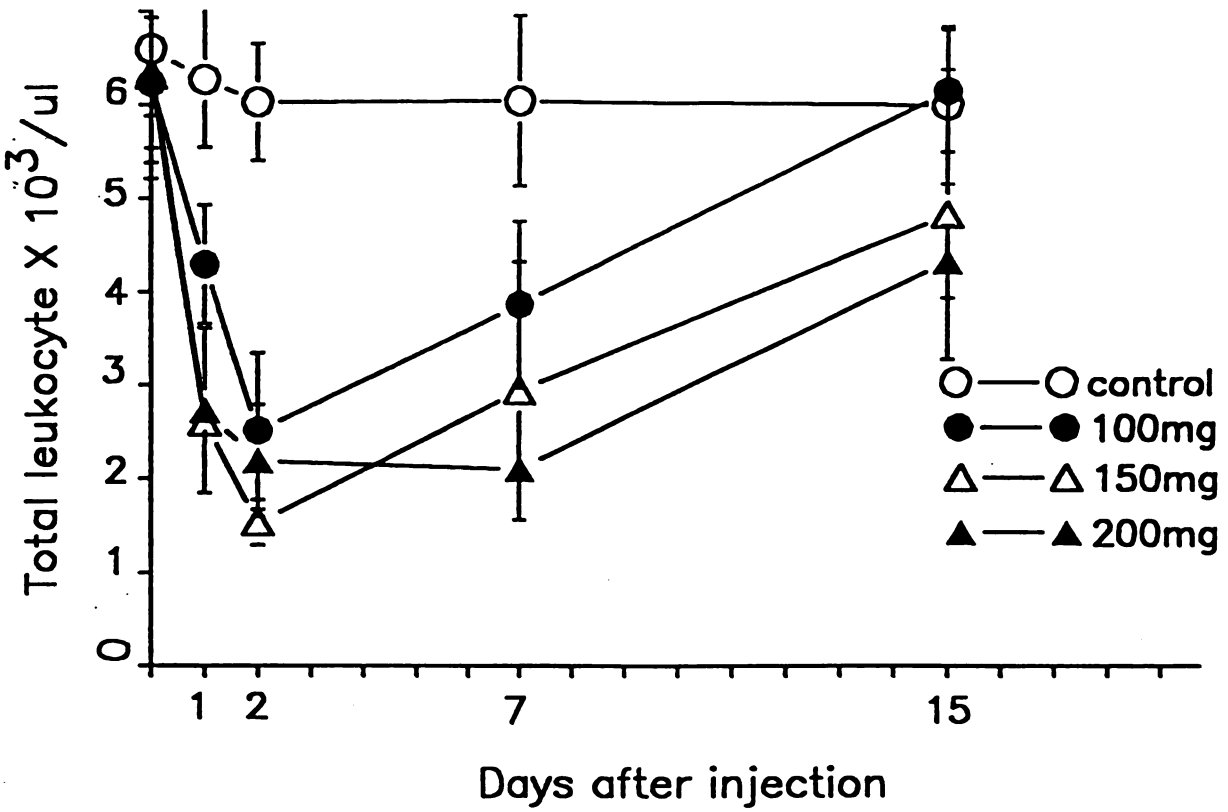

Figure 1: Changes in total leukocyte counts of mice following single intraperitoneal injections with cyclophosphamide (CY). Animals received a dose of 100, 150, or 200 milligram of CY per kilogram in a 0.2 milliliter volume. Controls were injected with the same volume of sterile saline. Blood sample for cell counts was collected on 0, 1, 2, 7, 15 days after CY treatment. Each point represents a mean cell count of 5 mice.

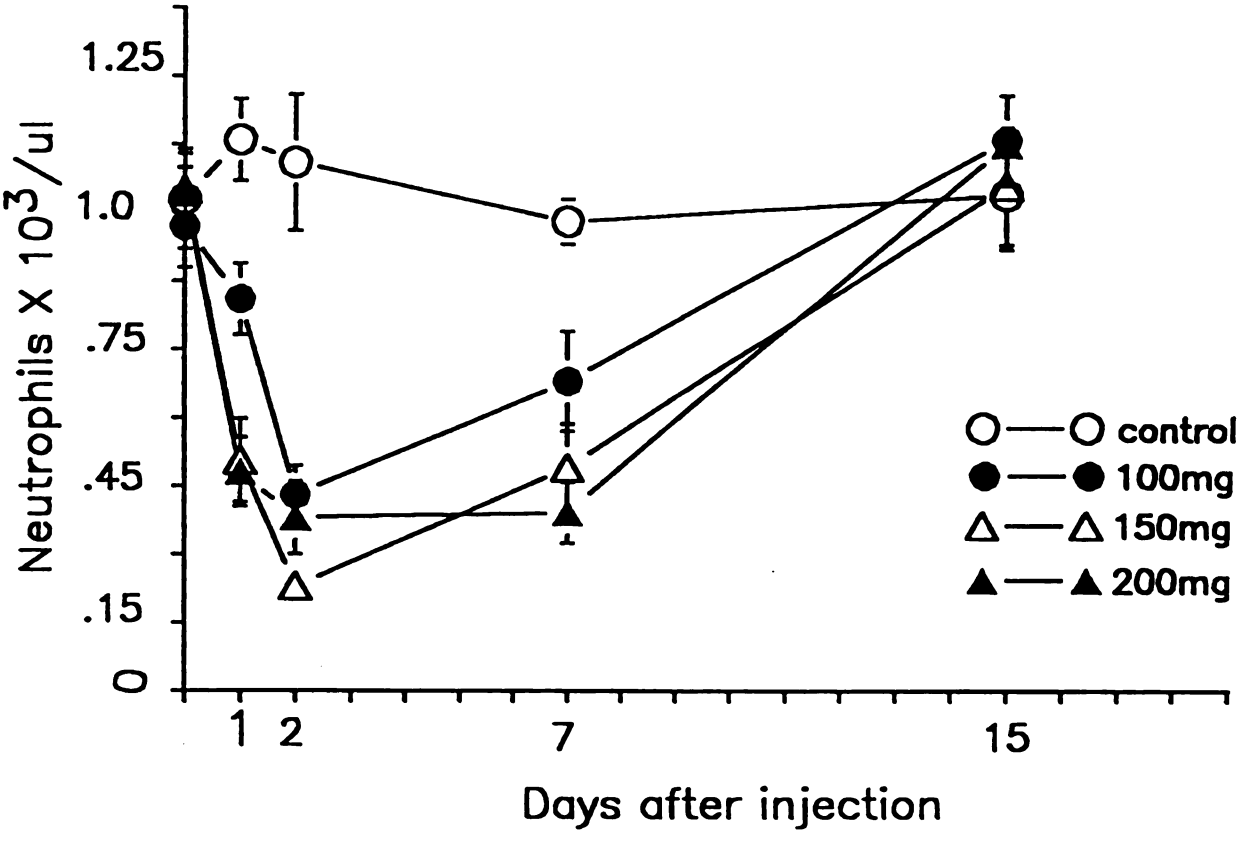

Figure 2: Changes in neutrophil counts of mice following single intraperitoneal injections with cyclophosphamide (CY). Animals received a dose of 100, 150, or 200 milligrams of CY per kilogram in a 0.2 milliliter volume. Animals were bled on 0, 1, 2, 7,and 15 days after drug administration for cell counts. Controls received the same volume of sterile saline. Each point represents a mean cell count of 5 mice.

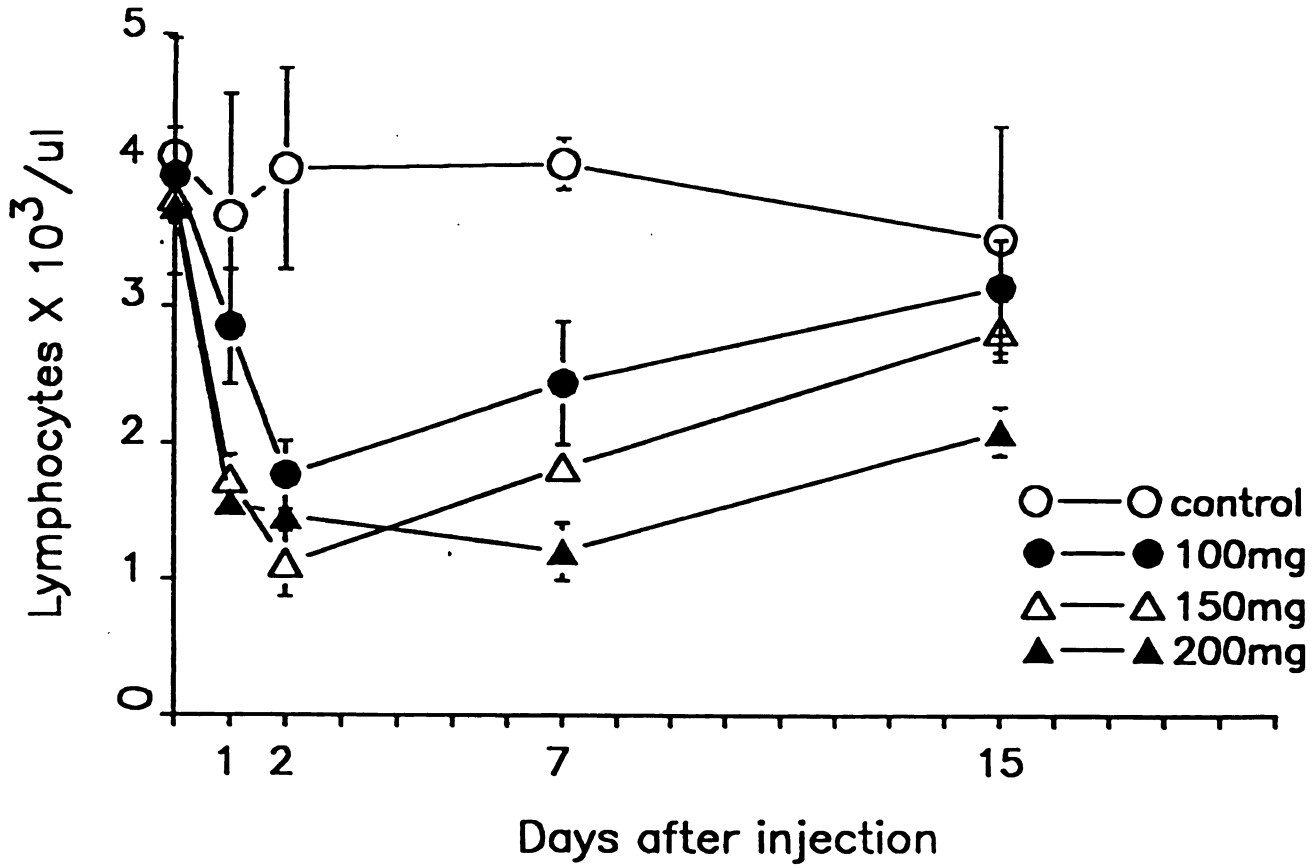

Figure 3: Changes in lymphocyte counts of mice following single intraperitoneal injections with cyclophosphamide (CY). Animals received a dose of 100, 150, or 200 milligrams of CY per kilogram in a 0.2 milliliter volume. Animals were bled on 0, 1, 2, 7,and 15 days after drug administration for cell counts. Controls received the same volume of sterile saline. Each point represents a mean cell count of 5 mice.

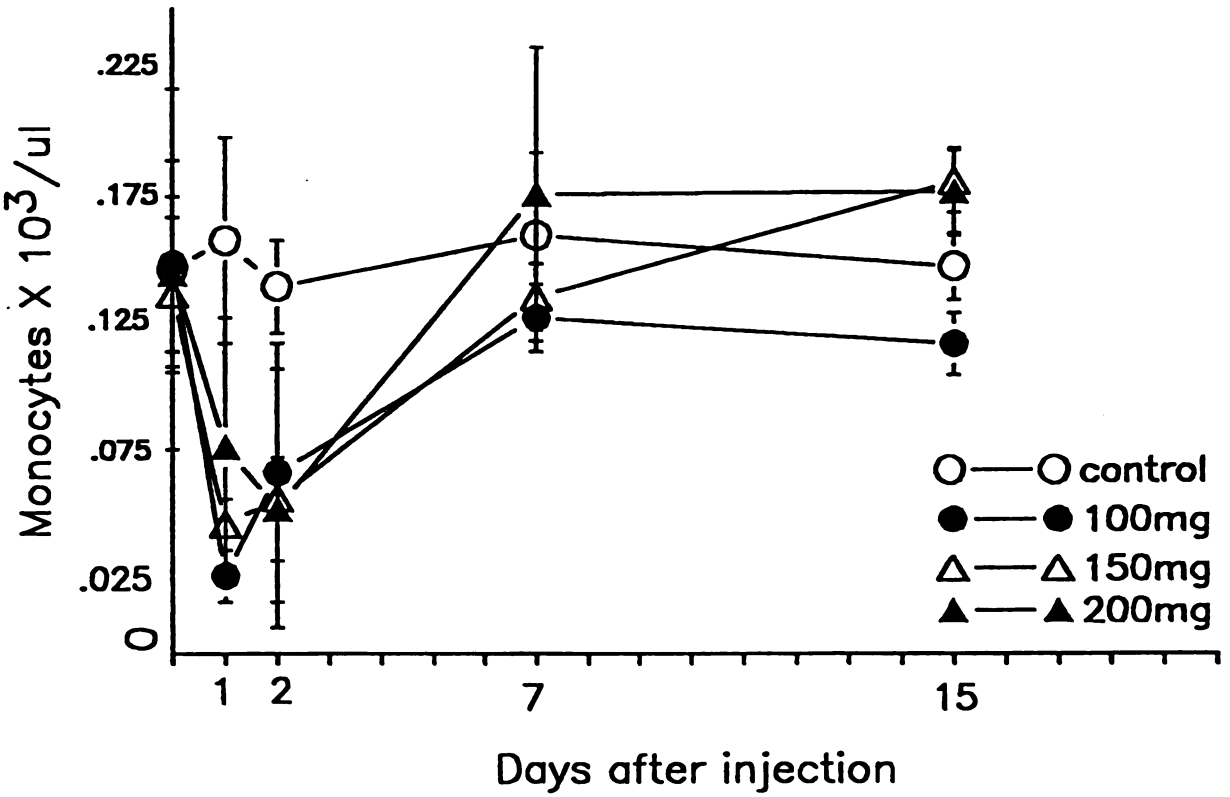

Figure 4: Changes in monocyte counts of mice following single intraperitoneal injections with cyclophosphamide (CY). Animals received a dose of 100, 150, or 200 milligrams of CY per kilogram in a 0.2 milliliter volume. Animals were bled on 0, 1, 2, 7,and 15 days after drug administration for cell counts. Controls received the same volume of sterile saline. Each point represents a mean cell count of 5 mice.

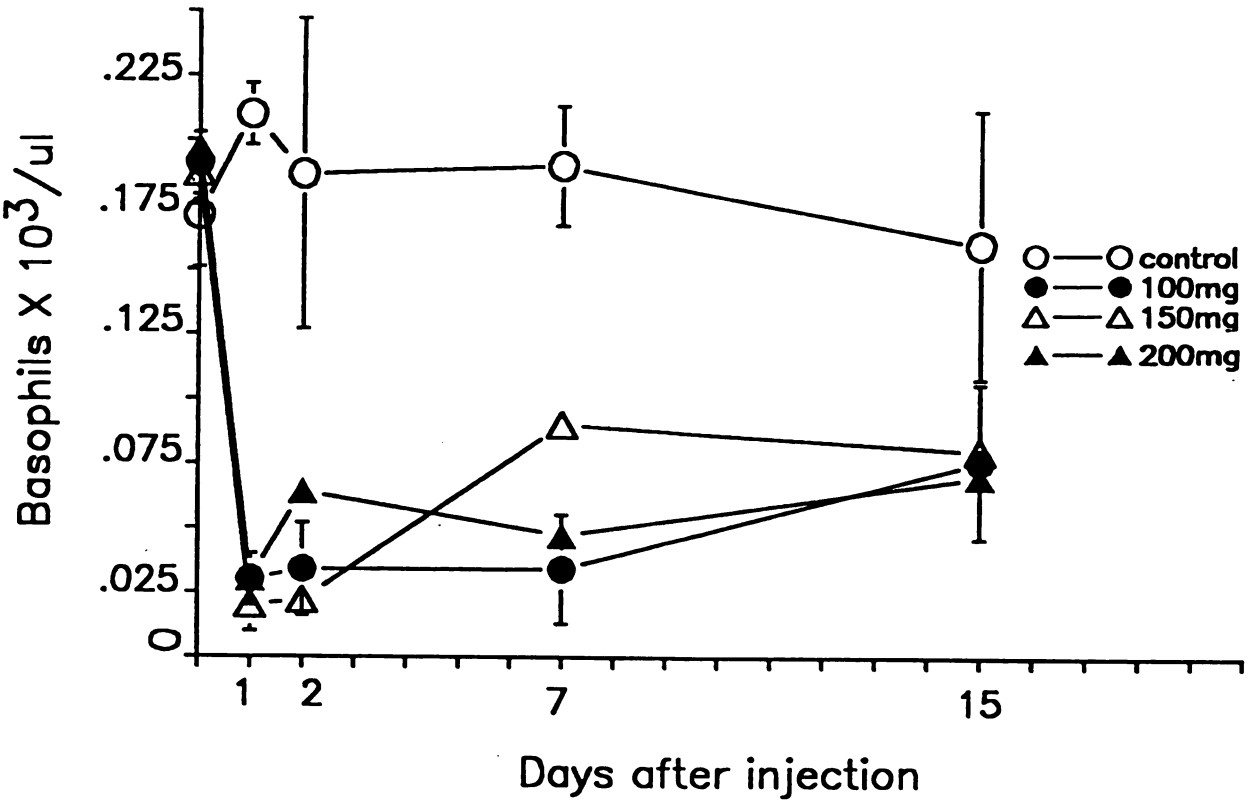

Figure 5: Changes in basophil counts of mice following single intraperitoneal injections with cyclophosphamide (CY). Animals received a dose of 100, 150, or 200 milligrams of CY per kilogram in a 0.2 milliliter volume. Animals were bled on 0, 1, 2, 7,and 15 days after drug administration for cell counts. Controls received the same volume of sterile saline. Each point represents a mean cell count of 5 mice.

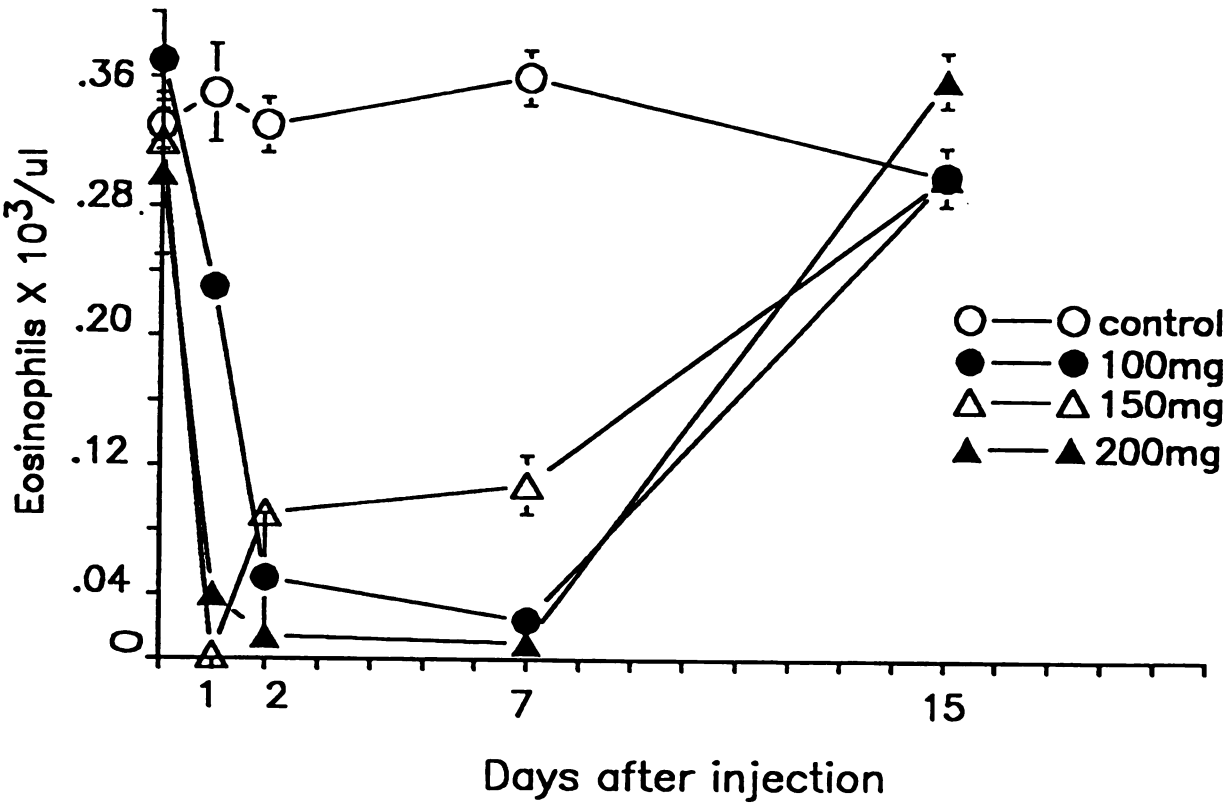

Figure 6: Changes in eosinophil counts of mice following single intraperitoneal injections with cyclophosphamide (CY). Animals received a dose of 100, 150, or 200 milligrams of CY per kilogram in a 0.2 milliliter volume. Animals were bled on 0, 1, 2, 7,and 15 days after drug administration for cell counts. Controls received the same volume of sterile saline. Each point represents a mean cell count of 5 mice.

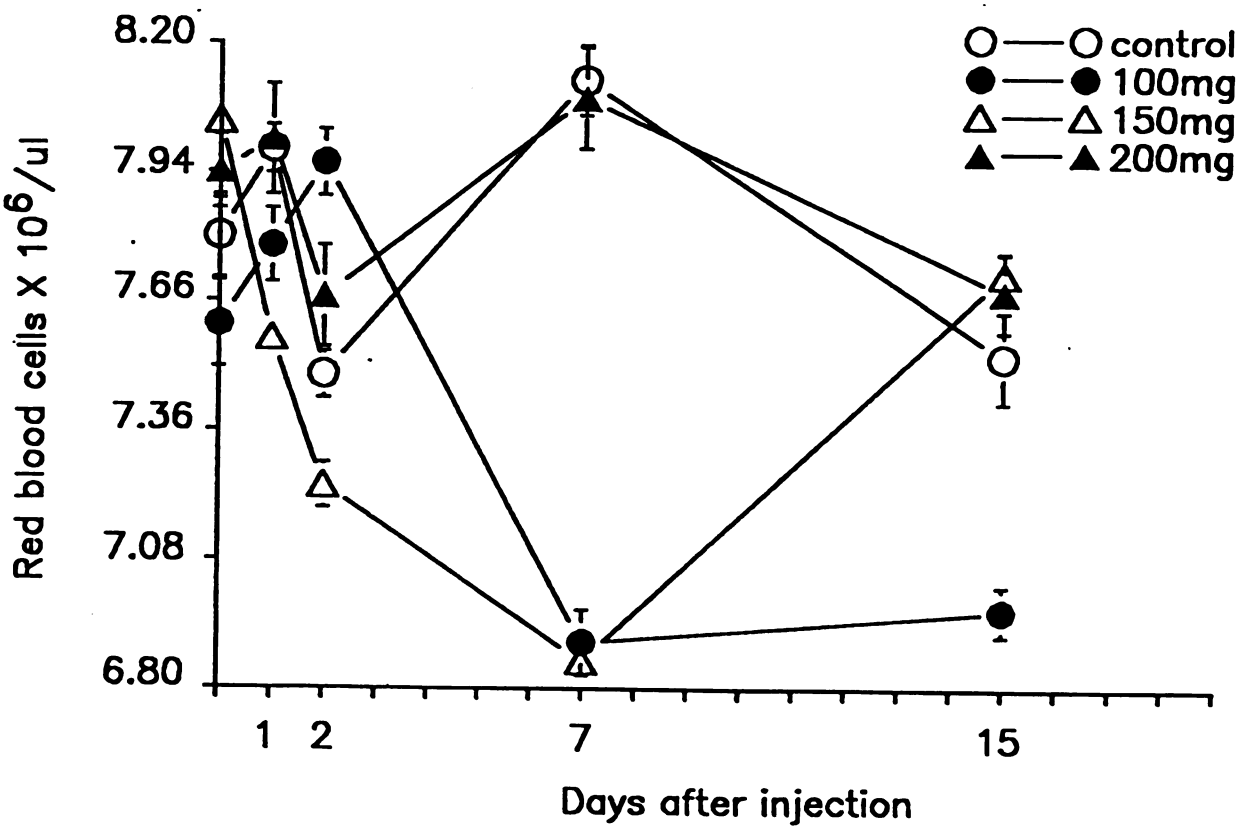

Figure 7: Changes in red blood cell counts of mice following single intraperitoneal injections with cyclophosphamide (CY). Animals received a dose of 100, 150, or 200 milligrams of CY per kilogram in a 0.2 milliliter volume. Animals were bled on 0, 1, 2, 7,and 15 days after drug administration for cell counts. Controls received the same volume of sterile saline. Each point represents a mean cell count of 5 mice.

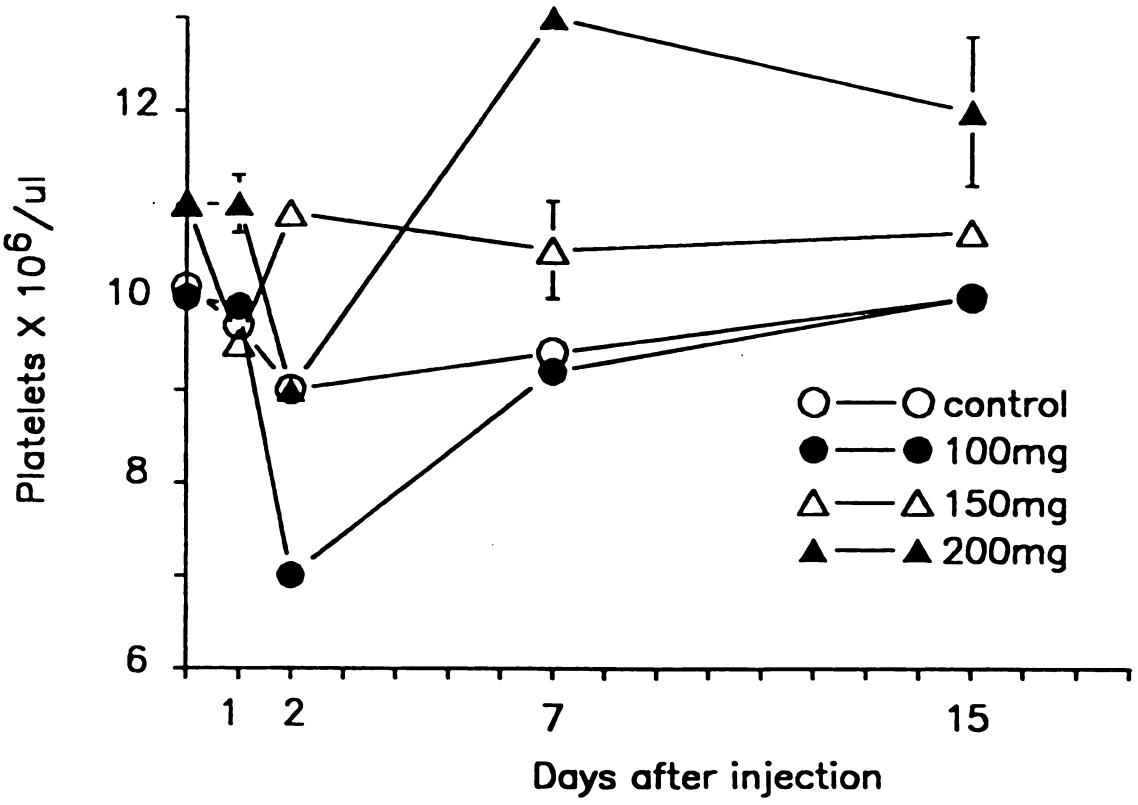

Figure 8: Changes in platelet counts of mice following single intraperitoneal injections with cyclophosphamide (CY). Animals received a dose of 100, 150, or 200 milligrams of CY per kilogram in a 0.2 milliliter volume. Animals were bled on 0, 1, 2, 7,and 15 days after drug administration for cell counts. Controls received the same volume of sterile saline. Each point represents a mean cell count of 5 mice.

Prior to the treatment studies in the murine model, different dosages (1.5 X 10^5, 1.75 X 10^5, 2.0 X 10^5, 2.5 X 10^5 and 3.0 X 10^5 cfu) of C. albicans, strain AK 785 were used to determine the lethal dose in the neutropenic murine model (data not shown). Preliminary results showed that the size of LD_{50} ranges from 1.75 X 10^5 to 2 X 10^5 cfu for the strain, whereas the maximum dose tested, 3 X 10^5 cfu was used in subsequent studies to insure 100% death between 3 and 8 days after the initiation of the infection.

Survival rates of mice receiving 100, 150, or 200 mg/kg of CY and non-CY treated mice intravenously challenged with 3 X 10^5 of strain AK 785 after 2 days of drug administration were examined (Figure 9). Among CY-treated groups, mice receiving the highest dose had a rapid mortality rate in which all animals died in 1 to 4 days following initiation of infection. Mortality in the animals receiving 150 mg/kg of CY occurred uniformly between 4 and 8 days after initiation of infection. On the other hand, mortality rate in animals receiving 100 mg/kg of CY remained above 70% but did not reach 100%. Control animals that received no CY had a delayed but steady death rate during the period between 6 to 12 days after the intiation of infection.

To further evaluate the effect of CY on susceptibility of neutropenic mice to the strain, they were challenged with 3 x 10^5 on days 1, 2, 7, or 15 days after drug

administration (Figure 10). There was a 100% mortality in mice challenged 2 days after CY treatment. In contrast, in mice challenged 1 or 7 days after CY treatment, a 100% mortality was not achieved over a short period of time, rather mortality was widely spread over time. Although death started occurring 8 days after infection, animals challenged 15 days after drug administration had up to 90% mortality. Non-neutropenic mice, that did not receive CY had consistent fatality rates with death starting to occur 8 days post infection.

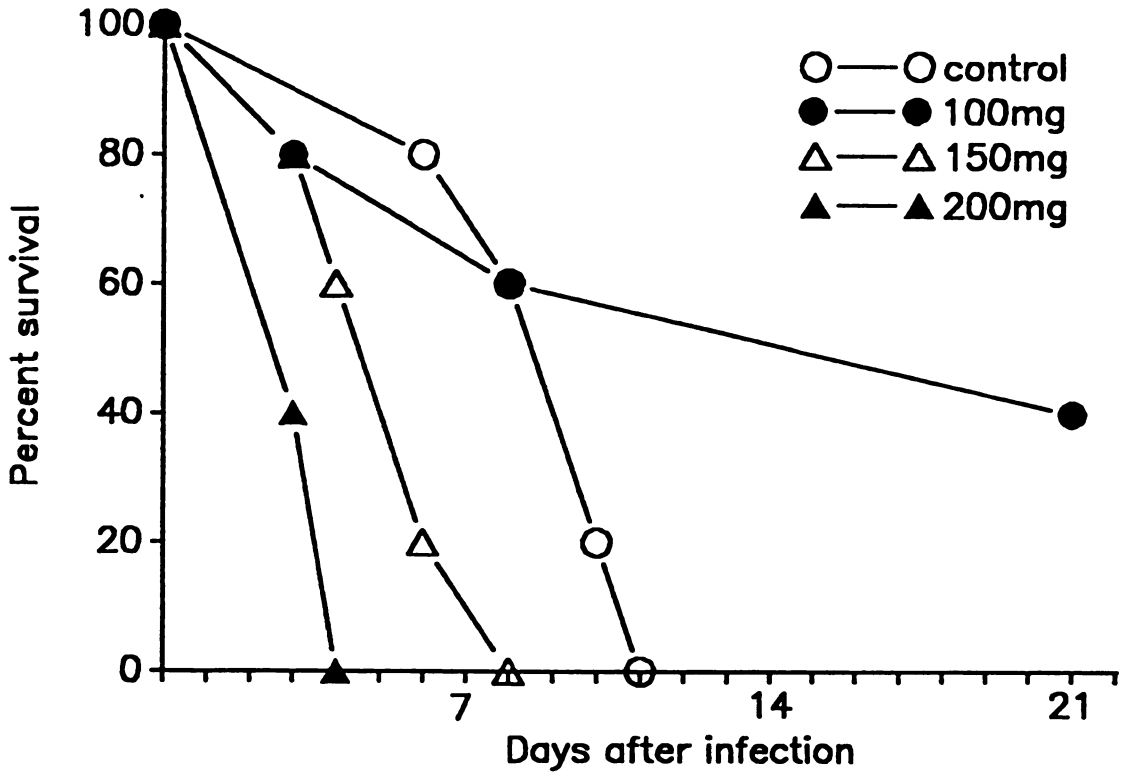

Figure 9: Survival rate of mice following challenge with 3 X 10^5 colony forming units of Candida albicans, strain AK 785. A dose of 100, 150, or 200 milligram of cyclophosphamide (CY) per kilogrm was administered intraperitoneally 2 days prior to initiation of infection. Control animals received no CY. Each treatment group consisted of 10 mice.

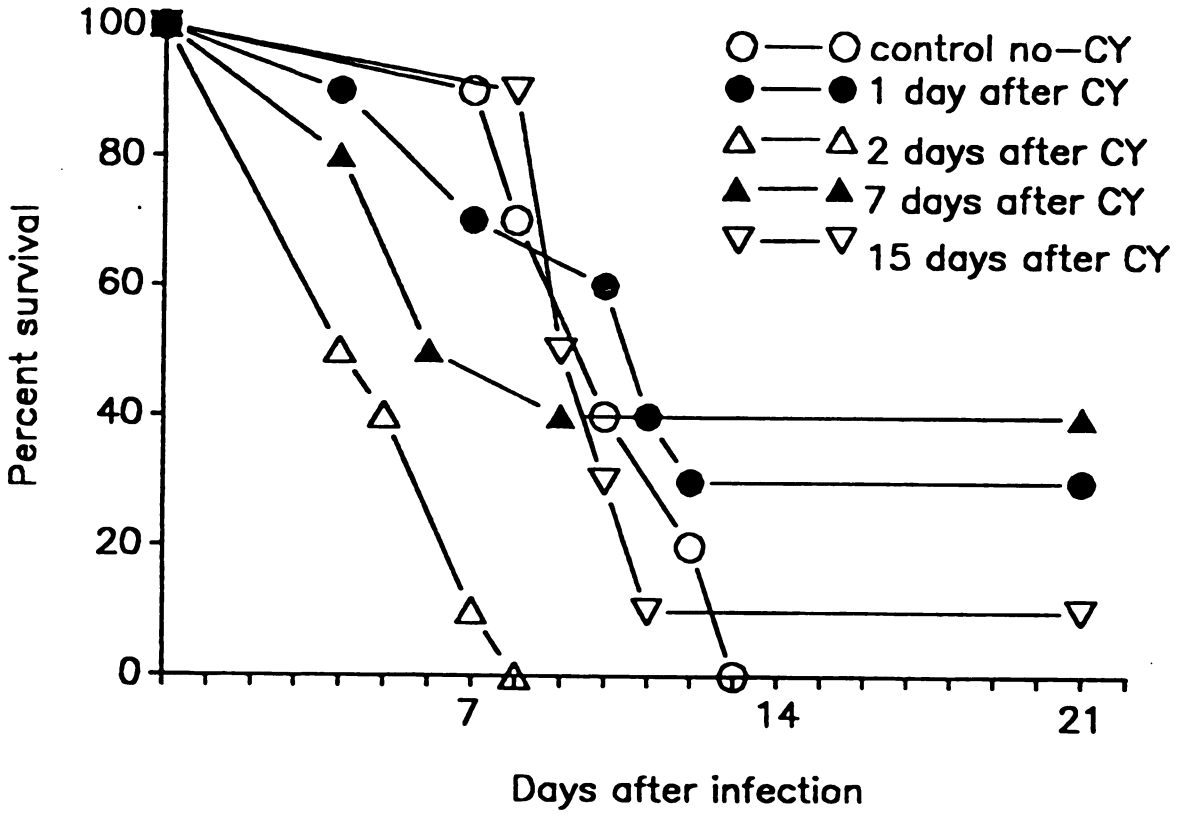

Figure 10: Mortality rate of neutropenic mice following challenge with 3 x 10^5 colony forming units of Candida albicans, strain AK 785 after 1, 2, 7, or 15 days after cyclophosphamide (CY) treatment. All mice received 150 milligrams of CY per kilogram one day prior to initiation of infection on day 1. Control animals received no CY but were challenged with the same inoculum of the strain.

Treatment: As shown in Figure 11, 100% (8/8) of the untreated neutropenic mice were dead on day 4 following initiation of the infection. In contrast, 50% (4/8) of mice that received LAMB-Ab survived to day 8 after initiation of infection. Moreover, 38% (3/8) of mice treated with LAMB-Ab survived throughout the study period. There was a significant difference between mortality in the LAMB-Ab treated animals and control animals ($P < 0.001$). There was also a significant difference in mortality between the LAMB-Ab treated animals and the animals treated with LAMB ($P < 0.01$) or fAMB ($P < 0.05$). Differences were not statistically significant between mortality in the LAMB treated animals and fAMB treated animals or between LAMB treated and control animals. There was a statistical difference in mortality between the fAMB treated and control mice ($P < 0.006$).

Regarding the non-neutropenic mice (Figure 12), the survival time of fAMB or LAMB treated mice was greater than those of control animals ($P < 0.001$). In the untreated group, 50 percent of deaths occurred by day 7 post infection. By day 14 post infection, all untreated control mice had died, whereas 67% of mice treated with LAMB, and 33% of the mice treated with fAMB survived to the end of the experiment. Non-neutropenic mice treated with LAMB survived significantly longer than those treated with free

AMB ($P < 0.05$).

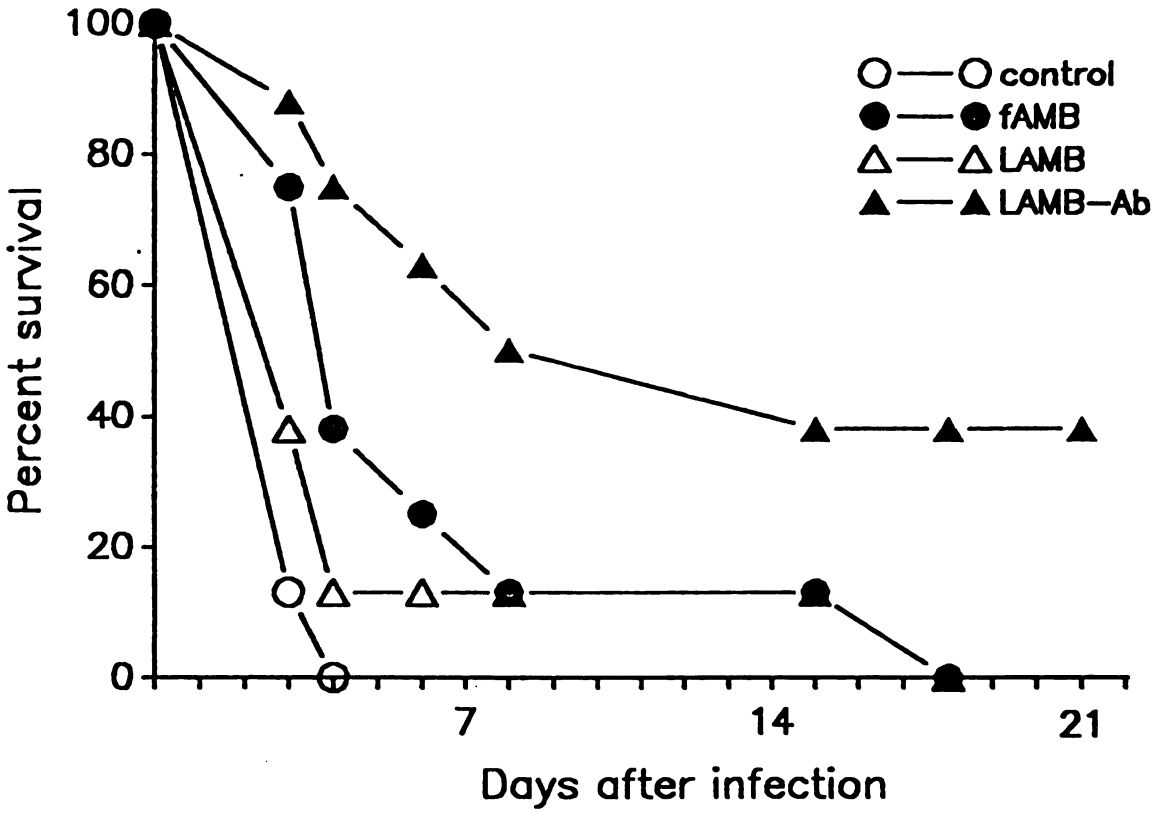

Figure 11: The percent survival of neutropenic mice treated with a single (0.6 milligram amphotericin B per kilogram) intravenous dosages of antibody-bearing liposomal amphotericin B (LAMB-Ab), liposomal amphotericin B (LAMB), or free amphotericin B (fAMB) two days following inoculation with 3 X 10^5 colony forming units of Candida albicans. Mice received 150 milligram of cyclophosphamide (CY) two days prior to infection. Control animals received CY and yeast but no antifungal therapy.

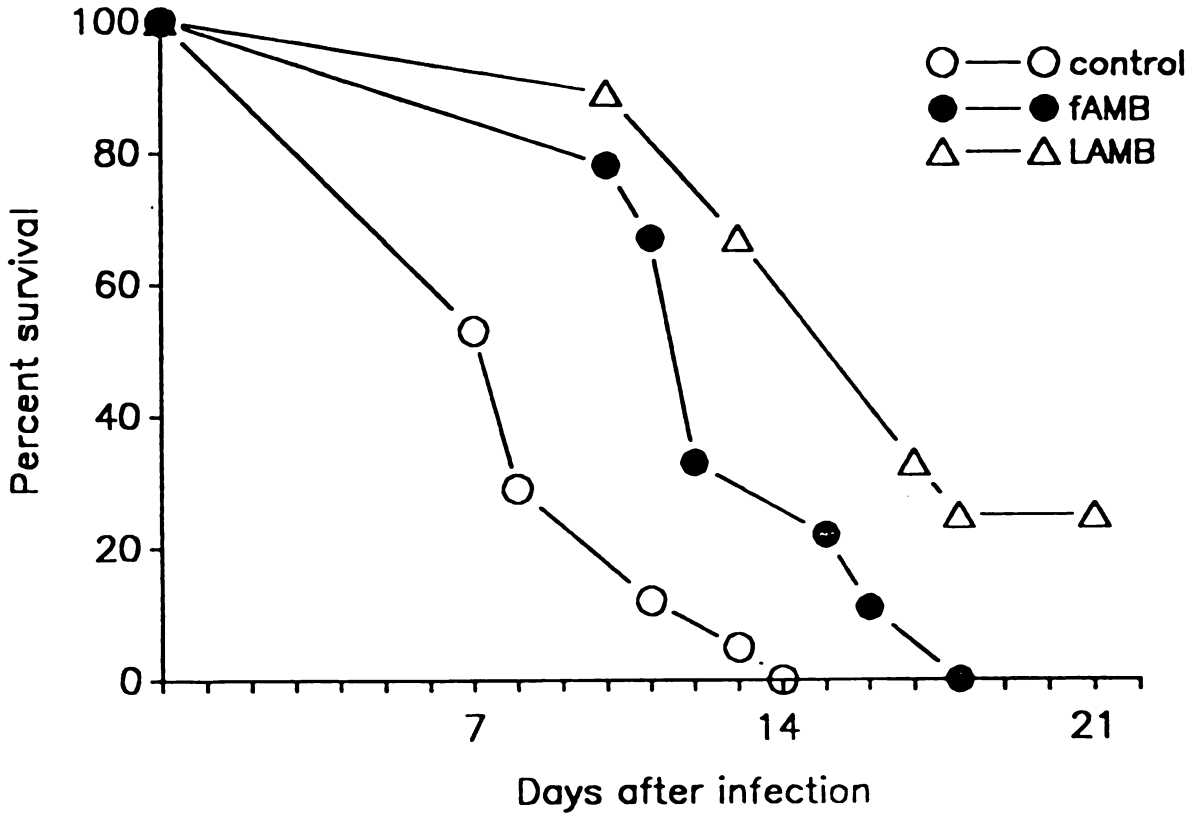

Figure 12 : Percent survival of non-neutropenic mice treated with a single dose (0.6 milligram amphotericin B per kilogram) of liposomal amphotericin B (LAMB) or free amphotericin B (fAMB) two days after initiation of infection intravenously with 3 x 10^5 cfu of Candida albicans. Control animals received no treatment after infection.

DISCUSSION

Though neutropenia is not always essential for establishment of murine candidiasis, the occurrence of systemic candidal infection and other opportunistic mycoses is more likely during periods of reduced counts of peripheral neutrophils (5). The development of a neutropenic mouse model and the initial use of this model to examine LAMB-Ab treatment of systemic candidiasis was herein reported.

In an attempt to determine the optimum dose of CY for neutropenia induction in our murine model, the effect of 3 different doses of CY were investigated. Induction of neutropenia in the model was evaluated by observing the change of cell counts through time after CY treatment. Our results demonstrate that the pattern of decreases and increases of total leukocytes on the specified days were relatively similar, the main difference being in quantity of leukocytes, which were dependent on CY administration doses. To document if similar changes are achieved in various cell populations, differential cell counts were performed. It was found that CY modified not only neutrophils, but also other leukocyte populations as well. Undetectable levels of monocytes, basophils and eosinophils

were recorded in some mice during the experiment. The highest decrease in the number of different leukocyte cell types including neutrophils, was obtained 1 to 2 days after CY treatment. This significant decrease in cell populations in all mice receiving the drug in the course of 2 days after CY administration, was followed with a full or partial recovery by days 7 or 15 depending on the dose used and cell type. It was shown that leukocytes start recovering to normal values in 7 days and continue to reach normal values through day 15 after CY treatment. These results are in agreement with previously reported findings where CY treatment leads to a marked decrease of granulocytes and lymphocytes 1 to 4 days after drug injection in experimental animals (5,10, 18). The pattern of increase observed is also similar to that of Bostani et al. (5), where the leukocyte number of CY treated mice return to normal values 9 days after drug treatment. Cyclophosphamide is known to affect most lymphoid cells, primarily B lymphocytes, helper and suppressor T lymphocytes and other rapidly dividing cells such as polymorphonuclear cells and monocytes. The effect of CY on red blood cells and platelets was was not clear in our investigation. However, earlier reports showed that red blood cells and platelets in animals were affected by cyclophosphamide resulting in a marked decrease in the number 2 days after the drug administration (10,18).

Experimental infections were carried out to observe if mortality rates parallels the decrease in cell counts. Infection was initated 2 days post-CY administration, a time corresponding in the mice model to the lowest cell counts. It was possible to establish uniformly fatal infection with C. albicans in the neutropenic mouse by examining the effect of numerous doses of CY administration followed by intravenous challenge of the etiologic agent.

Although no mortality was observed from CY administration, mice that received 200 mg/kg of CY appeared severely ill (loss of hair and weight). In addition, mice that were injected with 200 mg/kg of CY and infected with C. albicans were all dead by day 2 of the infection, whereas mice receiving 150 mg/kg of CY began dying on day 3 of the infection showed a consistent and uniform mortality rate for 4 to 5 days after initiation of infection.

As seen in the neutropenic murine model (Figure 9), survival was associated with the degree of neutropenia at C. albicans challenge. This observation confirms previous works that have shown animals injected with cyclophosphamide 2 to 4 days prior to challenge with etiologic agents were highly susceptible to infection (5). On the other hand, no rapid and steady mortality rate was found in mice challenged with C. albicans 7 or 15 days after CY administration (Figure 10) indicating that delaying infection beyond 7 days after CY-treatment does not provide persistent infection in the

model. This may be associated with late increase of neutrophil and other cells thus enhancing resistance to C. albicans infection in the model.

Early death of animals creates a difficulty in the use of the model for subsequent treatment studies. From our results, early death with the highest dose of CY in the murine made it impossible to use in our treatment studies. Therefore to avoid early death prior to initiation of antifungal therapy while sustaining reduced leukocytes as long as possible, a 150 mg/kg of CY was selected for subsequent treatment studies. Since the lowest number of neutrophils was observed on day 2 after the drug treatment, this time was selected to challenge mice with C. albicans in the current treatment studies.

In previous prophylactic and therapeutic studies, antibody bearing liposomal amphotericin B (LAMB-AB) in a dose of 0.6 mg AMB/kg showed an improved survival rate in non-neutropenic mice with systemic candidiasis (7). The same dose was implemented in the present study to evaluate its therapeutic effect in the treatment of sytemic candidiasis induced in a neutropenic murine model.

AMB entrapped within liposomes with anticandidal antibodies attached to their surfaces (LAMB-Ab) was used to treat neutropenic mice infected with C. albicans. Inoculation of 3 x 10^5 cfu of C. albicans resulted in the death of all untreated neutropenic mice by day 4 following

infection, whereas half the number of LAMB-Ab treated neutropenic mice survived to eight days after infection. LAMB-Ab improved the percent survival and the mean survival time of the neutropenic mice in comparison to those treated with either LAMB ($P < 0.01$) or free amphotericin B ($P < 0.05$). Neutropenic mice treated with LAMB, however, showed no significant difference in survival time when compared to control. Whether this was due to the small number of animals used in these studies, or secondary to the added stress placed on these compromised animals by the injection of lipid-laden drug, is uncertain.

LAMB treatment contributed to a prolonged survival of non- neutropenic mice more than did fAMB treatment ($P < 0.001$). These results are similar to the treatment outcome noted in previous studies of non-neutropenic mice treated with the same compound (7,9).

The results therefore suggest that the use of LAMB-Ab, prepared from crude rabbit antiserum is a promising system to improved the treatment of *C. albicans* infections, in both neutropenic and non-neutropenic hosts. However, further investigation is needed to insure that there is antibody-mediated AMB delivery to target yeast cells in the model. The second phase of this study was to purify immunoglobulin G molecules from the commercial rabbit antiserum and examine their role in facilitating the therapy of systemic candidiasis in the murine model.

ACKNOWLEDGEMENTS

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ARTICLE II

THE TREATMENT OF SYSTEMIC CANDIDIASIS IN A NEUTROPENIC MURINE MODEL USING IMMUNOGLOBULIN G-BEARING LIPOSOMAL AMPHOTERICIN B

Running Title: Treatment of systemic candidiasis

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ABSTRACT

Efficacy of immnunoglobulin G (IgG) bearing liposomal amphotericin B (LAMB-IgG), liposomal amphotericin B without IgG (LAMB) or free amphotericin B (fAMB/Fungizone) was investigated in the treatment of systemic candidiasis in a neutropenic mouse model. Treatment with a single dose (0.6 or 0.9 mg amphotericin B per kg body weight) of LAMB-IgG resulted in a significant increase in the survival rate of neutropenic mice infected with 3 x 10^5 cfu of Candida albicans compared to untreated controls, mice injected with IgG, or liposome alone. In addition, survival was better in neutropenic mice treated with LAMB-IgG than in neutropenic mice treated with the same dose of LAMB or fAMB. Moreover, 65% of all mice survived the infection after treatment with LAMB-IgG. Quantitative culture counts of organs showed that both fAMB and LABM-IgG formulations even at a dose of 0.3 mg AMB/kg, cleared C. albicans from the spleens, livers, and lungs but not from the kidneys. A decreasd number of C. albicans cells was recovered from the kidneys of mice that survived the infection. Results of our study suggest that LAMB-IgG is more effective than LAMB or fAMB in the therapy of disseminated candidiasis in neutropenic mice.

INTRODUCTION

Opportunistic fungal infections are becoming more common and life-threatening especially in patients with impaired immune system (1,3,4). Disseminated candidiasis most frequently caused by Candida albicans is one of the major causes of morbidity and mortality in the neutropenic patients (3,4). Disseminated candidiasis occurs frequently in individuals with haematologic malignancies, patients under broad-spectrum antibiotic therapies, in transplant recepients, or patients undergoing cytotoxic and immunosuppressive therapy (18,19). Other predisposing factors for the disease in humans include, use of intravascular catheters, post-surgical state, severe burns, hyperalimentation, and diabetes mellitus (18,19).

Although its severe toxicity to liver and kidneys along with other side effects limit its wider use, amphotericin B (AMB) remains the drug of choice in the treatment of disseminated mycotic infections including candidiasis (1,14). To overcome the toxicity of AMB, several investigators have been searching for different methods of amphotericin B delivery to the host in less toxic form without decreasing its activity to fungal cells. It is approximately a decade since researchers have explored the

use of liposome-encapsulated AMB in the treatment of fungal infections (14). Successful treatments of histoplasmosis (24), cryptoccosis (8) and candidiasis (14) with liposomal amphotericin B using experimental mice models have been reported. Furthermore, different methods of liposomal formulations also have been found to be effective in the therapy of fungal infections in humans (15,20,21). It has been demonstrated that liposome encapsulated-amphotericin B has reduced toxicity to mammalian cells compared to Fungizone as evidenced in both the in vitro and in vivo studies (17,23). Further characterization of liposomal amphotericin B has shown that lipid composition, lipid ratio, and liposome size are some of the determinant physical factors that influence the therapeutic index of amphotericin B (23).

Incorporation of antibodies into the surfaces of liposomes in an attempt to enhance delivery of drugs to specific cells or tissues have been reported (13,22). Based on this application, Dromer et al.(6) recently reported an improved survival rate of mice infected with Cryptococcus neoformans after treatment with amphotericin B incorporated into liposomes conjugated with cryptococcal specific antibodies. Hospenthal et al.(9,10) similarly reported that unpurified anticandidal antibodies attached to liposomes containing amphotericin B increased the survival rate of non-neutropenic mice with systemic candidiasis. The present

study was undertaken to investigate whether the increased therapeutic efficacy of liposomal amphotericin B in the treatment of systemic candidiasis observed in previous studies is truly due to antibodies rather than other serum components. The immunoglobulin G (IgG) portion of a commercial rabbit antiserum to Candida albicans was separated and used in targeting liposomal AMB to evaluate its efficacy compared to Fungizone in treatment of systemic candidiasis in mice made neutropenic using cyclophosphamide.

MATERIALS AND METHODS

Animals: White Swiss CD1 (4 weeks old) female mice each weighing 19 to 22 grams were purchased from Charles River Laboratories Inc. Portage, MI. The mice were housed five animals per cage and allowed free access to food and water in accordance with animal care regulations of University Laboratory Animal Resources at Michigan State University.

Drugs, lipids and antisera: Dimyristoyl phosphatidylcholine (DMPC), dimyristoyl phosphatidylglycerol(DMPG), Caprylic acid and cyclophosphamide were purchased from Sigma Chemical Co. St. Louis, Mo. Amphotericin B, for use in liposomes and Fungizone were provided by Squibb Pharmaceutical, New Brunswick, N.J. Adsorbed rabbit antisera to C. albicans for use in targeting liposomal amphotericin B was purchased from Difco, Detroit, MI.

Inoculum preparation: An isolate of C. albicans, strain AK 785, recovered from a patient with a clinical evidence of mucocutaneus candidiasis, donated by Michael Kennedy (The Upjohn Co., Kalamazoo, MI.) was used for animal inoculation.

A sample from the strain maintained at -20°C was

subcultured on Sabouraud glucose agar and incubated overnight at 37°C. Inoculum from this slant was transferred into 100 ml of tryptic soy broth containing 4% glucose and incubated 12 to 16 hr at 37°C on a rotary shaker. Yeast cells were harvested by centrifugation (2,000 x g for 10 minutes) and washed 3 times in phosphate-buffered saline (PBS). Yeast cells were counted in a hemocytometer and adjusted to the desired concentration with PBS for animal inoculation.

Separation of IgG: A non-chromatographic procedure described by McKinney et al.(16) was used to fractionate immunoglobulin G (IgG) from rabbit antiserum raised to whole cells of C. albicans. Briefly, 5 ml of adsorbed rabbit antiserum to C. albicans was diluted with 15 ml of acetate buffer (0.6 M, pH 4.0) followed by pH adjustment to 4.5 at room temperature. Caprylic acid (25 ul/ml) was slowly added dropwise to the solution with vigorous stirring. Then the solution was stirred for 30 minutes and centrifuged (10,000 X g for 30 minutes). After mixing the supernatant with 10X concentrated PBS in a 10:1 ratio and adjusting the pH to 7.4, ammonium sulfate (0.277 gm/ml to give 45% saturation) was added at 4°C. Following stirring for 30 minutes, precipitated IgG was recovered by centrifugation (5000 X g for 15 minutes). The pellet was resuspended in 2 ml of PBS and dialyzed overnight in 100 ml of PBS with 3 changes. The final product was heated to 56°C for 20 minutes then

centrifuged at 5000 X g for 20 minutes. Activity of the recovered product was checked by using the slide agglutination test to C. albicans.

Purity of fractionated IgG was determined by using standard sodium duodecil polyacrilamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining (12).

Palmilotyl-IgG preparation: The purified IgG was reacted with radiolabeled N-hydroxysuccinimide ester of palmitic acid (NHSP) to produce palmitoyl IgG for liposomal amphotericin B formulation (11). Briefly, NHSP and purified IgG in the presence of 1% deoxycholate (DOC) in PBS were mixed and incubated overnight at 37°C. After centrifugation at 3400 X g for 15 minutes, the supernatant was carefully loaded on a Sephadex G-75 column (1.9 X 20 cm) equilibrated with PBS. Palmitoyl-IgG was eluted in the void volume and concentrated by ultrafiltration (Amicon, XM-50). Radio-label counts and protein content were measured using Tracerlab 4 Pie Scanner and Lowry method respectively. The final protein concentration of the recovered product was about 1.2 gm/ml. After checking antibody activity of the palmitoyl IgG using slide agglutination test to C. albicans, the modified IgG was stored in 1.0 ml aliquots at -20°C for further use.

IgG-bearing liposome preparation: Liposome containing AMB was prepared by reverse-phase evaporation as previously described (11). Briefly, amphotericin B dissolved in

methanol was mixed with phospholipids in chloroform (DMPC/DMPG ratio, 7:3) and 1 ml PBS. Organic solvents were evaporated under nitrogen and heat (45°C) until the solution was approximately one ml. The suspension was then sonicated for 2 seconds at 1000 W and incubated for 1 hour at room temperature. One milligram of modified-IgG in the presence of 0.15% DOC was added to the reaction mixture and left at room temperature for 2 hours. The resulting solution was dialyzed (using spectaPOR 2 dialysis tubing) overnight at 4°C with 3 changes of 500 ml PBS. The product was recovered by centrifugation (30,000 x g, 15 minutes, 4°C). Liposomal AMB without IgG was prepared using the same procedures. Dosages of liposomal compounds were determined by AMB content via spectrophotometry at 405 nm.

Immunofluorescence assay: The immunofluorescence assay was performed as described before (11). Formalin-killed whole cells of C. albicans, strain AK 785 (5 X 10^8), were reacted with 0.2 ml of crude rabbit antiserum, LAMB-IgG, or palmitoyl-IgG (as a control) for 30 minutes at 37°C. Two drops of each suspension were placed on a microscopic slide coated with 1% poly-L-lysine and allowed to air dry. After 2X washing with PBS, 2 drops of 1:1 PBS diluted fluorescin isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Sigma) were added to each slide and allowed to react at 37°C for 30 minutes. Following several washings with PBS and rinsed for 1 minute in distilled water, slides were

mounted using 10% glycerol. Then the slides were observed for fluorescence with an Olympus BH2 series microscope with reflected light fluorescence attachment. Photomicrographs were taken with an automatic Olympus camera system using Ektachrome film.

Treatment studies: In previous studies, Hospenthal et al. (9,10) reported that a single dose of 0.6 mg AMB/kg of liposomal amphotericin B complexed with rabbit antiserum to C. albicans improved survival time for non-neutropenic mice with systemic candidiasis. Based on this information, the same AMB dose was included in either IgG-bearing liposomal amphotericin B (LAMB-IgG), liposomal amphotericin B without IgG (LAMB), or free form (fAMB/Fungizone) for evaluation in the treatment of systemic candidiasis using immunocompromised mice.

Experiment 1: The neutropenic condition in mice was induced by a single intraperitoneal injection of cyclophosphamide (150 mg/kg of body weight) two days before mice were intravenously inoculated with 3 x 10^5 cfu of C. albicans, strain AK 785 (2). Each mouse then was treated with a single dosage of 0.6 mg AMB/kg in 0.2 ml aliquot of LAMB-IgG, LAMB, or fAMB on day 2 following initiation of infection. Mice receiving liposome only, modified IgG only or untreated animals were used as controls. Each group consisted of 8 to 11 mice. Survival rate every 24 hours was recorded. Animals were evaluated up to 21 days after

infection.

Experiment 2: Neutropenic mice consisting of 8 to 10 animals in each treatment group received a single injection of LAMB-IgG or fAMB at either 0.3, 0.6, or 0.9 mg of AMB/kg 2 days after initiation of infection. Although 0.8 mg/kg of Fungizone is the maximum tolerated dose recommended for intravenous injection of infected mice on a daily basis (14), 0.9 mg/kg of Fungizone was included for comparative studies.

Organ culture: Organs were aseptically removed, homogenized in sterile distilled water with a tissue grinder, serially diluted and plated on mycosel agar. Colony counts were performed after 48 hours incubation of plates at 37°C. Viable cells of C. albicans in the organs were expressed as the $\log_{10}$ colony forming units per organ.

Statistical analysis: Survival distribution differences were evaluated by using a generalized Wilcoxon test (7). Comparison of means of $\log_{10}$ colony forming units (CFU) recovered from selected organs of treatment groups was performed by using analysis of variance (ANOVA) or least significant difference (LSD) test. Cell counts less than 2 Log_{10} were not considered as detectable levels in this study.

RESULTS

As shown in Figure 1, a successful separation of IgG was accomplished by using caprylic acid-ammonium sulphate precipitation method, where less antiserum contaminants were seen as determined by SDS-polyacrylamide gel electrophoresis and Coomassie staining. About 30 ug of purified IgG was purposefully loaded onto the gel (lane 2) in order to detect contaminating serum proteins. The same amount of non-specific IgG from Sigma (lane 3) was included for comparison purposes. The heavy chain part of IgG appeared intact (between 66.2 and 45.0 Kilodalton molecular markers), whereas light chain was separated in a diffused form between 24 to 29 kilodalton. In addition, antibody activity of IgG was not affected during separation as evidenced by the agglutination of whole cells of C. albicans and by detection of antibody-antigen reaction shown by the indirect immunofluorescence assay (Figure 2).

Experiment 1: Including relevant controls, we compared the efficacy of LAMB-IgG, LAMB, and fAMB (0.6 mg AMB/kg) on the survival of neutropenic mice after challenge with a lethal dose of C. albicans (Figure 3). LAMB-IgG treated mice showed a significant difference in mortality compared with mice treated with LAMB ($P < 0.002$), fAMB, or compared

with untreated controls, only IgG controls, or groups receiving only liposome ($P < 0.001$). By day 21 after challenge, 45% (5/11) of mice treated with LAMB-IgG and 10% (1/10) of mice treated with LABM survived the infection. LAMB treated mice also survived longer than untreated mice, mice that received IgG only, or liposome only ($P < 0.001$, for each control). Furthermore, statistical differences were observed in the survival of mice treated with LAMB and fAMB ($P < 0.03$) and between mice treated with fAMB and untreated controls ($P < 0.02$). But no significant differences were obtained in the survival of mice in untreated controls, or those receiving only liposome or modified IgG. Another group of mice treated with nonspecific IgG-bearing liposomal amphotericin B had similar treatment outcomes as noted in mice treated with LAMB (data not shown).

Organs (spleens, livers, lungs and kidneys) from untreated controls and mice from the treatment groups that died after receiving the drug were cultured to compare the residual burdens of C. albicans with that of survivors sacrificed after 21 days of infection. Viable yeast counts from organs of mice in untreated controls from experiment 1, are shown in Figure 4a. As expected, kidneys and spleens were organs with the highest and lowest cfu of C. albicans, respectively. Analysis of yeast counts using ANOVA and LSD tests indicated significant differences between counts for

both organs ($P < 0.05$). Yeast cell counts for lungs and livers were higher than for spleens, but lower than counts for kidneys. No statistical difference was observed between counts for lungs and livers.

The colony forming units of C. albicans, in spleens, lungs, livers and kidneys were quantified in 5 randomly selected mice that died after treatment with 0.6 mg/kg of fAMB or LAMB and 5 survivors from mice treated with the same dose of LAMB-IgG. Despite the occurrence of 100% death, C. albicans was not recovered from spleens, livers, or lungs of animals that were treated with either formulation except in 3 mice that had less than 53 cfu in the lungs and livers. In contrast, a high number of cfus of C. albicans was detected in the kidneys from mice that died after treatment with all formulations (Figure 4b) and showed no statistical difference when compared to the untreated controls. Mice that survived the infection after treatment with one dose of 0.6 mg AMB/kg of LAMB-IgG had a large number of viable cell counts in kidneys eventhough there was a statistically significant difference in counts compared to those of dead mice ($P < 0.05$). Among mice treated with LAMB formulation, one mouse that survived the infection had 8×10^5 cfu in the kidneys. All survivors appeared very healthy at the time they were sacrificed. In addition, no change/damage was detected macroscopically in the kidneys.

Experiment 2: The efficacy of either fAMB or LAMB-IgG

given in various doses was compared in the treatment of neutropenic mice after infection with C. albicans. All AMB concentrations given, except the group of animals receiving one dose of .3 mg/kg of fAMB (Fungizone) resulted in significantly delayed mortality compared to untreated controls (Figure 5a). Similar to experiment 1, statistical analysis using a generalized Wilcoxon test showed that there was a significant difference in mortality between control mice and mice receiving a dose of 0.6 mg/kg of Fungizone ($P < 0.001$). Although there were 37.5% surviving at the end of 21 days in mice treated with 0.9 mg AMB of fAMB, 62.5% of the group died within 2 days after treatment.

The treatment of mice with one dose of 0.3 mg of AMB/kg was more effective in delaying mortality than the treatment with the same dose of fAMB formulation, but less effective when compared to the higher doses of LAMB-IgG formulation. Mice treated with a dose of 0.6 or 0.9 mg/kg of the LAMB-IgG formulation prolonged survival compared to survival of controls ($P < 0.001$), or groups receiving 0.3 or 0.6 mg fAMB/kg ($P < 0.001$). There was also a significant difference in mortality of mice treated with 0.6 or 0.9 mg AMB /kg of LAMB-IgG compared to mice treated with 0.9 mg of Fungizone ($P < 0.03$). Furthermore, mice treated with a dose of 0.6 or 0.9 mg AMB of LAMB-IgG in dose-response study showed a higher percentage (75%) of survivors compared to the survivors (45%) in experiment 1.

Quantitative cultures of organs in the second experiment showed that all regimens including a dose of 0.3 mg of AMB in both formulations eradicated C. albicans from spleens, lungs, and livers. Similar to the results in experiment 1, levels of C. albicans ranging from 7 to 32 cfu were recovered from the livers of 3 mice.

Colony forming units of C. albicans recovered from kidneys of mice that died after treatment with various doses of both formulations and 12 survivors is shown in Figure 5b. No significant difference in counts for kidneys was found in animals that died after treatment, except those mice that died after receiving a dose of 0.9 mg AMB of Fungizone compared to non-treated controls. Again in experiment 2, no single animal was found free of C. albicans in the kidneys. However, mice treated with LAMB-IgG that survived the infection had significantly lower numbers of yeast cells in their kidneys compared to cells recovered from kidneys of dead animals ($P < 0.05$).

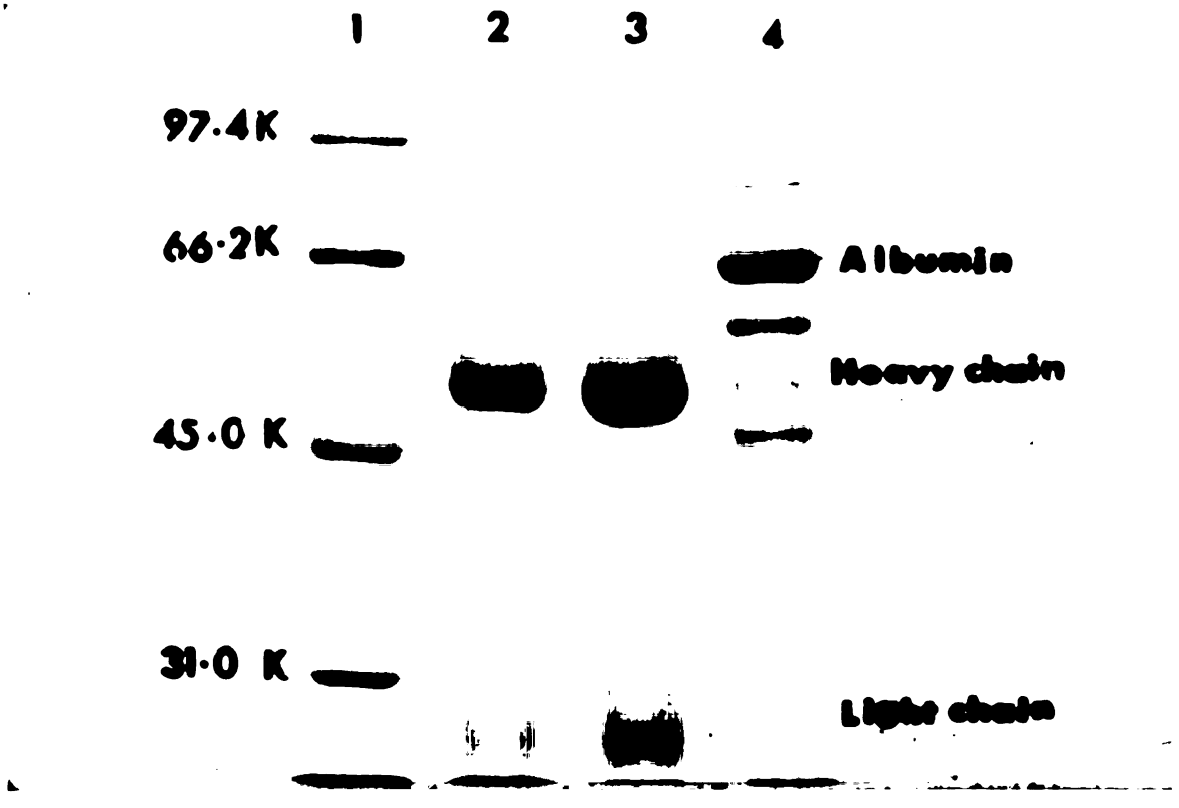
1
2
3
4
97.4K
66.2K
45.0 K
31.0 K
Albumin
Heavy chain
Light chain

Figure 1. Sodium duodecil sulfate polyacrilamide gel electrophoresis of proteins stained with Coomassie blue. Lane 1: molecular weight standards, lane 2: fractionated immunoglobulin G (IgG) from rabbit antiserum to Candida albicans. lane 3: Anti-chicken IgG developed in rabbit. lane 4: adsorbed rabbit antiserum to Candida albicans.

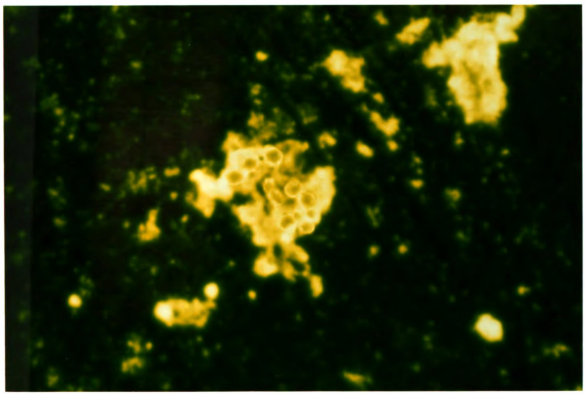

Figure 2. Micrograph of immunoglobulin G-bearing liposomal amphotericin B reacted with Candida albicans cells.

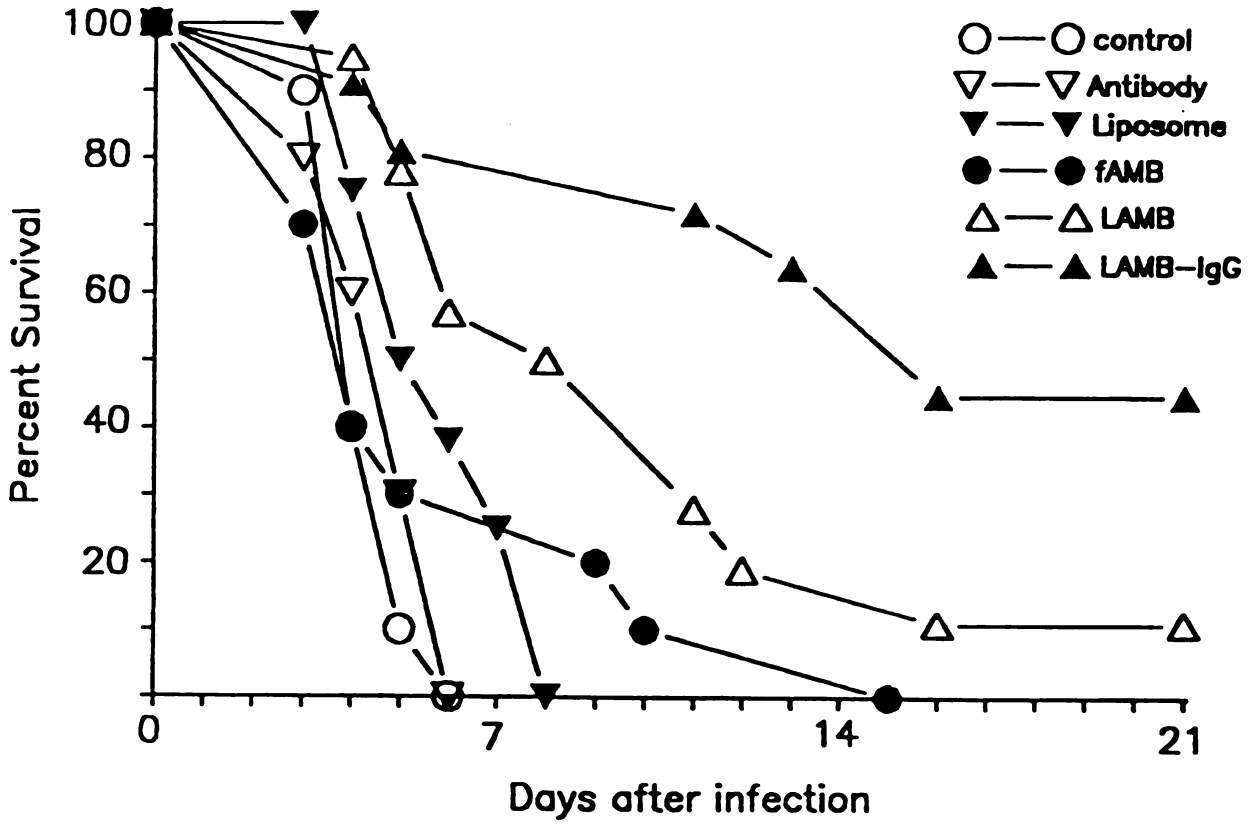

Figure 3. The percent survival of mice treated with single (0.6 milligram amphotericin B per kilogram) dosages of immunoglobulin G bearing liposomal amphotericin B (LAMB-IgG), liposomal amphotericin B (LAMB), free amphotericin B (fAMB), liposome only, modified antibody (IgG) and untreated controls two days following inoculation with 3 x 10^5 cfu of Candida albicans. Mice received 150 milligram of cyclophosphamide two days prior to infection. Each group consisted of 8 to 11 mice.

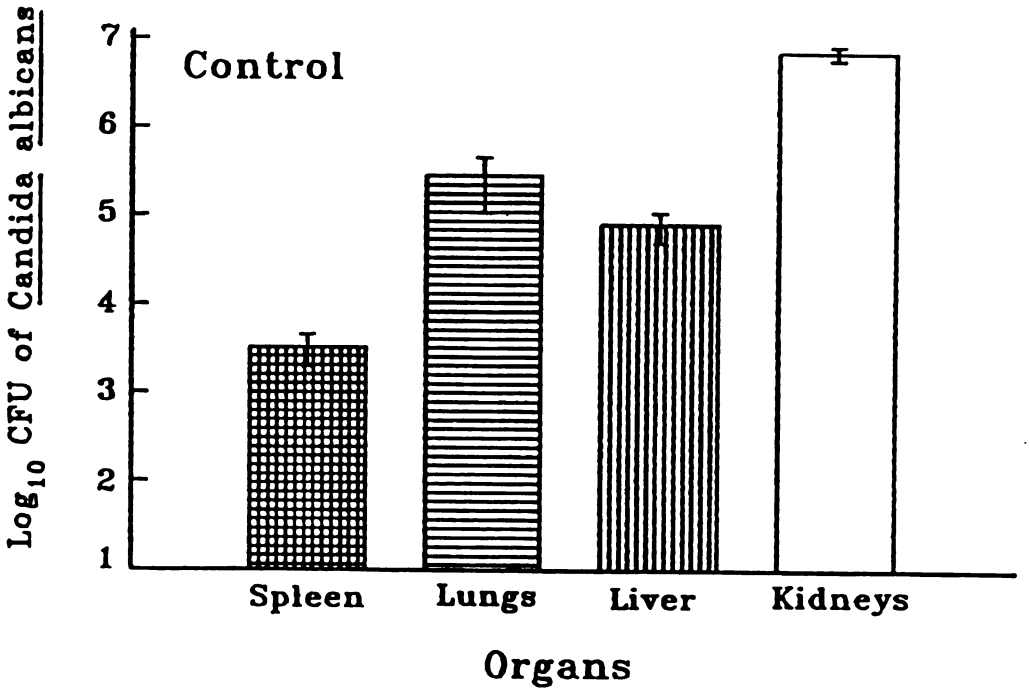

Figure 4a. Colony forming units (cfu) of Candida albicans recovered from organs of control animals that died 3 to 6 days after an intravenous challenge with 3 X 10^5 cells of C. albicans, strain AK 785. Bars represent the mean +/- standard deviation cfus in organs of 5 mice.

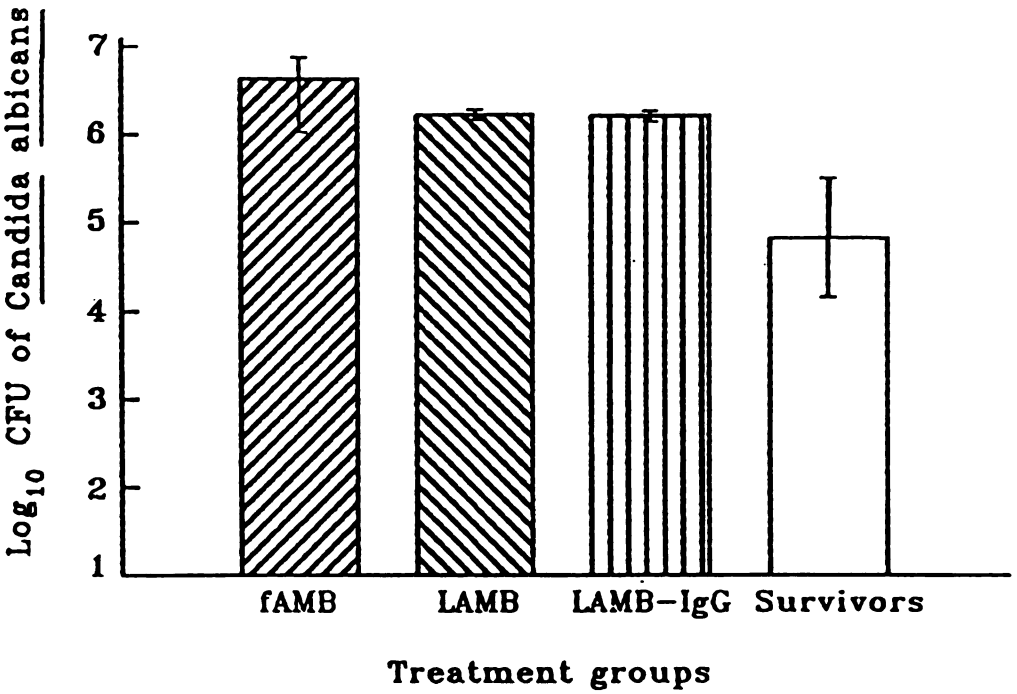

Figure 4b. Colony forming units (cfu) of Candida albicans recovered from kidneys of dead mice or surviving mice after challenge with 3 X 10^5 C. albicans cells and treated using free amphotericin B (fAMB), liposomal amphotericn B without immunoglobulin G (LAMB) or Immunoglobulin G-bearing liposomal amphotericin B (LAMB-IgG). Survivors from the mice treated with LAMB-IgG were sacrificed 21 days after initiation of infection. Bars represent the mean +/- standard deviation cfus of 5 or more mice.

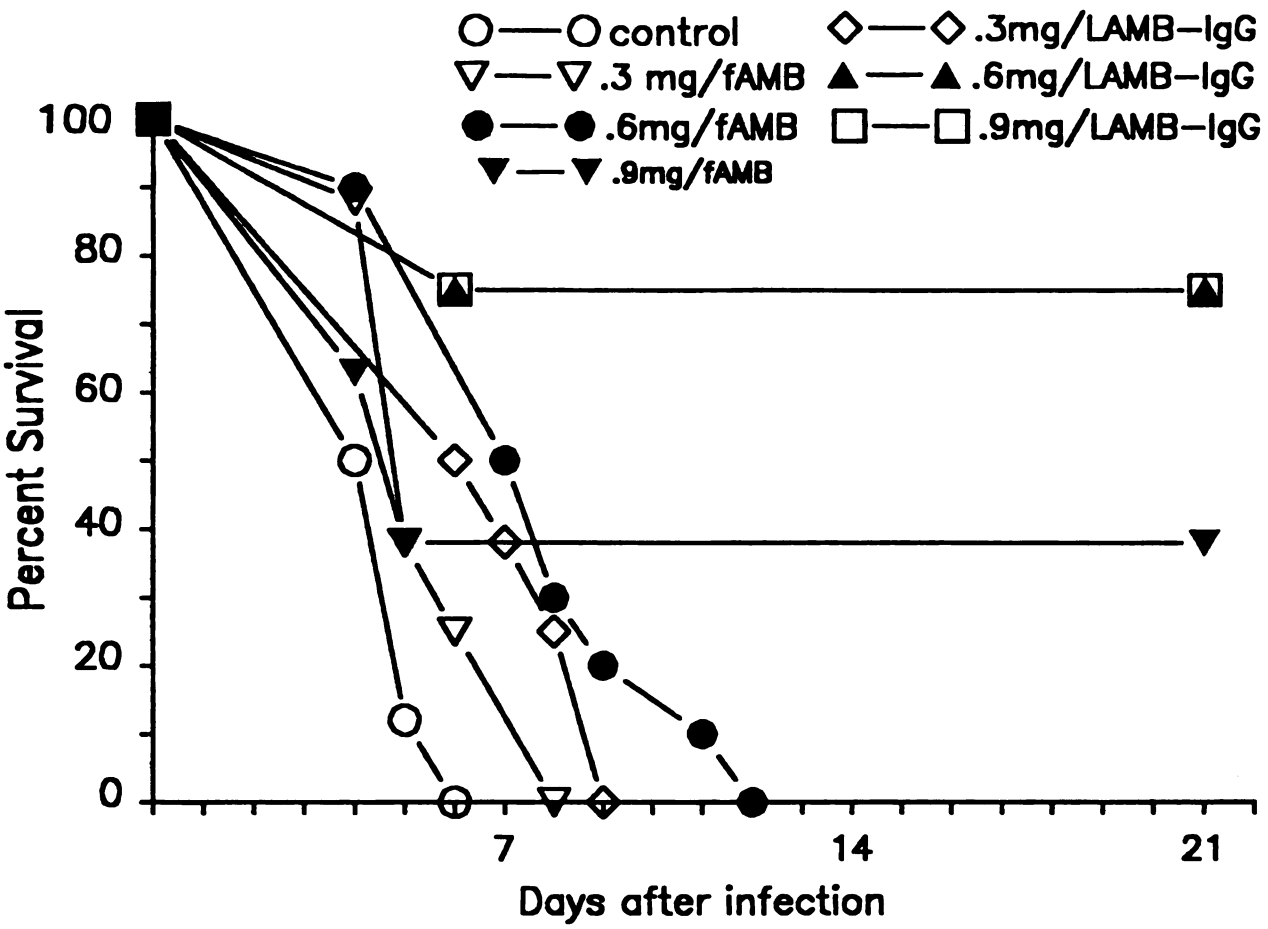

Figure 5a. The percent survival of mice treated with various doses of either immunoglobulin G bearing liposomal amphotericin B (LAMB-IgG) or free amphotericin B (fAMB), and untreated controls two days following inoculation with 3 x 10^5 cfus of Candida albicans. Mice received 150 milligram of cyclophosphamide two days prior to infection. Each group consisted of 8 to 10 mice.

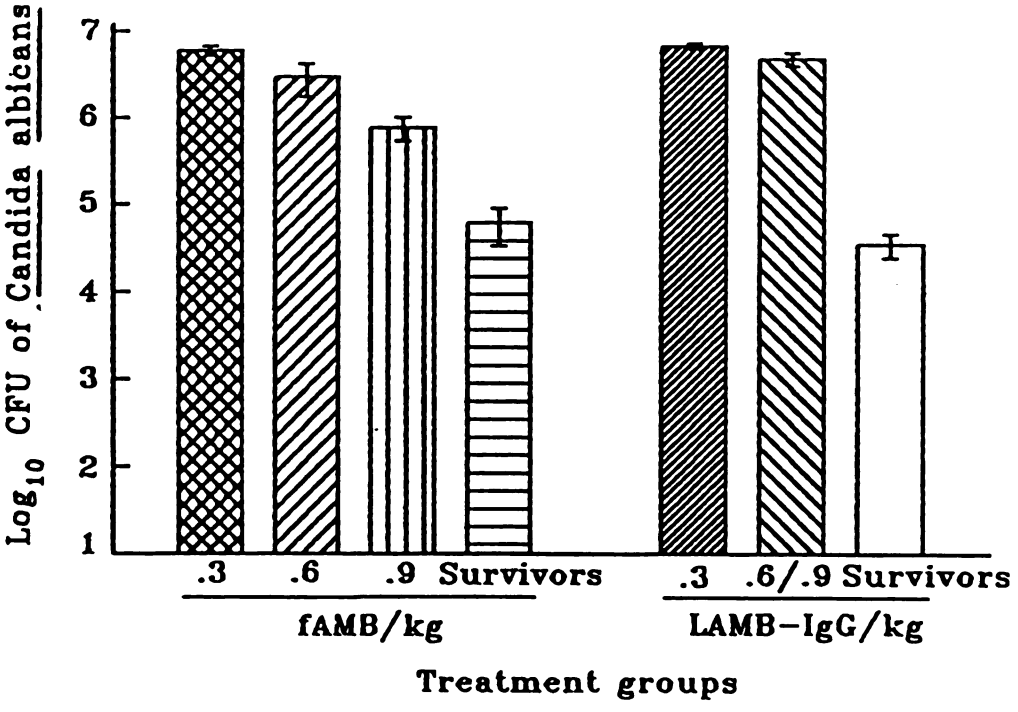

Figure 5b. Colony forming units (cfu) of Candida albicans recovered from kidneys of dead mice or survivors after challenge with 3 X 10^5 cells of C. albicans and treated using various formulations of free amphotericin B (fAMB) or immunoglobulin G-bearing liposomal amphotericin B (LAMB-IgG). Mice that survived after treatment with immunoglobunin G bound liposomal amphotericin B at a dose of 0.6 or 0.9 mg AMB/kg or 0.9 mg AMB of fAMB were sacrificed 21 days after initiation of infection. Bars represent the mean +/- standard deviation cfus of 3 to 12 mice.

DISCUSSION

The efficacy of IgG-bearing liposomal amphotericin B was compared with that of liposomal amphotericin B without IgG or free amphotericin (Fungizone) in the treatment of systemic candidiasis in a neutropenic murine model. Two separate experiments were carried out to compare the efficacy of both formulations administered at one dose of 0.6 mg of AMB /kg (experiment 1) or given at 3 doses (0.3, 0.6, 0.9 mg AMB/kg) for either formulation (experiment 2).

The application of IgG-bound liposomal AMB in the treatment of neutropenic mice with disseminated candidiasis resulted in a prolonged survival time compared to the untreated controls or mice treated with the same dose of LAMB or (fAMB) Fungizone. In both experiments, similar outcomes were obtained in mice treated with the same amount of AMB in either fAMB or LAMB-IgG except for the increased percent of survivors observed in experiment 2. Mice treated with LAMB-IgG had 45 and 75% of the total as survivors in experiments 1 and 2, respectively. This difference might be due variation in the counts of C. albicans given as inoculum at different times.

In a recent treatment study, Belay et al.(2) reported an improved survival time with 38% survival rate while using

the same neutropenic murine model after treatment with unpurified anti candidal antibodies bound to liposomal amphotericin. In the present study, the results indicate that liposomal amphotericin B targeted by IgG further delayed the death of neutropenic mice and beyond that an overall 65% of mice treated with LAMB-IgG survived the infection for 21 days after infection. However, treatment with 0.9 mg AMB/kg of LAMB-IgG as a high dose, showed no increased survival time over treatment with a dose of 0.6 mg AMB/kg of the same formulation.

Results in the dose-response experiment also indicated that treatment with a dose of 0.9 mg/kg of Fungizone resulted in a sharp mortality rate with 38% survivors. The death of 62% of mice in 2 days despite treatment with the high dose could be associated with the toxicity of Fungizone and not the infection alone. The residual burdens of C. albicans in these mice were also much lower than those of mice that died after receiving a dose of 0.3 or 0.6 mg/kg of Fungizone ($P < 0.05$). In previous studies it was demonstrated that 0.8 mg/kg of Fungizone is the maximum tolerated dose that can be given intravenously to mice in one day (14). In contrast, 3 out of 8 mice survived the infection in this study.

In order to examine the efficacy of the treatment regimens, the residual burdens of C. albicans in spleens, livers, lungs, and kidneys from dead mice and survivors was

determined. Treatment with various doses of AMB in LAMB-IgG or fAMB formulations, including the lower doses, resulted in clearance of C. albicans from the spleens, lungs, and livers of dead or surviving animals with a few exceptions harboring very low levels of the organism. The absence of significant residual burdens in the 3 organs, however, did not succeed in preventing mortality. To the contrary, all mice that died after treatment irrespective of the formulations and doses given had high residual burdens in the kidneys. These findings are in agreement with the fact that the kidneys are the most severely infected organs by C. albicans. A decreased number of C. albicans in the kidneys of survivors was found to parallel the increased rate of survivals seen in this study. Moreover, no mice free of C. albicans were found which is similar to the findings observed in previous studies by Hospenthal et al.(9).

Eventhough a study of tissue distribution and pharmacokinetics of our liposomal formulations has not been performed, the drug complex is mainly directed to the spleens, livers, and lungs compared to the kidneys. Other investigators have reported that the highest concentrations of AMB were detected in the spleens, livers and lungs in mice after administration of amphotericin B lipid complex (5).

Neither a dose-related increased survival nor decreases in the residual burdens of the organism was found with

regard to the doses, 0.6 and 0.9 mg of AMB/kg of LAMB-IgG formulation. Further dose-response studies using wider ranges of AMB content should be carried out to determine the optimal dose of LAMB-IgG that may clear C. albicans from mice.

The use of liposomes as a carrier of AMB has been well described by others to show an interaction of liposomes with fungal cells (17). The present study was intended to further add to selectivity of LAMB by attaching IgG specific C. albicans. The results show that IgG bearing liposomes containing amphotericin B have increased the efficacy in the treatment of systemic candidiasis in neutropenic mice compared to the other preparations suggesting that the targeting is due to the IgG and not other serum components. However, it is not possible to overrule the presence of nonimmune and nonspecific IgG molecules in the product recovered by caprylic acid-ammonium sulphate precipitation method.

The use of larger doses of AMB encapsulated in liposomes complexed with IgG may lead to a complete elimination of C. albicans from the organs including the kidneys. Therefore the effect of higher doses other than 0.9 mg AMB in LAMB-IgG or multiple smaller doses given on a daily basis should be further examined before a definite conclusion is made about value of the complex.

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