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AND CRYPTOCOCCUS NEOFORMANS

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INTRODUCTION

Cryptococcus neoformans, also previously named Saccharomyces neoformans by Sanfelice (76), Torula histolytica by Stoddard and Cutler (84) is a spherical yeast-like fungus which is found in all parts of the world. Recently the sexual state of the organism was described by Kwon-chung (54) and named Filobasidiella neoformans. The organism has been isolated from soil (20,26), particularly when enriched with pigeon droppings (27,50,59). It is also found in pigeon roosts and nests far removed from the soil, e.g., on window ledges and towers of urban buildings (25). Outbreaks of pulmonary infection among workers involved in demolishing old buildings have been reported (25).

Since C. neoformans remains viable in dried excreta of birds, dissemination is thought to be by the organism being aerosolized directly from soil or excreta materials (51,68). In fact, in one study, pigeon droppings were found to be highly contaminated with viable C. neoformans (5×10^7 viable organisms per gram) (51). Although several species of laboratory animals are quite susceptible to C. neoformans infection, birds such as pigeons are highly resistant to it. The explanation may be that the organism

is unable to grow at the normal body temperature of birds which is about 40 to 42°C.

The organism normally reproduces by budding at any point on the surface although rare sexual reproduction has been described (54). The small buds which break off from the parent cells vary in size, ranging from 4 to 20 μm in diameter. The most distinct morphological characteristic of C. neoformans is the presence of a mucoid polysaccharide capsule surrounding the cell. The thickness of the capsule varies with the isolate and is also dependent on the environment in which the organism is growing (24b,60), hence, the actual size of the fungus varies. It has been reported that greater amounts of capsular material are produced when the cells are grown in a low content of carbohydrate (24b). It has been shown that the capsular polysaccharide is anti-phagocytic (53) and is able to induce immunological unresponsiveness (67). These findings attracted debates over the question of whether there is a correlation between the capsule size of C. neoformans and its virulence. Recently, Dykstra et al. (24b) demonstrated that virulence of C. neoformans was not affected by the capsular size of the organism. There was no significant difference in the mortality rate of mice when infected either

with large- or small-encapsulated isolates of C. neoformans.

Early reports on the analysis of the chemical composition of the capsular polysaccharide revealed 4 basic components of the polysaccharide namely, xylose, mannose, glucuronic acid and galactose (11, 74a,b), however, recent reports described by Cherniak et al. (18) and Bhattacherjee et al. (8) failed to detect galactose in their polysaccharide preparations. Although C. neoformans evokes only a feeble humoral immune response in infected humans (7), hyperimmunized rabbits yield capsule-specific antisera that allows the differentiation of four serotypes, A, B, C and D (29, 26, 89).

Fluorescent antibody cross-staining and adsorption studies have shown that serotype A contains all of the antigenic determinants of group D in addition to an A-specific determinant (18, 89). Recently, Bhattacharjee et al. (8) reported that serotypes B and C have similar capsular polysaccharide structures, these results confirmed earlier findings of Evans (29) that B and C serotypes are serologically cross reactive. There are biochemical differences between the A-D group polysaccharides and those of B-C group (6b). These two serogroups also have different geographic distributions. Serotype A is widespread throughout the world, D is found to be rare in the United States

but common in Europe. Serotypes B and C are rare with most cases of infection occuring in Southern California (6a). Another difference among the two serogroups is that serotypes A and D are most commonly found in association with pigeon droppings, while the ecological niche of B and C is still unknown (8).

The disease caused by C. neoformans is called cryptococcosis. The nature of the infectious particle has attracted much debate over the past few years. It is generally believed that the causative agent is a ubiquitous small yeast particle (26, 30, 68,83)but recently the basidiospores of the sexual stage of F. neoformans has been suggested as candidate for infectious particle (24a,54). It is believed that the portal of entry of the infectious particles is via respiratory route (15,41) although the disease has been induced in experimental animals via the skin (25) and oral routes (45,85).

The primary infection in the lungs (pulmonary cryptococcosis) presents no characteristic picture (25,41). Frequently, the patients present a history of prolonged respiratory infection with cough, low grade fever, mild chest pain, and easy fatigibility. These symptoms may be

transient, easily overlooked and subside spontaneously (25). However, if the host resistance is low and the organism's virulence is high, the infection progresses to a clinically recognizable or systemic form (22,25,77, 78, 91). It has been reported that systemic cryptococcosis is manifested generally as meningitis due to the predilection of the organism for the brain and meninges. Invasion of the central nervous system is presumably by hematogenous spread (25). Patients with cryptococcal meningitis frequently complain of intermittent headaches and vomitings. Patients also experience low grade fever, malaise and weight loss. The duration of central nervous system cryptococcosis varies from a few months to 15 or 20 years (25). In chronic cryptococcosis remissions followed by episodes of the disease are common.

It has been reported that host susceptibility is increased in patients with impairment of the reticuloendothelial system as seen in Hodgkin's diseases, lymphosarcoma, leukemia, sacoidosis and tuberculosis (22,77,78,91). Susceptibility is also enhanced if the patients are treated with corticosteroids, or immunosuppressive drugs (61). Currently, meningeal cryptococcosis is the most frequently reported form of the

disease; although many investigators believe that the pulmonary form is very common but is seldom diagnosed (25). Littman and Schnerson (59) speculated that 5000 to 15,000 clinical or subclinical cases of pulmonary cryptococcosis may occur annually in New York City alone. Because the organism is ubiquitous and the disease can be severe when progressed into systemic form, detailed studies on the pathogenicity, mode of infection, methods of diagnosis and therapy and particularly, studies on the host-parasite relationships are warranted.

Until 1957 there was no successful treatment for most forms of cryptococcosis. Cryptococcal meningitis was almost always fatal. However with the use of Amphotericin B, the prognosis has improved (14, 75, 82). A new drug, 5-fluorocytosine which is not nearly as toxic as Amphotericin B is now being used in patients. However, this drug is usually not effective in central nervous system cryptococcosis, because the organism becomes resistant to it. Treatment schedules for Amphotericin B varies but most physicians start treating with 10 mg/day given intravenously with an increase in dosage every day until 60 to 100 mg/day is reached. The drug is sometimes given every other day to decrease the associated side effects (56). Reports have been described that in most patients

receiving adequate doses of Amphotericin B, some side effects develop (64). Renal toxic reaction is most common, and cardiac toxic reaction also occurs and is usually associated with hypokalemia (64). In a recent report, transfer factor (TF), a dialyzable extract of immune lymphocytes which has a molecular weight less than 10,000 has been used for treatment of systemic cryptococcal abscesses (46). Transfer factor therapy was initiated in doses of 3 ml intramuscularly per week and administration was continued for 16 months. The patient was asymptomatic 16 months after therapy. In some past occasions, transfer factor has been a useful adjunct of Amphotericin B in other cases of pulmonary and meningeal crytococcosis (19) as well as other diseases (52,80).

A number of defense mechanisms that enable man and animals to resist a cryptococcal infection have been described. These mechanisms were comprised of both innate and acquired immunity to this infection. Innate immune mechanisms such as the effect of cationic tissue proteins (34,35), phagocytosis by macrophages and monocytes (12, 13, 65), action of complement components (31,63), and fungicidal activities of granulocytes (23, 79) have been described. However, many of these mechanisms (12-13,31-35) do not provide definite sustained protection to the host in

the disease. In recent years much attention has been directed to the acquired immune mechanisms of the host with aims to determine whether these mechanisms are effective in controlling the infection. These acquired host immune mechanisms include both humoral (36,40,47, 48,49) and cell-mediated responses (1,2,3,4,16,21,22,37, 42-44, 57, 58), but the former mechanism, the protective role of antibodies in cryptococcal infection, is questionable. Several workers have been unable to protect experimental animals from a challenge infection with anti-cryptococcal antibodies (40,62); although, others, report that passive transfer of antibodies increased the survival time of infected animals (36). Monga et al. (66) recently provided evidence showing that antibodies were not involved in protection of mice infected with C. neoformans. They infected B-cell-deficient mice, which were prepared by administering rabbit anti-mouse-μ anti-serum to new born animals. The mortality pattern of the mice, the viable counts of cryptococci in different organs, the delayed-type hypersensitivity reactions, and antigen levels in the sera were monitored both in the infected B-cell-deficient and normal control animals. They were unable to detect any differences in the normal and B-cell-deficient animals.

This report provides strong evidence that humoral immune responses are not protective in cryptococcosis. In contrast to this, increasing evidence has been described suggesting that cell-mediated immunity might be the primary means of host defense in the infection (1,2,3,4,16,21, 22,37,42-44, 57, 58). Early workers such as Adamson and Cozad (4) and Diamond and Bennett (22) provided some insight to this problem.

Adamson and Cozad (4) showed that treatment of mice infected with C. neoformans with anti-lymphocyte serum shortened the survival of the infected mice, and 150 times more viable cryptococci were present in the spleens, livers and lungs in the antiserum treated group of animals. Diamond and Bennett (22) showed that patients with disseminated cryptococcosis had a lower lymphocyte transformation response to C. neoformans, their results suggested that this defective function of the lymphocytes may lead to an increased susceptibility to cryptococcal infections.

Since then, several studies have taken more direct approaches in attempts to show cell-mediated immunity is the major mechanism of resistance. These studies are addressed here in this report.

Abrahams et al. (3) showed that when peritoneal leukocytes

from immunized mice were mixed with a challenge dose of live C. neoformans and were injected subcutaneously in an air sac chamber established on the dorsal side of the mice, the leukocytes inhibited the multiplications of the pathogen. Diamond (21) demonstrated that guinea pigs which had been given C. neoformans-sensitized lymphocytes and a challenge of viable cryptococci survived longer than the infected control animals.

Recently, evidence described in the athymic or nude mouse model not only further substantiated the importance of the role of cell-mediated immunity in protection but also put forward indirect evidence that the thymus-dependent immune system, possibly the T lymphocyte functions, might be the major mechanism of host resistance against cryptococcal infection (16, 44). Cauley and Murphy (16) demonstrated that nude mice were more susceptible to C. neoformans infection. They showed that nude mice infected with the pathogen had more viable cryptococci in their spleens, livers and lungs than the phenotypically normal mice. Graybill et al. (44) further showed that nude mice receiving a transplant of thymus tissue had prolonged survival times after challenge with live C. neoformans when compared with untreated nude mice challenged in the same manner.

Lim and Murphy (57, 58) demonstrated that delayed-type hypersensitivity responses to cryptococcal culture filtrate antigen as well as resistance to further challenge with viable cryptococci could be passively transferred to normal naive recipient mice with C. neoformans-sensitized splenic and nylon wool column enriched splenic lymphocytes. The delayed-type hypersensitivity responses and the ability to confer immunity to recipient mice were abolished when the donor cells were treated with anti-Thy-1 antiserum and complement. Results suggested that effector cells were sensitized T lymphocytes.

T lymphocytes have been known to have a number of mechanisms to effect killing or elimination of the infectious agents (5,9,10,17,55,69,70,71). These mechanisms include: (A) Direct cytotoxic effects on target cells by effector T cells. These target cells can be the infected host cells or the pathogen itself (10,17,55,69,70). (B) The production of soluble mediators known as "lymphokines" which are capable of killing or inhibiting the growth of infected host cells or pathogens directly or indirectly (28,38,39,72,73). The lymphokines have a wide spectrum of biological activities (38, 81, 87, 88) that include: (i) lymphotoxin (LT), which is non-specific in action and is capable of lysing a whole

variety of cell types including cells from different animal species (73); (ii) macrophage activating factor (MAF) which can render macrophages more active by enhancing non-specific phagocytic and microbicidal activities (9,28); (iii) chemotactic factor which attracts monocytes, granulocytes, eosinophils and lymphocytes to the foci of the infection so as to allow these cells to exert their effect on the infectious agent (28); and (iv) macrophage inhibition factor (MIF) which reacts with macrophages and prevents their migration (90), hence, the macrophages can fully exercise their effects on the infectious agent.

Although many T lymphocyte actions have been reported on varieties of cell types of infectious agents (5,9,10,17, 55, 69, 70, 71), their effects on C. neoformans have not been completely investigated. Lymphokines were shown to exert direct killing on yeast cells of Saccharomyces cerevisiae, Candida albicans (72) and recently on Blastomyces dermatitidis (39); however, their effect on C. neoformans has not been described. It was the objective of this study to first develop an in vitro assay for detecting C. neoformans-sensitized lymphoid cells in the spleens of mice previously immunized with heat-killed C. neoformans, and to examine

more directly the effect(s) of these lymphoid cells and their soluble mediators on the pathogen by monitoring their interactions in an *in vitro* system. With the *in vitro* system, further characterization of the effector cells and their specificities of action could be elucidated.

As mentioned earlier, very little was known concerning the exact host defense mechanism of man and animals which effectively eliminates a cryptococcal infection. These studies as described may provide a deeper insight and understanding of the host-parasite interactions in order to resolve the exact role played by the T lymphocytes in cryptococcosis which in turn may allow one to predict or project what is possibly happening in human cryptococcosis. When the T cell mechanism in resistance to cryptococcosis is completely resolved, it will be possible to design ways of potentiating or enhancing the host immune response to prevent or eliminate a cryptococcal infection.

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PAPER I

IN VITRO INTERACTIONS OF IMMUNE LYMPHOCYTES AND *CRYPTOCOCCUS NEOFORMANS*

SECTION I

INTRODUCTION

Cryptococcosis is a mycotic disease caused by an encapsulated yeast-like fungus, *Cryptococcus neoformans*. The distribution of this organism is world wide, commonly found in pigeon droppings (31,41). The route of infection in humans and animals is believed to be through inhalation of the small yeast particles; although inhalation of basidiospores, the spore of the perfect stage, *Filobasidiella neoformans*, has been suggested recently (16,36,58) as a possibility. The most common clinical forms of cryptococcosis are pulmonary and meningeal. It is believed that the pulmonary form of the disease is transitory and precedes the systemic form of the disease (17). Whether the organism disseminates and is manifest as meningitis depends on the numbers and virulence of the invading pathogen and the immunological status of the infected individual (12,57,65). It has been shown that patients with underlying immunodeficient disease such as Hodgkin's diseases, leukemia

or tuberculosis or patients being treated with immunosuppressive drugs such as corticoidsteriod are highly susceptible to cryptococcal infections (42,65). Such findings have resulted in the direction of attentions to investigations of the host defense mechanisms in cryptococcosis. The requirement for greater understanding of the host-parasite relationships in cryptococcosis is necessary in order to develop better methods for diagnosis, prognosis and treatment of the disease.

A number of defense mechanisms have been described that enable man and animals to resist cryptococcosis. These defenses are comprised of both humoral and cell-mediated immune mechanisms (1,2,8,9,11a-14,21-23,26,29,39 40,44,50,65). In recent years, increasing evidence suggests that cell-mediated immunity (CMI) is the primary means of host defense (1,2,9,12,21-23,40,50). The murine cryptococcosis model has provided insights on the interactions of the host's immune systems and C. neoformans (39,40). The studies of C. neoformans infections in the athymic or nude mouse model substantiated the importance of the role of the T lymphocyte in protection against the infection (9,22). Lim and Murphy (40) recently showed that the delayed-type hypersensitivity (DTH) response to cryptococcal culture filtrate (CneF) antigen as well as resistance to

further challenge with viable cryptococci could be passively transferred to normal naive recipient mice with C. neoformans sensitized T lymphocytes; thereby, establishing that CMI plays an important role in host defense in cryptococcosis.

It was the objectives of the present study to develop an in vitro assay system for detecting effector C. neoformans-sensitized lymphoid cells and to examine more directly the role of these cells in inhibiting the growth of C. neoformans by monitoring their interactions in the in vitro system. With this system, further characterization of the effector cell populations and their specificities of action were elucidated.

SECTION II

MATERIALS AND METHODS

Mice. Inbred CBA/J mice purchased from Jackson Laboratory, Bar Harbor, Maine, were bred in the animal facilities in the Department of Botany and Microbiology, University of Oklahoma. Mice of both sexes, aged 10 to 12 weeks were used in all experiments of this study.

Organisms and target cells. C. neoformans isolate 184A, described by Murphy and Cozad (53) was used in this study both for the sensitization of animals and for the in vitro growth inhibition assays. The cultures were grown on modified Sabouraud's agar (MSAB) slants and were maintained by biweekly passage on the same medium. Candida albicans and Saccharomyces cereviseae were obtained from the stock culture collection of the Department of Botany and Microbiology, University of Oklahoma and were also maintained on MSAB slants. L929 mouse fibroblast and YAC-1 lymphoma cells were used in the microcytotoxicity ^{51}Cr release assay for the determination of cytotoxic activity of the lymphocyte populations.

Cryptococcal antigen. The cryptpcoccal culture filtrate (CneF) antigen used in this study was prepared according to the procedures described by Cauley and Murphy (9).

This preparation contained 4.0 mg protein and 4.0 mg carbohydrate per ml as determined by the Lowry procedure (45) and phenol-sulfuric acid method (15), respectively. Bovine serum albumin solution was used as a standard and Monitrol I (Dade Division American Hospital Supply Corp., Miami Fla.) as a control for the protein determination. Mannose and mannan served as standard and control, respectively, in the carbohydrate determination.

Antisera. Anti-Ia^k and anti-mouse immunoglobulin (anti-MIg) sera were provided by the courtesy of Dr. John Moorhead, University of Colorado Medical School, Denver, Colorado. These sera have been described and characterized previously by Moorhead and coworkers (51,52). Briefly, anti-Ia^k serum was prepared by giving A.TH mice multiple i.p. injections of A.TL spleen cells. Both ^{51}Cr and trypan blue dye exclusion tests revealed that the anti-Ia^k serum, at a final dilution of 1/160 plus complement killed 45-60% of CBA/J spleen cells; whereas, less than 1% of Balb/c spleen cells were killed with the same dilution. The anti-MIg was raised in rabbits to Salmonella adelaide coated with mouse anti-Salmonella adelaide antibodies. The antiserum contained antibodies directed against both heavy and light chain determinants of all mouse immunoglobulins. Rabbit anti-MIg serum was inactivated at

56°C for 30 min and absorbed with mouse erythrocytes and thymocytes. The anti-Thy-1 serum was provided by Dr. Paul Barstad, Department of Botany and Microbiology, University of Oklahoma. The serum was prepared by immunizing goats with a preparation of purified Thy-1 antigen (66). The goat anti-Thy-1 serum had a titer of 1:16,000 on CBA/J thymocytes when assayed in trypan blue dye exclusion assay. A 1:14 dilution of low toxicity rabbit serum which had 5-13% cytotoxicity on CBA/J thymocytes as determined by the trypan blue dye exclusion method was used as the complement source. A 1:100 dilution of anti-Thy-1 serum with rabbit complement killed 75-86% of the CBA/J thymocytes whereas 1:500 dilution killed 98-100% and 1:1000 dilution killed 64-71% of the same cell preparation. Functional tests with mitogens showed that pretreatment of CBA/J splenic cells with dilution of 1:100, 1:500 and 1:1000 of anti-Thy-1 serum plus rabbit complement inhibited the phytohemagglutinin (PHA) response by 72%, 91% and 63%, respectively, but had no effect on the endotoxin lipopolysaccharide (LPS) response. These results indicated that the antiserum was specifically killing the T lymphocytes without affecting the B lymphocytes. Dilutions of 1:10 of the anti-Ia^k, anti-MIg or normal mouse sera and 1:100, 1:500 or 1:1000 dilutions of the anti-Thy-1 serum were used for

treatment of splenic and T-enriched lymphocyte populations.

Antiserum treatment of cells. For treatment of cells, 1×10^7 cells were pelleted in 12 x 75 mm tubes,(Falcon plastic Oxnard, Calif.), 1 ml of the diluted antiserum was added and the tubes were mixed by gentle shaking. After incubation at 4°C for 1 h, the cells were washed before adding 1 ml of a 1:14 dilution of rabbit complement. The cell suspensions were then incubated for 10 min on ice followed by 35 min at 37°C. The cells were washed 3 times, resuspended in original 1 ml volume medium and the viability was determined.

Sensitization of animals. C. neoformans was first grown on fresh MSAB slants for 3 days and the cells were harvested in sterile physiological saline (SPSS) and were washed 3 times in SPSS. The washed cells were heat killed by incubating at 60°C for 60-90 min depending on the number of cryptococci. After heat killing, viability was determined by plating 0.1 ml aliquots of the suspension on MSAB plates. Mice were injected subcutaneously with an emulsion prepared by mixing equal volumes of heat-killed C. neoformans and complete Freund's adjuvant (CFA). Each mouse was given a total of 1×10^7 heat-killed cryptococci in two 0.05 ml volumes at two sites on the upper abdomen as described by Graybill and Taylor (21).

Control mice received either SPSS-CFA emulsion in the same fashion or remained unsensitized.

Delayed-type hypersensitivity (DTH) response. Footpad testing for DTH responsiveness to CneF was performed according to the procedures described by Cauley and Murphy (9). A net increase of 0.3 mm or more in footpad swelling was considered a positive response.

Splenic lymphocyte preparation. Spleens were collected from sensitized or control groups of mice. Single cell suspensions were prepared by pressing spleens through a 100 mesh stainless steel screen into sterile Dulbecco's phosphate buffered saline (DPBS) solution. Red cells were lysed by treating the cell pools with Tris-NH_4Cl buffer (pH 7.2) (7); then the cells were washed 3 times in DPBS solution before being resuspended in RPMI-1640 culture medium (Grand Island Biological, New York) supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin and 10% fetal calf serum (FCS, Flow Laboratories, Virginia).

Nylon wool enrichment for T and B lymphocytes. Nylon wool (Leukopak Leukocytes Filter, Fenwal Laboratories, Deerfield, Ill.) was washed and used according to the methods of Julius et al (30) and Handwerger and Schwartz (24) for

separation and enrichment of T and B cells, respectively. The recovery of non-adherent cells (T cells) ranged from 25 to 38% and consisted of 98 to 100% T lymphocytes as determined by trypan blue dye exclusion after treatment with with anti-Thy-1 serum and complement; less than 1.5% B lymphocytes as determined by staining with rabbit anti-MIg and fluorescein-labelled goat anti-rabbit Ig; and less than 0.8% macrophages based on non-specific esterase staining (34). Forty percent of the cells put on the column were recovered in the adherent (B cells) cell populations. They consisted of 70-84% immunoglobulin positive cells and 10-15% T lymphocytes as determined by methods described above. Viability of both non-adherent and adherent cell populations exceeded 95% as determined by trypan blue dye exclusion.

In vitro growth inhibition assay.

Effector cells (splenic, T, or B enriched lymphocytes) in medium RPMI-1640 supplemented with penicillin and streptomycin and 10% FCS were incubated together with target cells (C. neoformans, C. albicans or S. cerevisiae) in 16 x 125 mm tissue culture tubes (Corning glass, Corning, N.Y.) at 37°C in an atmosphere of 5% CO_2-95% air for 24 h unless otherwise specified. Target cell suspensions were adjusted to 1 x 10^5 cells/ml and 0.1 ml was added to the tissue culture tubes. Effector cells were then added from a stock

suspension of 1 x 10^7 cells/ml to achieve final effector to target cell ratios (E:T) of 100:1, 200:1, 300:1, 400:1 or 500:1. The final volume of each assay tube was 1.1 ml. Assays with each ratio were performed in duplicate. The controls consisted of target cells (0.1 ml) incubated with 1.0 ml RPMI-1640 culture medium in the absence of effector cells. After a 24 h incubation (unless otherwise specified), aliquots of the reaction mixtures were removed, diluted and plated in duplicate on MSAB plates. Colony counts were made 3 days after plating. The effects of lymphoid cells on the target cells such as C. neoformans or other yeasts cells were expressed as percent inhibition of growth which was determined by the following formula :

$$\% \text{ Inhibition of growth} = \frac{\#\text{ colonies (control - test)}}{\#\text{ colonies in control}} \times 100$$

The protocol for setting up the in vitro growth inhibition assay is shown in Fig. 1.

Microcytotoxicity assay. Target cells L929 mouse fibroblast and YAC-1 lymphoma cells were grown in RPMI-1640 medium containing penicillin and streptomycin and 2 or 10% FCS for L929 or YAC-1 cells, respectively. Cells were adjusted to 1 x 10^7 cells/ml and were labelled with 200 µCi of ^{51}Cr (New England Nuclear, Boston, Mass.) for 1 h (47).

After 3 washings to remove excess unbound ^{51}Cr, the cell suspensions were adjusted to 2 x 10^5 cells/ml in culture medium. Effector splenic or T-enriched lymphocytes suspensions obtained from sensitized or control mice on day 8 after immunization were adjusted to 1 x 10^7 cells/ml. A volume of 0.1 ml each effector cell pool and target cell suspension was transferred into wells of U-bottom tissue culture multi-well plates (Linbro, Hamden, Connecticut). After a 4 h incubation at 37°C in an atmosphere of 5% CO_2-95% air, the cells were pelleted and 0.1 ml of each supernatant was transferred into a tube and counted in a gamma-counter (Packard, Chicago, Ill.). Percentage specific ^{51}Cr release was calculated according to the following formula :

$$\% \ ^{51}Cr \text{ released} = \frac{T - S}{M - S} \times 100$$

where T represents counts per minute (cpm) of ^{51}Cr released in the presence of effector cells; S represents the spontaneous ^{51}Cr released determined by incubating target cells in media; and M represents the maximum ^{51}Cr released upon addition of 2N HCl. All determinations were done in triplicate.

Pretreatment of C. neoformans-sensitized splenic cells with CneF antigen or Actinomycin D. Splenic cells were prepared from mice at day 8 after immunization with heat-

killed cryptococci-CFA emulsion. For treatment with CneF antigen, 1 x 10^7 C. neoformans-sensitized or normal splenic or T-enriched lymphocytes were incubated with CneF antigen containing 100 µg protein and 100 µg carbohydrate in 2 ml RPMI-1640 culture medium at 37°C for 24 h. Cells were washed 3 times, and the viability of the cells was determined by trypan blue dye exclusion test before the cells were put into the in vitro growth inhibition assay. For treatment with Actinomycin D, C. neoformans - sensitized splenic cell suspensions of 1 x 10^7 cells/ml were prepared in tissue culture tubes (Corning Plastics, Corning, New York). Actinomycin D was added to cell suspensions to give final concentrations of 0.5, 1.0, 1.5 or 2.0 µg/1 x 10^7 cells. The mixtures were cultured at 37°C in 5% CO_2-95% air for 24 h. After treatment cells were washed 3 times, and their viability was determined by trypan blue dye exclusion test before the cells were put into the in vitro growth inhibition assay.

Statistical analysis. The means, standard errors of the means and statistical evaluations of the data were analysed with a Hewlett-Parkard calculator model 9810A.

SECTION III

RESULTS

DTH profile and the development of effector cells. At day 2, 4, 6, 8, 16 and 24 after immunization, 4 to 5 mice from sensitized or control groups were footpad tested with CneF antigen to determine DTH responsiveness. Mice from control groups did not show increased footpad swelling in response to CneF antigen; however, mice sensitized with heat-killed cryptococci-CFA emulsion began showing positive DTH responses by day 6 after immunization. The intensity of the responses continued to increase significantly and reached a maximum between 8 and 16 days post immunization (Fig. 2)

Kinetic studies on the development of effector cells capable of inhibiting the growth of C. neoformans were performed using the in vitro inhibition assay. Splenic cells for this assay were collected at the same time periods after immunization that DTH responses were measured. Effector to target cell ratios (E:T) of 100:1, 200:1, 300:1, 400:1 and 500:1 were used in the experiments, and the cell suspensions were incubated at 37°C for 24 h. The results are shown in Fig. 3. Effector cells from control mice did not inhibit the growth of C. neoformans; however,

effector cells from mice sensitized 6 days previously were inhibitory to the cryptococci. By day 8 after immunization, the growth inhibition activity attained a maximal level and continued high through day 24. The highest inhibition of growth was observed at E:T ratios above 300:1; whereas E:T ratios less than 300:1 gave negligible or poor activities. For convenience, in further experiments effector cells were collected on day 8 after immunization, and an E:T ratio of 300:1 was used unless otherwise specified.

Effects of T and B enriched lymphocytes on C. neoformans. To determine whether the effector cells, which were capable of inhibiting the growth of C. neoformans, were T or B lymphocytes, sensitized splenic lymphocytes collected on day 8 after immunization were separated on a nylon wool-column into adherent (B) and non-adherent (T) pools and were tested for growth inhibitory ability on the pathogen. Effects of immune B-enriched cells on C. neoformans were determined at E:T ratios of 100:1 and 300:1, whereas effects of unfractionated immune splenic and T-enriched cells on the pathogen were determined at E:T ratios of 100:1 through 500:1. Results showed that unfractionated C. neoformans-sensitized splenic cells were able to inhibit the growth of C. neoformans at E:T ratios of 300:1, 400:1 and 500:1 (Fig. 4); whereas the sensitized nylon wool T-

enriched populations inhibited the growth of the pathogen at all E:T ratios examined. The percent growth inhibition values of T-enriched population were not significantly different among each E:T ratio. The nylon wool adherent or the B-enriched populations were unable to inhibit the cryptococci at an E:T ratio of 100:1, and exhibited a significantly lower ability to inhibit the growth of C. neoformans at an E:T ratio of 300:1 than the unfractionated splenic or T-enriched effector cell populations ($p<0.01$).

The percentage of inhibition exhibited by the T-enriched lymphocytes appeared to be higher than those of the unfractionated splenic populations at all E:T ratios examined especially at E:T ratios of 100:1 and 200:1. Normal groups of effector cells did not exhibit inhibition of growth of C. neoformans at all E:T ratios examined.

Kinetics of growth inhibition. Inhibition of growth of C. neoformans by sensitized effector cells was analyzed at various times after effector and target cells were mixed. Growth inhibition patterns exhibited by sensitized groups of splenic and T-enriched cell populations were identical in that inhibition of growth was observed as early as 4 h, then growth inhibition increased gradually with time, peaking at 24 h. By 48 h the ability to inhibit the growth of the target cells dropped significantly. Control cell populations

showed no effect on C. neoformans at any time periods. The inhibition of growth was higher with T-enriched cells as effectors than with whole unfractionated splenic cells. The inhibitory activities of the unfractionated sensitized splenic and T-enriched cells were significantly different ($p < 0.05$) after 24 h incubation, the time when both effector cell populations displayed maximum effect on the organism (Fig. 5).

Supplement with medium and lymphoid cells. In all experiments performed in this study, it appeared that the maximum inhibition of growth of C. neoformans occured at 24 h, but by 48 h the growth inhibitory activities had substantially waned. The loss of inhibitory activity could have been due to either death of effector cells or due to the lost of cellular function resulting from the depletion of essential nutrients in the reaction medium. To see if the inhibitory ability could be maintained or restored beyond 24 h, equal volumes of fresh medium or an additional 3×10^6 sensitized effector cells were added to the reaction mixtures at 12 and 24 h, and were allowed to incubate up to 48 h. Inhibition of growth of C. neoformans was enhanced significantly at 48 h in those groups in which the mixtures received supplements of either fresh medium or effector cells (Table 1). A two-fold or greater inhibition of growth at 48 h was observed

in those groups receiving either supplement.

Pretreatment of effector cells with CneF antigen. Sensitized effector cells pretreated for 24 h with CneF antigen containing 100 μg protein and 100 μg carbohydrate were used in the in vitro growth inhibition assay. The CneF antigen suppressed the growth inhibitory activity of the immune cells (Table 2). This suppression was not due to cytotoxic effects of the CneF antigen on the effector cells since the effector cells treated with CneF in this manner were 98% viable as determined by trypan blue dye exclusion (results not shown).

Further characterization of effector cells. Earlier experiments in this study strongly indicated that the nylon wool non-adherent cells were the major effector cells responsible for the inhibition of growth of C. neoformans. This finding was further substantiated by the observations that the ability to inhibit the growth of cryptococci by nylon wool non-adherent cells could be abolished by pretreatment of the effector cells with anti-Thy-1 serum and rabbit complement (Fig. 6). A 1:100 or 1:500 dilution of the anti-Thy-1 serum completely abolished the inhibitory activity of the effector cells and a 1:1000 dilution of anti-Thy-1 serum which had been shown to reduce T cell response to PHA by 63%, reduced the C. neoformans growth inhibition activity of splenic and T-enriched lymphocytes by approximately 25 and 42%, respectively.

Pretreatment of effector splenic or T-enriched cells with a 1:10 dilution of anti-Ia^k serum and complement also abolished their growth inhibitory activity. However, pretreatment of the same effector cell populations with a 1:10 dilution of normal mouse serum or anti-MIg serum and complement or complement alone had no effect on their growth inhibitory abilities (Fig. 7).

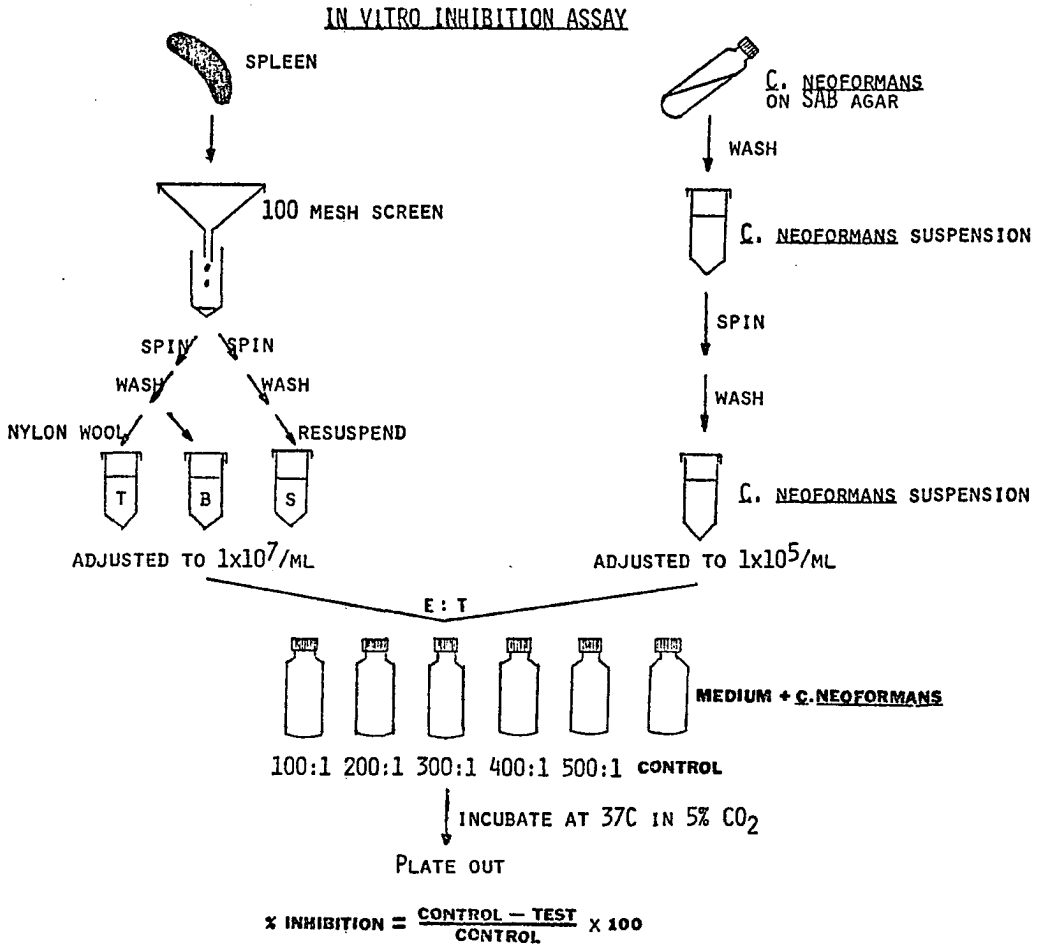
Growth inhibition specificity. The effector cells capable of inhibiting the growth of C. neoformans were tested for their growth inhibitory effects on other yeasts. Candida albicans and Saccharomyces cerevisiae were incubated with sensitized and normal T-enriched lymphocytes at an E:T ratio of 300:1 under the same conditions described previously in this study. Sensitized T-enriched lymphocytes inhibited the growth of C. neoformans but had no effect on C. albicans. However, unexpected results were observed with S. cerevisiae, this organism was inhibited in excess of 90% by normal T lymphocytes as well as sensitized T lymphocytes (Table 3).

Effect of Actinomycin D treatment on the ability of C. neoformans-sensitized splenic lymphocytes to inhibit the growth of C. neoformans. The objective of this study was to provide evidence for the fact that RNA was required for growth inhibition of C. neoformans. Sensitized

splenic lymphocytes pretreated with actinomycin D had a significantly decreased ability to inhibit the growth of C. neoformans than did the untreated sensitized splenic lymphocytes (Fig. 8). The decrease in the ability to inhibit the growth of the pathogen by the sensitized splenic cells was dependent on the amount of actinomycin D used for treatment. Treatment with 2.0 μg/1 x 10^7 cells resulted in the complete abolishment of the growth inhibitory activity; whereas, treatment with 0.5 μg of the drug reduced the growth inhibitory ability from 78.0 to 54.3%.

Cytotoxicity assays. To determine whether the sensitized lymphoid cells had non-specific cytotoxic effects on cell lines, sensitized splenic and T-enriched lymphocytes prepared from immunized and normal unsensitized mice were assayed for cytotoxic activities on L929 or YAC-1 cells. Results are shown in Table 4 and revealed that neither effector cell population exhibited significant cytolytic effects on L929 or YAC-1 cells.

Fig. 1. Diagramatic illustrations of the protocol for the *in vitro* growth inhibition assay. Spleens were collected at various times after immunization from *C. neoformans*-sensitized or unsensitized mice. Single cell suspensions from the spleens were prepared by pressing spleens through a 100 mesh stainless steel screen. Red cells were lysed with Tris-NH_4Cl buffer before passing through nylon wool columns for T (non-adherent) and B (adherent) cell enrichment.
E:T = Effector to target cell ratio.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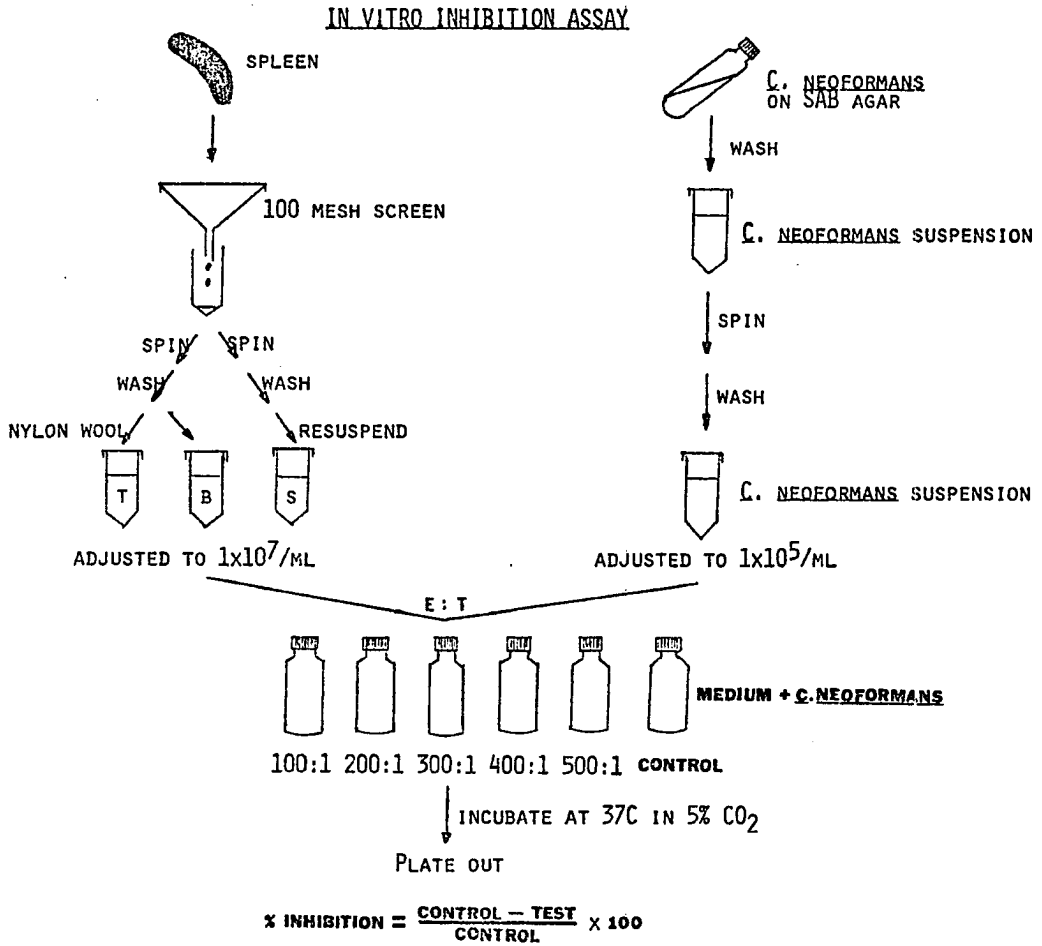
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PLATE OUT
% INHIBITION = CONTROL − TEST / CONTROL X 100

Fig. 2. Profile of the development of delayed-type hypersensitivity (DTH) responses to cryptococcal culture filtrate antigen in mice after immunization with 1 x 10^7 heat-killed cryptococci-CFA emulsion. After premeasurement of footpads, 0.03 ml CneF antigen or saline was injected into the left or right rear footpad of each mouse, respectively. Footpads were remeasured after 24 h. A net increase of 0.3 mm or more in footpad thickness was considered a positive DTH response. Vertical bars indicate the standard errors of the means.

C. neoformans + CFA

Saline + CFA

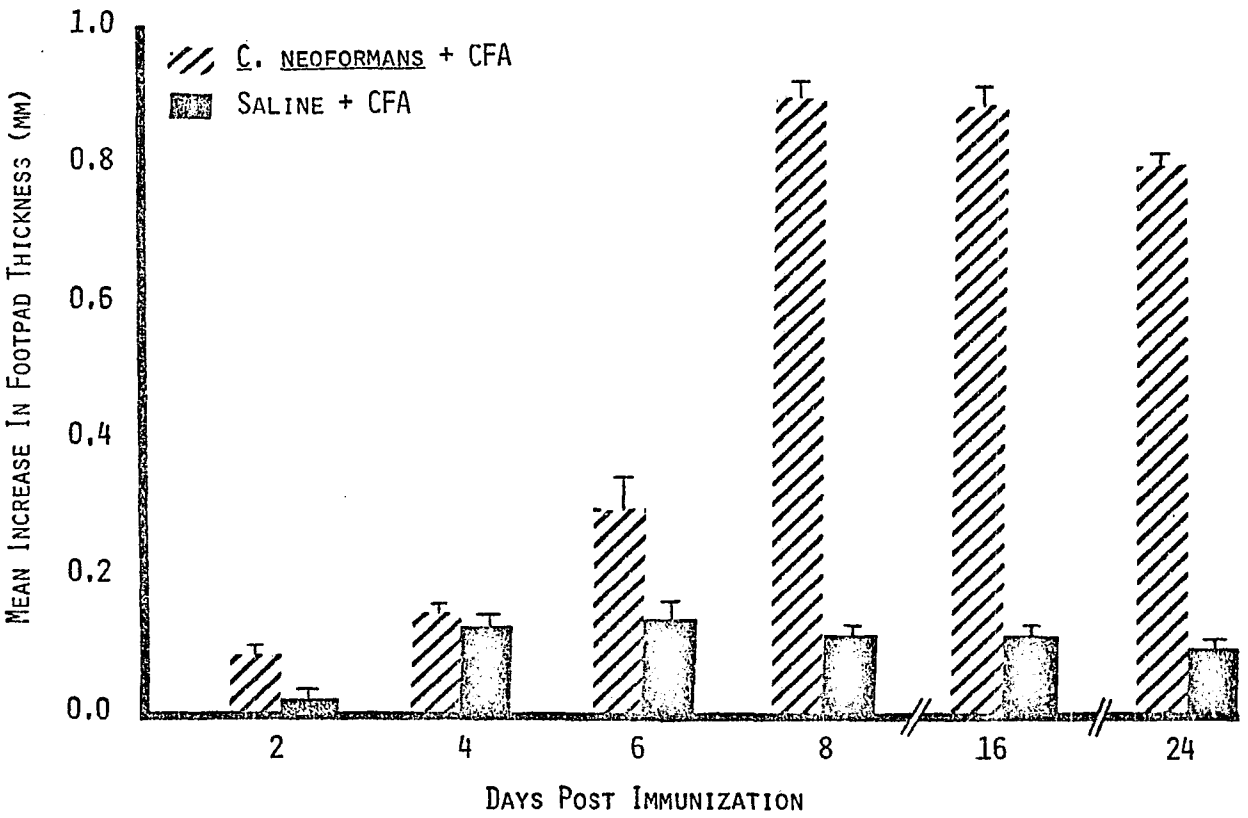
1.0
0.8
0.6
0.4
0.2
0.0
C. NEOFORMANS + CFA
SALINE + CFA
MEAN INCREASE IN FOOTPAD THICKNESS (MM)
2
4
6
8
16
24
DAYS POST IMMUNIZATION

Fig. 3. Profile of the development of effector splenic cells which were capable of inhibiting the growth of C. neoformans as measured with an in vitro growth inhibition assay.

———— C. neoformans-sensitized splenic cells

..... SPSS-CFA sensitized splenic cells

---------- Normal unsensitized splenic cells

Incubation time was 24 h at 37°C

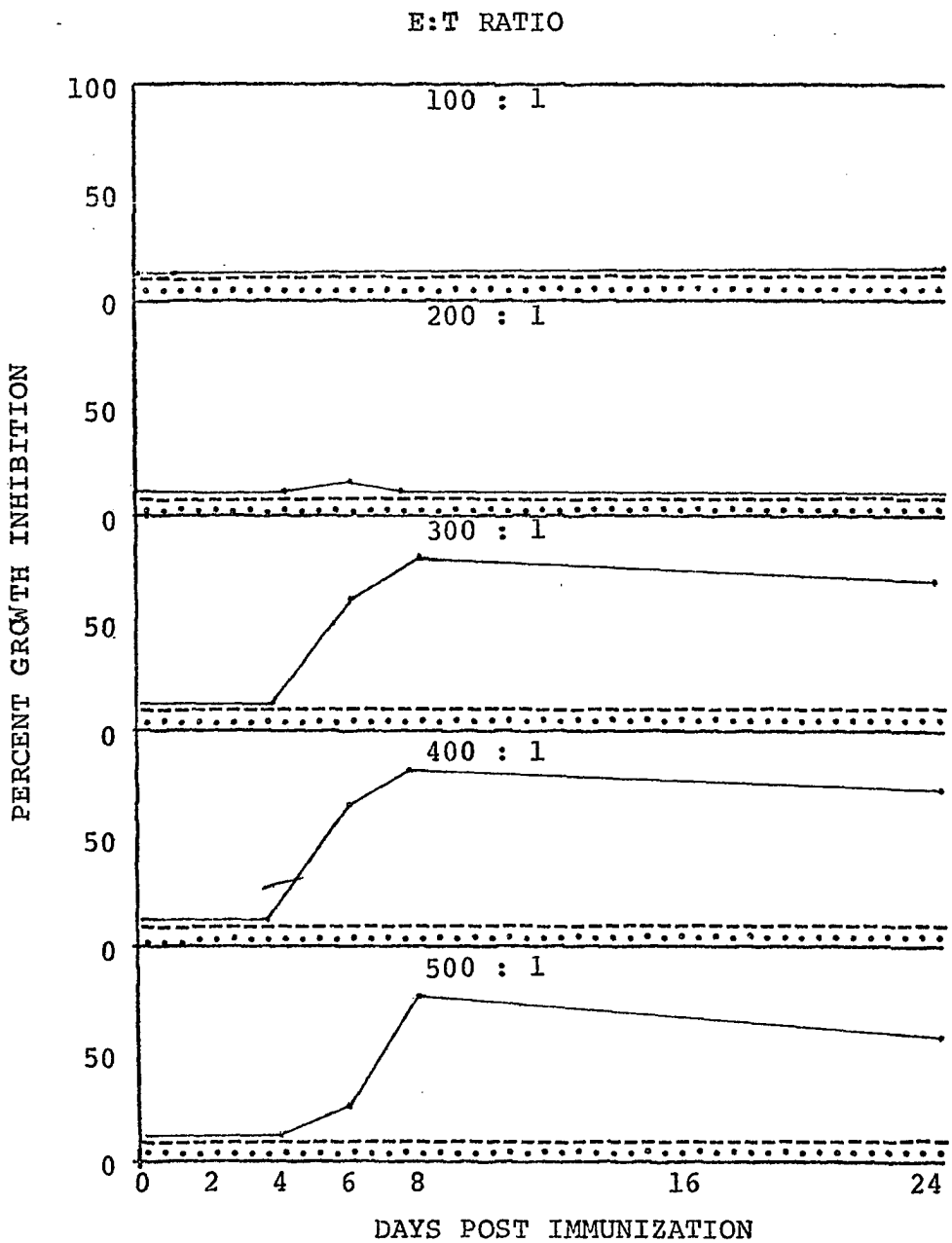
E:T RATIO
100 : 1
200 : 1
300 : 1
400 : 1
500 : 1
100
50
0
50
0
50
0
50
0
50
0
PERCENT GROWTH INHIBITION
0
2
4
6
8
16
24
DAYS POST IMMUNIZATION

Fig. 4. Comparison fo the ability of sensitized splenic, B- and T-enriched lymphocytes to inhibit the growth of C. neoformans at various effector : target cell ratios. Effector lymphoid cells were collected from mice on day 8 after immunization and were incubated with target cells at 37°C for 24 h.
Vertical bars represent standard errors of the means.

C. neoformans-sensitized unfractionated splenic lymphocytes

C. neoformans-sensitized splenic T-enriched lymphocytes

C. neoformans-sensitized splenic B-enriched lymphocytes

Normal unfractionated splenic lymphocytes

Normal splenic T-enriched lymphocytes

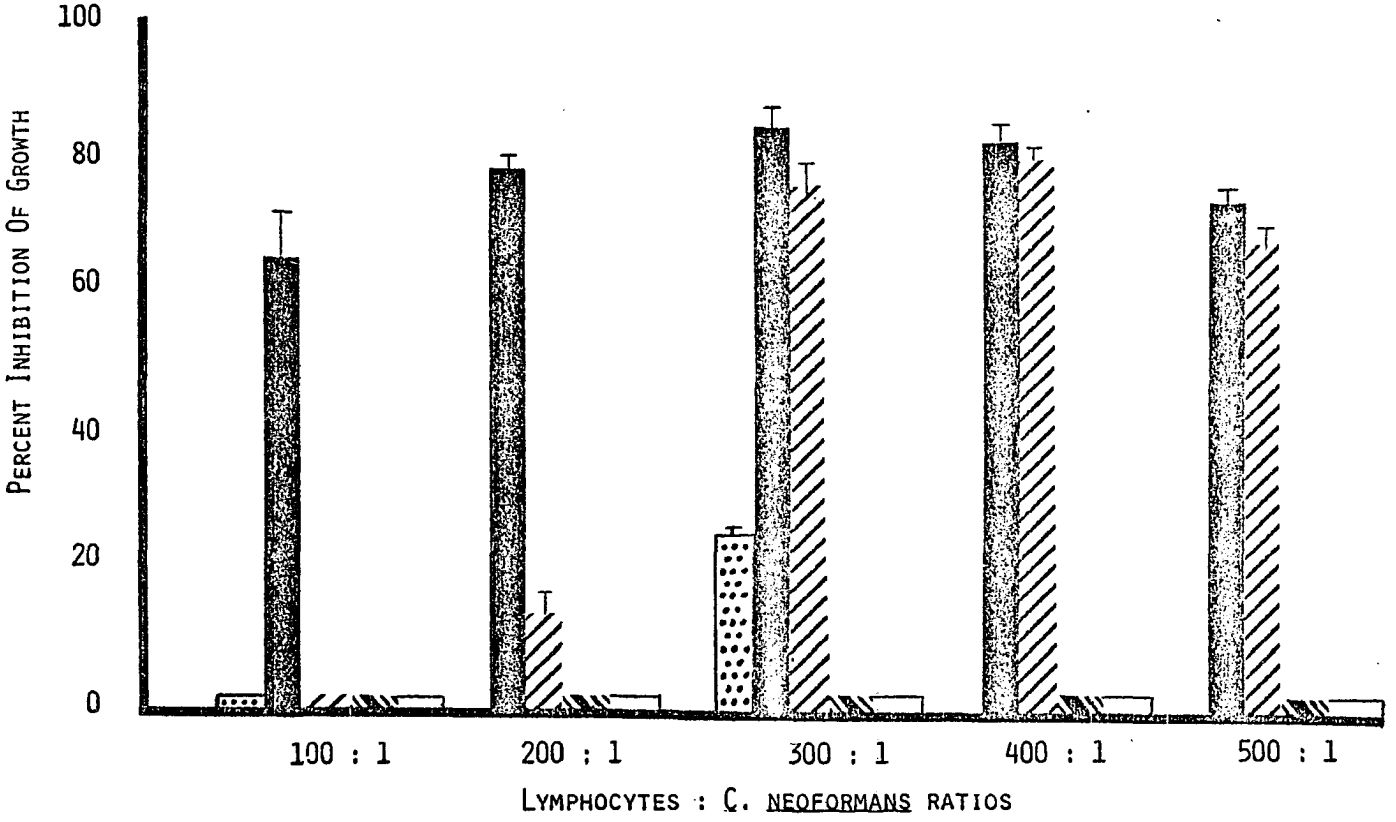
100
80
60
40
20
0
PERCENT INHIBITION OF GROWTH
100 : 1
200 : 1
300 : 1
400 : 1
500 : 1
LYMPHOCYTES : C. NEOFORMANS RATIOS

Fig. 5. Kinetic studies of the growth inhibition effects by immune splenic and T-enriched lymphocytes on C. neoformans as targets. Effector cells, collected 8 days after immunization with heat-killed cryptococci or saline, were incubated with C. neoformans at an E:T ratio of 300:1 at 37°C in 5% CO_2-95% air for various period of times.

o—o Immune T-enriched lymphocytes

o—o Immune splenic lymphocytes

▲—▲ Normal T-enriched lymphocytes

ϕ—ϕ Normal splenic lymphocytes

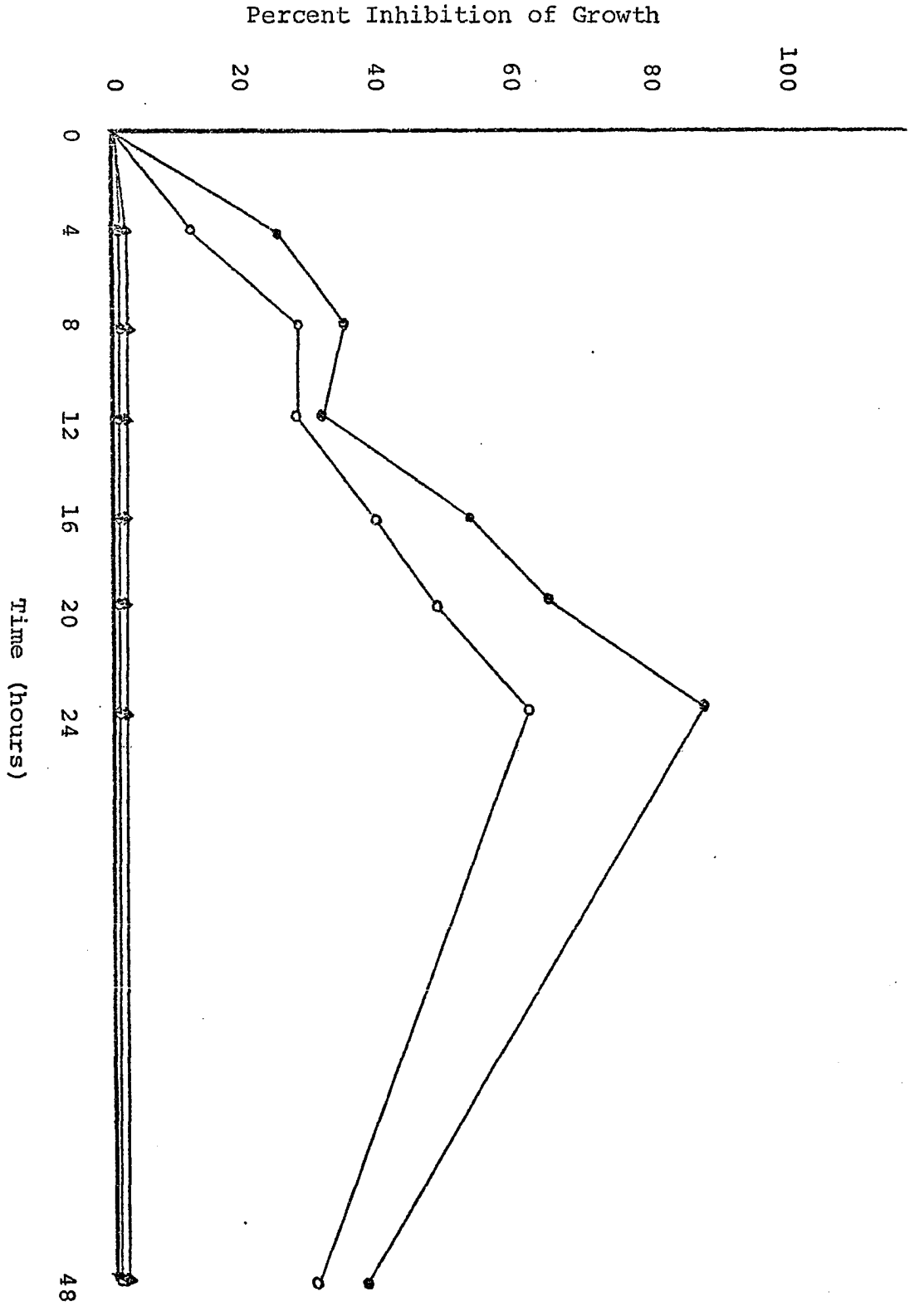
Percent Inhibition of Growth
0
20
40
60
80
100
Time (hours)
0
4
8
12
16
20
24
48

Fig. 6. Effects of anti-Thy-1 and rabbit complement on the ability of sensitized splenic and T-enriched cells to inhibit the growth of C. neoformans.
Effector cells were collected on day 8 after immunization. Effector to target cell ratio was 300:1. Incubation time was 24 h at 37°C.

Immune splenic lymphocytes

Immune splenic T-enriched lymphocytes

C' Rabbit complement

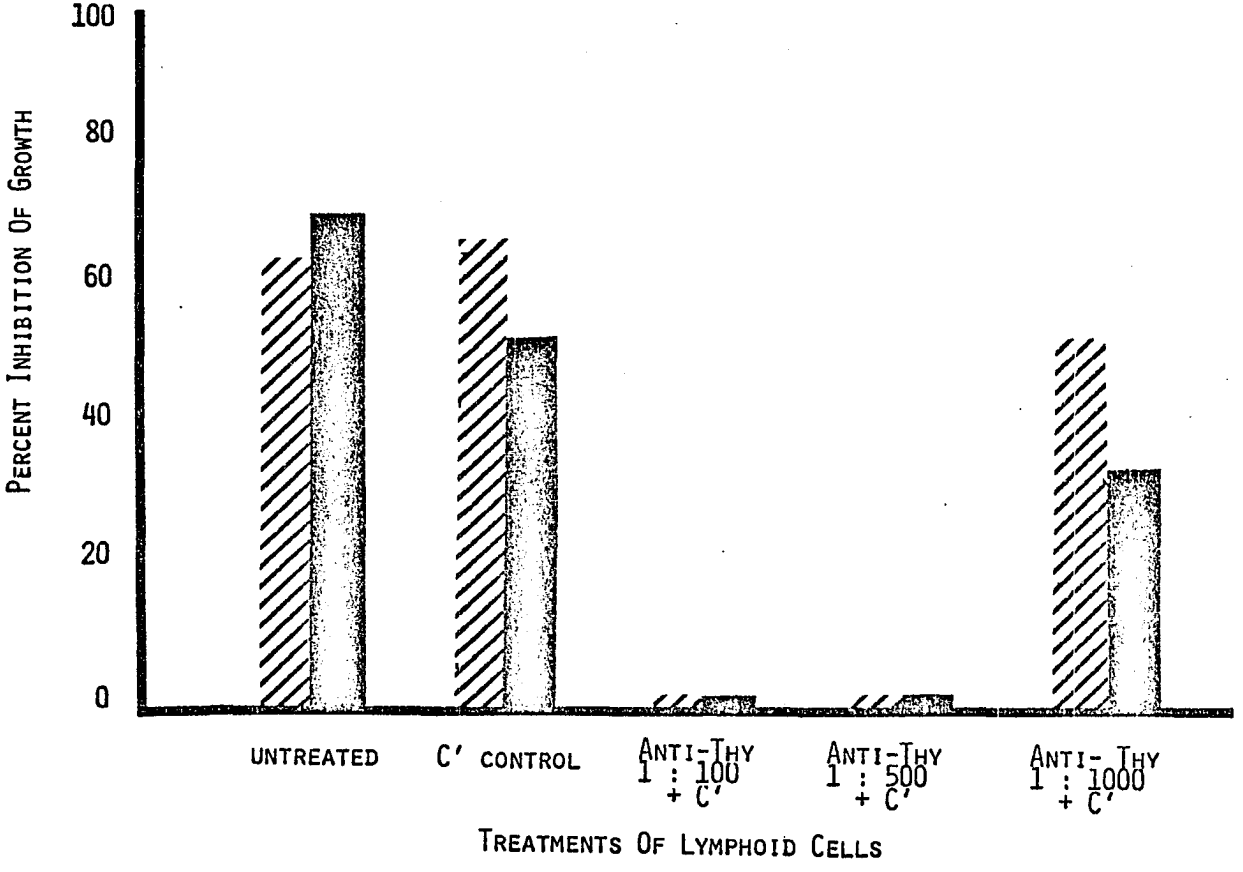
100
80
60
40
20
0
PERCENT INHIBITION OF GROWTH
UNTREATED
C' CONTROL
ANTI-THY 1 : 100 + C'
ANTI-THY 1 : 500 + C'
ANTI- THY 1 : 1000 + C'
TREATMENTS OF LYMPHOID CELLS

Fig. 7. Effect of normal mouse serum, anti-Ia^k serum and anti-MIg serum plus complement on the ability of sensitized effector splenic and T-enriched lymphocytes to inhibt the growth of C. neoformans.
Effector cells were collected from mice on day 8 after immunization. Effector to target cell ratio was 300:1. Incubation time was 24 h at 37°C.

▒ C. neoformans-sensitized splenic lymphocytes

▨ C. neoformans-sensitized T-enriched lymphocytes

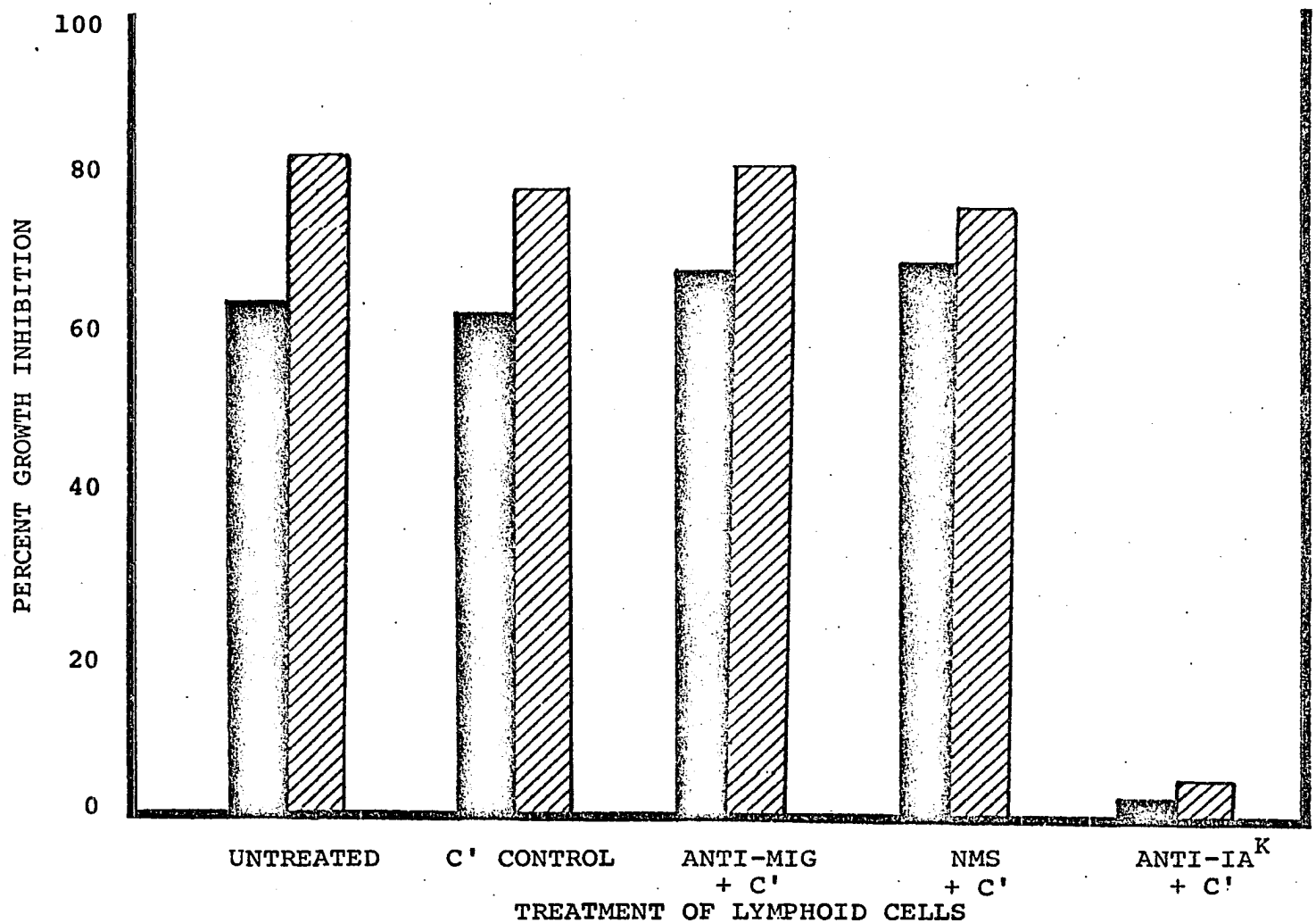
100
80
60
40
20
0
PERCENT GROWTH INHIBITION
UNTREATED
C' CONTROL
ANTI-MIG + C'
NMS + C'
ANTI-IA^K + C'
TREATMENT OF LYMPHOID CELLS

Fig. 8. Effect of Actinomycin D treatment on the ability of sensitized effector lymphoid cells to inhibit growth of C. neoformans. Effector splenic cells were collected from the C. neoformans-sensitized mice at day 8 after immunization, cells were subjected to treatment with various concentrations of Actinomycin D for 3 hours before washing and putting in the in vitro growth inhibition assay.

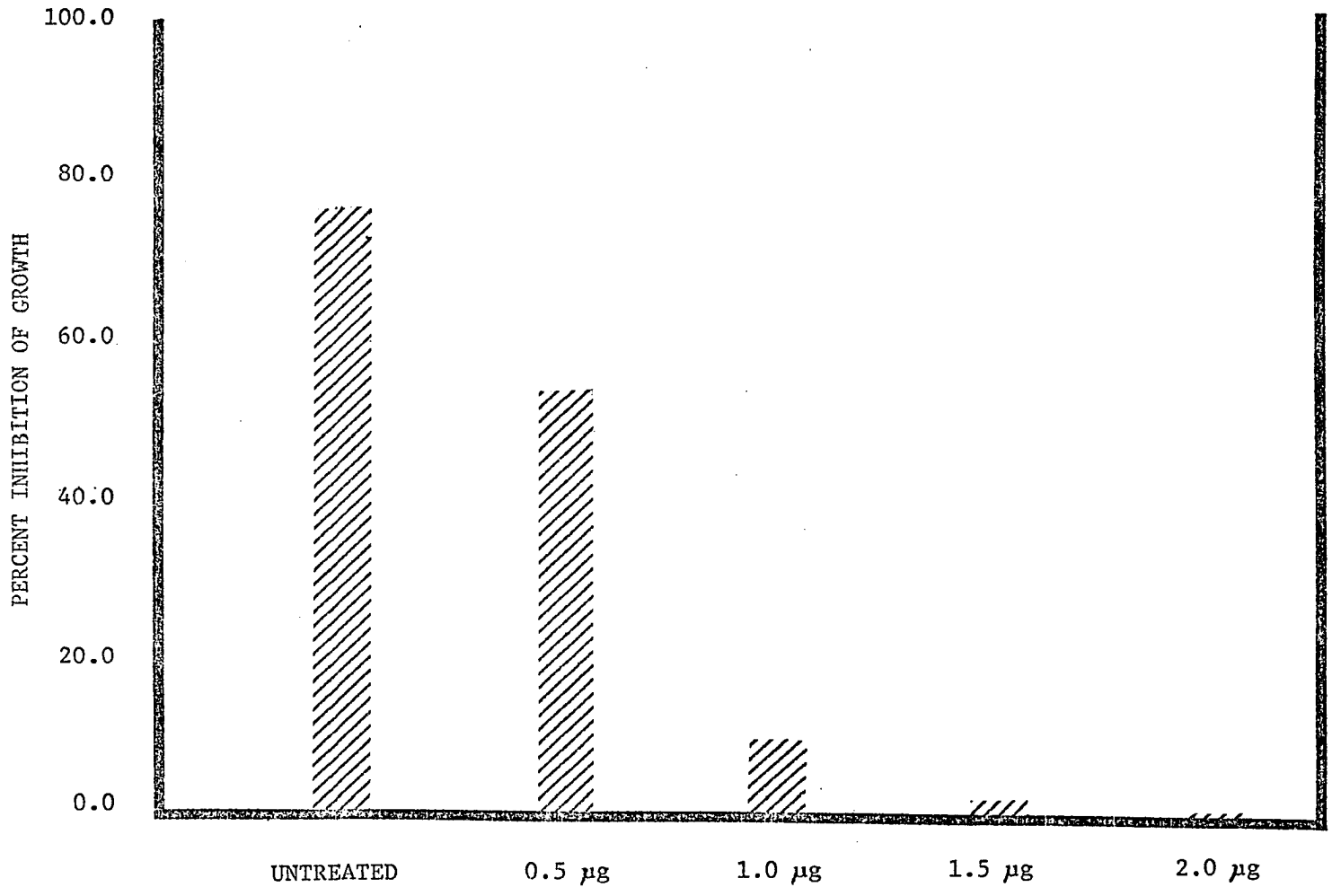
100.0
80.0
60.0
40.0
20.0
0.0
PERCENT INHIBITION OF GROWTH
UNTREATED
0.5 μg
1.0 μg
1.5 μg
2.0 μg
TREATMENT OF LYMPHOID CELLS WITH
ACTINOMYCIN D

Table 1. The effect of the addition of fresh medium or fresh immune effector cells on the inhibition of growth of *C. neoformans*. On day 8 after immunization, splenic and T-enriched lymphocytes were collected from *C. neoformans*-sensitized animals and were incubated with *C. neoformans* at E:T ratio of 300:1 in tissue culture tubes at 37°C. After 12 and 24 h of incubation, the reaction mixtures were supplemented with either equal volumes of fresh medium or 3×10^6 fresh *C. neoformans*-sensitized splenic or T-enriched lymphocytes. All reaction mixtures were allowed to incubate up to 48 h.

EFFECT OF THE ADDITION OF FRESH MEDIUM OR FRESH IMMUNE LYMPHOID CELLS ON THE INHIBITION OF GROWTH OF C. NEOFORMANS

CELL POPULATION	SUPPLEMENTS AT 12 & 24 H	% INHIBITION AT 24 H	% INHIBITION AT 48 H
IMMUNE SPLEEN	NONE	86	36.3
	FRESH MEDIUM	ND	70.03
	FRESH IMMUNE CELLS	ND	88.5
IMMUNE SPLENIC T-ENRICHED	NONE	83.7	33.2
	FRESH MEDIUM	ND	64.8
	FRESH IMMUNE CELLS	ND	75.6

ND = NOT DONE

Table 2. The effect of pretreatment of effector cells with cryptococcal culture filtrate (CneF) antigen on their ability to inhibit the growth of C. neoformans.
Immune splenic or T-enriched lymphocytes were collect on day 8 after immunization and were pretreated with CneF antigen containing 100 µg protein and 100 µg carbohydrate before using in the in vitro growth inhibition assays. Reaction mixtures were incubated for 24 h at 37°C.
S.E.M = Standard error of the means.

EFFECT ON THE ABILITY TO INHIBIT GROWTH OF C. NEOFORMANS BY LYMPHOID CELLS AFTER PRE-TREATMENT WITH C. NEOFORMANS CULTURE FILTRATE ANTIGEN

CELL POPULATION	PRE-TREATMENT WITH CNEF	% INHIBITION OF GROWTH ± S.E.M.	
		LYMPHOID CELL : C. NEOFORMANS CELLS	
		100 : 1	300 : 1
IMMUNE SPLEEN	+	0.0*	66.0 ± 3.13
	-	11.3 ± 3.99	81.85 ± 0.30
NORMAL SPLEEN	+	0.0	10.5 ± 3.17
	-	0.0	0.0
T-ENRICHED FROM IMMUNE SPLEEN	+	2.4 ± 0.85	34.5 ± 1.22
	-	63.05 ± 5.04	74.5 ± 2.35
T-ENRICHED FROM NORMAL SPLEEN	+	0.0	0.0
	-	0.0	0.0

Table 3. Effects of normal and immune splenic T-enriched lymphocytes on the growth of various yeasts.
Effector cells were collected from mice on day 8 after immunization with *C. neoformans*.
An E:T ratio of 300 : 1 was used in this assay
Incubation time was 24 h at 37°C.

EFFECT OF NORMAL AND IMMUNE T-ENRICHED LYMPHOCYTES
ON THE GROWTH OF VARIOUS FUNGAL YEASTS

CELL TYPES	EXPT	C. NEOFORMANS	C. ALBICANS	S. CEREVISIAE
IMMUNE	1	86.1[a]	0.0	97.8
	2	71.4	-2.8[b]	94.7
NORMAL	1	0.0	0.0	97.2
	2	0.0	-22.5	94.4

[a] Values expressed % inhibition of growth.

[b] Negative values indicated number of viable C. albicans in the test groups were greater then those in the control groups.

Table 4. Effect of effector splenic or T-enriched cells prepared from mice immunized with either heat-killed cryptococci-CFA or SPSS-CFA on specific ^{51}Cr release by L929 and YAC-1 cells. Effector cells were collected on day 8 after immunization. Splenic or T-enriched cells and target cells were incubated at E:T ratio of 50:1 for 4 h at 37°C before aliquots of supernatants were taken for counting.

% SPECIFIC ^{51}CR RELEASE BY TARGET CELLS

CELL TYPES	LYMPHOCYTE	L929	YAC-1
IMMUNE	SPLENIC	0.00	0.00
	T-ENRICHED	5.78	0.00
NORMAL	SPLENIC	0.00	1.74
	T-ENRICHED	0.00	6.94

SECTION IV

DISCUSSION

An *in vitro* growth inhibition assay was developed in this study which not only allowed a direct examination of the effect of sensitized lymphoid cells on *C. neoformans* but also provided a tool to further investigations on the kinetics of interactions of the effector cells and the pathogen. Using this assay system, further characterization of the effector cells and elucidation of the specificity of effector cell actions was accomplished. In addition, this work reaffirmed with an *in vitro* technique the importance of CMI, particularly the lymphocyte function in host resistance to cryptococcosis.

In this study, sensitized lymphoid cells capable of inhibiting the growth of *C. neoformans* were detected with the *in vitro* growth inhibition assay from *C. neoformans*-sensitized animals, and were found to develop coincidently with the increase of DTH responsiveness of the animal. The parallel developments of DTH responsiveness and the increase in the effect of lymphoid cell functions, such as the resistance to further challenge with *C. neoformans*

(21, 22, 40), the restriction of proliferation of viable cryptococci in various tissues (9,39, 40) or the production of macrophage migration inhibition factor (25,39) have also been demonstrated in other host-*C. neoformans* studies. It has been shown in some studies that DTH activities and other cellular immune functions such as cytotoxic responses to sheep red cells or lymphocyte proliferative responses are mediated by different T lymphocyte subclasses (28, 51). Whether the DTH responses and growth inhibition activities described in this study were manifested by the same or different T cell sub-population was not established. Nevertheless, these findings provided further evidence that DTH responsiveness and effective immunity are co-existing features in murine cryptococcosis, thus, an increase in DTH responses to *C. neoformans* during cryptococcosis was indicative of a stimulated CMI function, as assayed by the ability of T lymphocytes to specifically inhibit the growth of *C. neoformans*.

Two methods were used in this study to demonstrate that T but not B lymphocytes were the major effector cells responsible for inhibiting the growth of *C. neoformans*. First, T and B lymphocytes were enriched on nylon wool columns and were tested for growth inhibitory activities

on C. neoformans in the in vitro assay system. Results showed that the enrichment of T lymphocytes from C. neoformans-sensitized splenic cell pools enhanced the growth inhibitory activities. These enhanced growth inhibitory activities were evident by the fact that T-enriched lymphocytes were able to inhibit the growth of the pathogen at a lower E:T ratio than that required by the sensitized unfractionated splenic cells. Although the B-enriched cell populations maintained a slight ability to inhibit the growth of C. neoformans, this was considerably less than the inhibitory ability of the unfractionated splenic or T-enriched populations. The ability of B-enriched cells to weakly inhibit the growth of C. neoformans could have been due to a small number of sensitized T lymphocytes contaminating the adherent population in the process of their recovery from the nylon wool column. Although macrophages have been shown to be capable of engulfing C. neoformans in human or animal cryptococcosis (4, 5, 49), they were not likely to have played a role in this system since the depletion of macrophages from C. neoformans-sensitized splenic cell pools on nylon wool column did not affect the ability of the cells to inhibit the growth of the pathogen. Second, the effector cells were shown to be T lymphocytes by the fact that the growth inhibitory

ability of the sensitized splenic lymphocytes was not affected by treatment with anti-MIg and complement but was completely abolished by treatment with anti-Thy-1 serum and complement. These results were in full agreement with that reported by Lim and Murphy (40) who demonstrated that the ability of splenic enriched T lymphocytes to transfer resistance to recipient animals could be abolished only by treatment with anti-Thy-1.2 serum and complement but not by depletion of B and other adherent cells with nylon wool columns.

Recently, Monga *et al.* (50) also provided indirect evidence showing that B cells are not involved in protection of mice infected with *C. neoformans*. They demonstrated that there was no difference between the B-cell deficient and normal control animals with respect to their mortality patterns, viable cryptococci counts in different organs, DTH responses to cryptococcal culture filtrate antigen, or antigen levels in the sera when both groups of mice were infected identically with *C. neoformans*. Mice without B lymphocyte functions handled the disease in an equivalent manner as animals with B cell function; therefore, some cells other than B cells must be responsibe for the protection or elimination of the pathogen.

The study on the comparison of the growth inhibitory

ability of sensitized splenic and T-enriched lymphocytes provided data suggesting that an optimal effector to target cell ratio is essential for effective growth inhibition on C. neoformans. In particular, a certain number of sensitized T lymphocytes must be present in the effector cell populations before inhibition of growth of C. neoformans can be achieved. This speculation was made by 2 observations: (1) Results in this study demonstrated that there was little or no inhibition of growth of C. neoformans by sensitized unfractionated splenic cell pools at E:T ratios of 100:1 and 200:1; whereas considerable amount of growth inhibition of the pathogen was detected with an enriched population of T lymphocytes (98-100% T cells) at the same E:T ratios. (2) Preliminary experiments were performed in which normal splenic cells (filler cells) were added to the 100:1 and 200:1 sensitized splenic effector-target cultures to bring the total number of lymphoid cells equivalent to those in the E:T ratio of 300:1. Equivalent levels of growth inhibition was achieved with E:T ratios of 100:1 or 200:1 plus filler cells as compared to those obtained with E:T ratio of 300:1 without filler cells (data not shown). The interpretation of these results could be that the filler cells might have helped to condition the medium (that is the production of certain essential growth factors into the medium) thus

allowing a faster cell growth or proliferation of the effector T lymphocytes in the effector-target cell mixtures. This cellular proliferation might have resulted in an increase in numbers of effector T lymphocytes, hence, growth inhibitory function proceeded when certain numbers of T lymphocytes in the mixture were attained. Being an enriched population, the T cell numbers at an E:T ratio of 100:1 or 200:1 might already be sufficient for growth inhibition of C. neoformans, hence conditioning of the medium was not required for growth inhibition. Further investigation is needed to confirm this speculation.

The kinetics of growth inhibition exhibited by both sensitized splenic and T-enriched lymphocytes was very similar in that they exhibited growth inhibitory activities as early as 4 hours after effector and target cells were mixed, and the activities increased gradually and peaked at 24 hour. By 48 hour, this activity in both effector groups dropped significantly. The drop in growth inhibitory activity between 24 and 48 h could have been due to either death of effector cells or the loss of cellular functions resulting from the depletion of essential nutrients in the reaction medium. This appears to be what was happening in the cultures since the growth inhibitory activities of the sen-

sitized effector cells could be restored with either the addition of fresh medium or sensitized effector cells. In an *in vivo* situation, there is a constant and plentiful supply of fresh nutrients and sensitized effector cells circulating to the focal point of infection; therefore the proliferation of *C. neoformans* would be restricted effectively.

In human or animal cryptococcsis, cryptococcal polysaccharide antigen is frequently found in all body fluids of the patients or animals (6, 9, 39). Murphy and Cozad (53) have demonstrated that cryptococcal polysaccharide antigen when injected into mice induced immunological unresponsiveness (paralysis) to subsequent challenge-immunization with the same material. Recently, Murphy (53a) also demonstrated that cryptococcal antigen can induce suppressor cells which can specifically inhibit the DTH response in mice, however, the direct effect of the antigen on the effector cell (immune T lymphocytes) has not been studied. Therefore, one of the objectives of this study was to investigate the effect of pretreatment of effector cells with cryptococcal culture filtrate (CneF) antigen and determine whether the treatment alters their ability to inhibit the growth of *C. neoformans in vitro*. Results showed that the pretreatment of effector cells with CneF antigen reduced their growth inhibitory activities. This reduction was not likely due to cytotoxic effects of the antigen on the effector cells because no killing was observed

when treated cells were examined with trypan blue dye exclusion tests. Cryptococcal polysaccharide has been shown capable of inhibiting phagocytosis of C. neoformans by preventing the attachment of the yeast to macrophages (35). Recently, McGaw and Kozel (46) presented evidence suggesting that the cryptococcal polysaccharide acts as a barrier between the opsonized C. neoformans (cell wall-associated IgG) and the macrophage Fc receptors, thus preventing phagocytosis. Possibly, the cryptococcal culture filtrate antigen (which contains 4.0 mg carbohydrate/ml antigen) might have exerted a similar effect such as masking the specific antigen-recognition determinants on the surface of the sensitized effector cells, thus preventing them from interacting and exerting their growth inhibition functions on the target cells. If this hypothetical action of the antigen were correct, it could explain the observation that increasing severity of the disease in cryptococcosis is associated with increasing cryptococcal antigen titer in the body fluids of the host (20,63). One thing that needs to be addressed here is that the reduction of growth inhibitory activities of the effector cells observed as a result of treatment with CneF was not likely due to the blocking of macrophage function by the antigen; because identical effects were also observed in the CneF-treated T-enriched populations where macrophages had been depleted.

Diamond and Allison (11b) demonstrated that human peripheral blood leukocytes were capable of killing C. neoformans in the presence of anticryptococcal antibody in vitro (Antibody dependent cell-mediated cytotoxicity, ADCC assay). The effector cells responsible for the killing were identified as monocytes and granulocytes but suprisingly, no killing of C. neoformans was observed when a population of enriched T lymphocytes obtained from normal donors was used in the assay. The absence of killing by T lymphocytes in their system could have been due to the fact that normal T lymphocytes rather than sensitized ones were used in the assay. Since results in this study have demonstrated that only C. neoformans-sensitized T lymphocytes but not the unsensitized ones were effective in inhibiting the growth of the pathogen, they could have detected killing of C. neoformans by T lymphocytes if these lymphoid cells had been taken from C. neoformans-infected or sensitized donors.

Another objective in this study was to investigate whether the C. neoformans-sensitized T effector cells were specific for C. neoformans or whether they had non-specific inhibitory abilities. To do this, sensitized T-enriched lymphocytes were tested simultaneoulsy for growth inhibitory effects on C. neoformans, C. albicans and S. cerevisiae. Results showed that C. neoformans-sensitized T lymphocytes were capable of inhibiting the growth of C. neoformans but had no effect on the growth of C. albicans nor were they able to lyse L929 fibroblasts and YAC-1 cells. Taken together these results indicated that the effector cell action was specific.

The observation that S. cerevisiae was inhibited equally well by normal lymphoid cells as by sensitized T lymphocytes was unexpected. However it seems reasonable when one considers the S. cerevisiae is not a pathogen while C. albicans and C. neoformans are. The potential of normal cells to kill S. cerevisiae in these experiment is possibly the demonstration of an important innate mechanism which eliminates the so called non-pathogenic organisms.

Characterization of the effector cells was made possible using the in vitro growth inhibition assay. Results revealed that the effector T lymphocytes were Ia^+ as determined by the fact that their growth inhibitory abilities could be completely abolished upon treatment with anti-Ia^k serum and complement. It has been reported that T lymphocytes responsible for the DTH response, the T_{DH} cells, in the 2,4-dinitrofluorobenzene (DNFB) system were Ia^- T cells (51). Similar findings have been reported in other systems(28,60). In contrast to the T_{DH} cell type, cytotoxic T lymphocytes (T_C) generated in the mixed lymphocyte culture (MLC) reactions resembled the T_{DH} cells with respect to their specificity of action, having Thy-1 antigens on their cell surface and being nylon wool non-adherent; however, T_C cells differed from T_{DH} cells in that T_C cells were not able to transfer DTH responsiveness to recipient animals and in

some cases, T_C cells were shown to have Ia antigens on their surface (16,33,43). In this study, the effector T (T_{EFF}) lymphocytes capable of inhibiting the growth of C. neoformans appeared to differ from the T_{DH} cells with respect to the Ia antigen, but resembled the T_C cells in that it was Ia^+. The T_{EFF} differed from the T_C cells in their ability to lyse tumor cells.

Recently, Yu et al. (59) reported that the percentage of Ia^+ T lymphocytes was elevated in the circulation of patients with various disease such as rheumatoid arthritis, infectious mononucleosis or graft-vs-host diseases. Elevation of Ia^+ T lymphocytes also occured in individuals after stimulation with antigens such as tetanous toxoid, purified protein derivative (PPD) (59) or mitogens (38,59). It has also been reported that Ia^+ T cells in large numbers appeared on approximately 7 days after stimulation with pokeweed mitogen (59). The Ia^+ cells in this study that were responsible for the inhibition activity on C. neoformans may be a cell similar to the ones mentioned above. Speculation could be made that during a cryptococcal infection there is an induction of Ia^+ T lymphocytes which could specifically inhibit the growth of C. neoformans during the course of cryptococcosis accounting for the protection of the host against the infection. Further studies must be done to

establish whether Ia^+ T lymphocytes can be found and whether their numbers are increased in circulation during cryptococcosis.

The complete abolishment of growth inhibitory activities of the effector cells as a result of treatment with Actinomycin D at a concentration of 1.5-2.0 µg/l $x10^7$ cells which is known to inhibit RNA synthesis demonstrated that RNA synthesis was necessary for the growth inhibitory process. Actinomycin D is an irreversible inhibitor of RNA synthesis which, through intercalation in DNA double strand, prevents the activity of the RNA polymerase (56) from functioning; the absence of RNA synthesis may cause an eventual cessation of protein synthesis. RNA and protein synthesis have been shown to be required for many immune functions, e.g., in phagocytosis (32), antibody-dependent cytolysis of chicken erythrocytes (37), generation of cytotoxic cells in vitro (54), lymphocyte proliferation (48,61) and lymphokine production (10, 27, 62); however, exactly, what role RNA and protein synthesis play in these immune functions or the one described in this work is unclear at this time. Since RNA and protein synthesis are necessary for lymphokine production (10, 27, 62), and lymphokines have been demonstrated to have fungicidal effect on other yeast cells (19, 55), it

is possible that lymphokines were being produced by the effector cells in this system and were inhibiting the growth of C. neoformans. In fact, in the companion paper lymphokines have been shown to inhibit the growth of the pathogen.

Although the investigation of the exact mechanism(s) by which T lymphocytes inhibit the growth of C. neoformans was not within the scope of the present study, whether a direct cell-cell contact or the involvement of membrane interaction between the effector cells and the target cells is required for the growth inhibition remains unclear, however, this problem could be resolved by an ultrastructural examination of the interactions between the effector cells and the pathogen.

Cell mediated immunity has been known to be a most important defense mechanism not only in cryptococcosis but also in other systemic fungal infections such as coccidioidomycosis, blastomycosis and histoplasmosis (2,3,64). The effector cells responsible for host resistance to these pathogens have not been well defined, the in vitro growth inhibition assay developed in the present study may provide a tool for resolving this problem. Another potential usage of this assay system is its application in human cryptococcosis with which the peripheral blood lymphocytes from patients with cryptococcal infection could be monitored for their inhibitory

function on the pathogen, hence, this may provide a better prognostic tool for this disease.

SECTION V

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PAPER II

EFFECT OF LYMPHOKINES ON CRYPTOCOCCUS NEOFORMANS

SECTION I

INTRODUCTION

It has been well established that cell mediated immunity (CMI) plays a major role in defending the host against a Cryptococcus neoformans infection (1,2,4,5,8,12-14,19). Lim and Murphy (19) recently demonstrated that the effector cell responsible for host defense in murine cryptococcosis was in a T lymphocyte population from the spleens of C. neoformans-sensitized mice. They showed that these effector cells were able to passively transfer to normal naive recipient animals delayed-type hypersensitivity (DTH) to C. neoformans as well as resistance to further challenge with viable cryptococci. The preceding report (8) provided further evidence that C. neoformans-sensitized T lymphocytes are capable of exerting an inhibitory effect on the growth of this pathogen in vitro. The mechanism by which T lymphocytes kill or eliminate an infectious agent can be by direct contact or through the production of soluble factors known as lymphokines (17, 18, 27, 28). These factors which are produced in minute quantities have been shown

to have biologic effects on a variety of cell types including T lymphocytes themselves (3,7,9-11,17,18,27 28, 30-32). These biologic effects include cytotoxic activities on tumor and other cells by lymphotoxin (LT) (9,29, 35); activation of macrophages which possess enhanced microbicidal activity by macrophage activating factor (MAF) (24); and inhibition of migration or movement of macrophages by migration inhibition factor (MIF) (25). Although MAF and MIF factors exhibit different functions on macrophages, recent evidence suggested that they are identical molecules (24). Other functional lymphokines include T-cell growth factor (TCGF) which is able to enhance proliferation of splenic lymphocytes (3, 31) and to induce cytotoxic lymphocytes in both thymocytes and nude mouse spleen cell cultures (3,10, 31, 32). Enhancement of cytotoxicity of sensitized lymphocytes by lymphokines which exhibited interferon activities has also been described (20).

Lymphokines have been shown to possess fungicidal activities. Pearsall et al (27) demonstrated that lymphokines prepared from phytohemagglutinin (PHA)-stimulated lymphocytes were able to exert direct killing on Saccharomyces cerevisiae and Candida albicans. Gorcyca (9) recently demonstrated that lymphokines prepared from PHA- and blastomycin-stimulated

lymph node cells exhibited not only fungicidal activities on Blastomyces dermatitidis but also displayed lymphotoxin activities on L929 mouse fibroblast cells.

Since the effect of lymphokines on C. neoformans has not been investigated, it was the primary objective of the present report to study the susceptibility of C. neoformans to lymphokines produced by C. neoformans-sensitized splenic lymphocytes stimulated with C. neoformans specific antigens. In this report, the biological activities of the lymphokines were assayed and the specificities of action of the lymphokines on C. neoformans were determined. As mentioned earlier, lymphokines were found to be able to enhance lymphoid cellular functions such as proliferative and cytotoxic responses (3,20,31,32); therefore, the effect of pretreatment of sensitized or normal splenic lymphocytes with lymphokines was determined by measuring their growth inhibitory ability on C. neoformans before and after lymphokine treatment.

SECTION II

MATERIALS AND METHODS

Mice. Inbred CBA/J mice purchased from Jackson Laboratory, Bar Harbor, Maine, were bred in the animal facilities in the Department of Botany and Microbiology, University of Oklahoma. Mice of both sexes, aged 10 to 12 weeks were used throughout this study.

Organisms. Cryptococcus neoformans 184A described by Murphy and Cozad (23) was used for sensitization of animals and served as target cells for the in vitro growth inhibition assay. A suspension of heat-killed cryptococci (8) was used for in vitro stimulation of lymphokines by C. neoformans-sensitized splenic lymphocytes. Candida albicans and Saccharomyces cerevisiae were obtained from the stock culture section of the Department of Botany and Microbiology, University of Oklahoma. All cultures were maintained on modified Sabouraud's agar (MSAB) slants as described previously (8).

Cryptococcal antigen. The cryptococcal culture filtrate (CneF) antigen used in this study was prepared according to procedures described previously (4). Protein and carbohydrate concentrations of this antigen preparation were 4.0 mg/ml as determined by Lowry procedure (21) and phenol-sulfuric acid method (6), respectively.

Bovine serum albumin solution was used as a standard and Monitrol I (Dade Division, American Hospital Supply Corp., Miami, Fla) as a control for the protein determination. Mannose and mannan served as standard and control respectively, in the carbohydrate determination.

Sensitization of animals. Mice were immunized according to procedures of Graybill and Taylor (13), using a mixture of heat-killed C. neoformans and complete Freund's adjuvant (CFA) emulsion. Control mice were injected either with sterile physiological saline solution (SPSS)-CFA emulsion or remained unimmunized.

To sensitize mice to Mycobacterium, the animals were injected with 0.2 ml of CFA subcutaneously on the upper left and right side of the abdomen. One week after the first injection, mice were boosted with 0.1 ml CFA intraperitoneally.

Delayed-type hypersensitivity (DTH) response. DTH responsiveness to CneF was measured at various times after immunization according to the procedures described by Cauley and Murphy (4).

Production of lymphokines. Lymphoid cells for lymphokine production were collected from the spleens of mice which had been immunized 8 days previously. Splenic and T-enriched lymphocytes were prepared according to procedures previously described (8,16). Both cell populations were adjusted

to a concentration of 1 x 10^7 cells/2.5 ml RPMI-1640 medium containing 100 units/ml penicillin, 100 µg/ml streptomycin and 10% fetal calf serum (FCS, Flow Laboratories, Mclean, Virginia). These cell populations in 2.5 ml volumes were cultured with either 0.1 ml CneF antigen containing 100 µg of protein and carbohydrate or with 0.1 ml of 3.3 x 10^4 heat-killed cryptococci in tissue culture flasks (Corning Plastics, Corning, New York) at 37°C under an atmosphere of 5% CO_2 and 95% air. After 48 h of incubation, the cell suspensions were centrifuged, the supernatants were collected and filter sterilized through a 0.45 µm millipore filter (Millipore Corp. Bedford, Mass) before storing at -20°C until assayed for growth inhibitory and macrophage migration inhibition factor (MIF) activities.

To prepare MIF from CFA-injected mice, splenic cells were obtained from mice 7 days after the last injection of CFA and were cultured with purified protein derivative (PPD, Parke Davis & Co., Detroit, MI) at a concentration of 2.5 µg/1 x 10^7 cells at 37°C for 48 h. The supernatants from this cultures were collected and filter sterilized.

In vitro growth inhibition assay. The in vitro growth inhibition assay as described previously (8) was used for 2 studies in this report. (i) To assess direct effects

of the lymphokines on the target yeast cells, a suspension of 1 x 10^4 target cells of C. neoformans, C. albicans or S. cerevisiae were incubated with 2.0 ml of the lymphokine preparation at 37°C for 24 or 48 h in 5% CO_2-95% air.

(ii) To evaluate the effects of lymphokines treatment on the ability of C. neoformans-sensitized or normal splenic cells to inhibit the growth of C. neoformans, sensitized or normal splenic cells were treated with lymphokines prior to being used as effector cells in the growth inhibition assay. A 300:1 E:T ratio was employed. Effector and target cell mixtures were incubated at 37°C for 24 h in 5% CO_2-95% air.

Pretreatment of C. neoformans-sensitized or normal splenic cells with lymphokine preparations. Splenic cells were prepared on day 8 after immunization with heat-killed cryptococci-CFA or remained unimmunized. Two ml of lymphokine preparation was added to 0.1 ml of 1 x 10^8 cells/ml splenic cell suspension, and the mixture was incubated for 24 h at 37°C in 5% CO_2-95% air. After treatment, the cells washed 3 times and viability of the cells were determined with trypan blue dye exclusion before being used in the in vitro growth inhibition assay.

Lymphotoxin assay. L929 mouse fibroblasts were used as the target cells in the lymphotoxin assay. The cells

were grown in RPMI-1640 supplemented with penicillin and streptomycin and 2% FCS and were adjusted to 1 x 10^7 cells/ml. Target cells were labelled with 200 µCi of ^{51}Cr (New England Nuclear, Boston, Mass.) for 1 h (22). One tenth ml of the labelled target cells and 0.1 ml of the lymphokine preparation were transferred into wells of a U-bottom tissue culture multi-well plate (Linbro, Hamden, Connecticut). After 4 h incubation at 37°C under 5% CO_2-95% air, the cells were pelleted and 0.1 ml of each supernatant was transferred to tubes for counting in a Packard gamma-counter. Percent specific ^{51}Cr release was calculated according to the formula described in the previous report (8).

Macrophage migration inhibition assay. Lymphokines prepared from C. neoformans-sensitized or normal splenic or T-enriched lymphocyte suspensions were tested for macrophage migration inhibition factor (MIF) activities according to procedures described by Harrington and Stastny (15) using light mineral oil stimulated peritoneal macrophage from normal mice.

SECTION III

RESULTS

DTH response. CBA/J mice immunized with heat-killed cryptococci-CFA emulsion or SPSS-CFA or those remain unimmunized were footpad tested for DTH responsiveness to cryptococcal culture filtrate (CneF) antigen at days 2, 4, 6, 8, 16 and 24 after immunization. Results obtained in this study were similar to those reported earlier (8) in that peak swelling of footpads of C. neoformans-sensitized mice occured between 8 and 16 days after immunization. Normal mice did not show increased footpad swelling in response to CneF antigen. Therefore for convenience, splenic lymphocytes collected from mice on day 8 after immunization were used in the in vitro growth inhibition assay and for lymphokines production.

Effect of lymphokines on C. neoformans. Supernatants from cultures of normal or sensitized splenic or T-enriched lymphocytes stimulated with CneF antigen or heat-killed cryptococci were tested for growth inhibitory activity with C. neoformans. Table 5 shows the results of this experiment. At 24 h, 88.5 and 75.2% inhibition of growth of C. neoformans by lymphokines prepared from sensitized

splenic cells stimulated with CneF or heat-killed cryptococci (HKC), respectively were observed. Higher inhibition of growth of the organism was observed at 48 h. Significantly lower growth inhibition was observed when lymphokines prepared from C. neoformans-sensitized T-enriched lymphocytes were used ($p<0.01$). Supernatants from normal lymphocyte populations stimulated with CneF or HKC did not exhibit inhibitory effects on C. neoformans.

Effect of lymphokines on different yeasts. C. neoformans, C. albicans or S. cerevisiae were incubated with lymphokines prepared from CneF antigen-stimulated C. neoformans-sensitized splenic lymphocyte cultures for 24 and 48 h. Results presented in Table 6 showed that the growth of C. neoformans was inhibited by lymphokines obtained from cultures of sensitized splenic cells stimulated with CneF antigen but C. albicans was unaffected in the presence of the same lymphokine preparation. Germ tubes were observed in the cultures of C. albicans during the entire incubation period. When S. cerevisiae was used as the target cells in the assay, its growth was almost completely inhibited. Lymphokines prepared from cultures of normal or saline-treated splenic cells with CneF had no effect on any of the 3 yeasts.

Effects of lymphokine treatment on the growth inhibitory ability of C. neoformans-sensitized and normal splenic lymphocytes. One of the objectives in this study was to see if lymphokines prepared by stimulating various lymphocyte sources with different C. neoformans antigens, could enhance the growth inhibitory activities of the C. neoformans-sensitized or normal splenic cells. Results in Table 7 revealed that neither the growth inhibitory ability of normal or C. neoformans-sensitized splenic lymphocytes was enhanced by lymphokine treatment, but, instead, a slight reduction of growth inhibitory activities of the sensitized effector cells was observed after treatment when compared with the results of the untreated groups.

Assay for lymphotoxin activity. Supernatants from cultures of CneF antigen stimulated splenic lymphocytes were tested for lymphotoxin activities with L929 mouse fibroblasts. A ^{51}Cr release assay was employed for the detection of cytotoxic activity. Table 8 shows the results of this experiment which indicated that lymphokine preparations prepared from either sensitized or normal splenic lymphocytes stimulated with CneF antigen did not have detectable lymphotoxin activities on L929 cells.

Assay for migration inhibition factor (MIF). Lymphokines prepared from C. neoformans-sensitized splenic lymphocytes were assayed for MIF activities with light mineral oil stimulated peritoneal macrophages from normal mice. Supernatants from CFA-sensitized splenic lymphocytes stimulated with PPD served as positive controls for MIF activity. Results in Table 9 showed that C. neoformans-sensitized lymphocytes when stimulated with appropriate antigen produced lymphokines which exhibited MIF activities. Negligible amounts of MIF activity was detected in supernatants prepared from cultures of CFA-sensitized lymphocytes stimulated with CneF antigen. Results here indicated that MIF was produced from sensitized splenic lymphocytes only when stimulated with homologus antigens.

Table 5. Effect of lymphokines on the growth of *C. neoformans*. Lymphokines produced by stimulating normal or *C. neoformans*-sensitized splenic lymphocytes with either cryptococcal culture filtrate (CneF) antigen or heat-killed *C. neoformans* (HKC) were cultured with *C. neoformans* *in vitro* at 37°C for 24 and 48 h.

LYMPHOKINE PREPARED FROM	% INHIBITION AT 24 H	% INHIBITION AT 48 H
IMMUNE SPLENIC CELLS + CNEF	88.5	93.0
NORMAL SPLENIC CELLS + CNEF	0.0	0.0
IMMUNE T-ENRICHED CELLS + CNEF	21.5	0.0
IMMUNE SPLENIC CELLS + HKC	75.2	77.3
NORMAL SPLENIC CELLS + HKC	0.0	0.0

Table 6. Effects of lymphokines on the growth of various yeasts. Lymphokines were produced by stimulating normal or *C. neoformans*-sensitized splenic lymphocytes with CneF antigen and culturing the lymphokines with the yeast target cells for 24 or 48 h at 37°C.

LYMPHOKINES PREPARED FROM CULTURES OF	EXPT. #	TIME (H)	% INHIBITION OF GROWTH		
			C. NEOFORMANS	*C. ALBICANS*	*S. CEREVISIAE*
C. NEOFORMANS-SENSI-SPLENIC CELLS + CNEF	1	24	80.0	0.0	83.7
	2		88.5	0.0	94.5
	1	48	80.4	0.0	89.4
	2		93.0	0.0	97.2
NORMAL SPLENIC CELLS + CNEF	1	24	0.0	0.0	0.0
	2		0.0	0.0	0.0
	1	48	0.0	0.0	0.0
	2		0.0	0.0	0.0

Table 7. Effects of lymphokine treatment on the growth inhibitory ability of *C. neoformans*-sensitized and normal effector cells.
Sensitized lymphoid cells were collected from mice on day 8 after immunization and were cultured for 48 h with either CneF antigen or HKC to produce lymphokines.
Lymphokine preparations were incubated with *C. neoformans*-sensitized or normal effector cells for 24 h at 37°C prior to washing the cells and putting them into the *in vitro* growth inhibitory assay.

SOURCE OF LYMPHOKINE	% INHIBITION OF GROWTH AT 24 HOURS — LYMPHOCYTES BEING TREATED: C. NEOF.-SENSITIZED SPLENIC CELLS	% INHIBITION OF GROWTH AT 24 HOURS — LYMPHOCYTES BEING TREATED: NORMAL SPLENIC CELLS	ACTION OF LYMPHOKINES ALONE
C. NEOF.-SEN. SPLENIC CELLS + CNEF[1]	71.8	0.0	82.4
C. NEOF.-SEN. SPLENIC CELLS + HKC[2]	68.2	0.0	74.1
NORMAL SPLENIC CELLS + CNEF	66.7	0.0	0.0
NORMAL SPLENIC CELLS + HKC	77.9	0.0	0.0
C. NEOF.-SEN. T-CELLS + CNEF	72.2	0.0	21.5
NONE[3]	80.4	0.0	N.A.

[1] Cryptococcal culture filtrate antigen

[2] Heat-killed cryptococci

[3] Culture medium alone

N.A. = Not applicable

Table 8. Assay for lymphotoxin activity using a ^{51}Cr release assay.
Suspensions of 1×10^6 target cells were incubated with 0.1 ml of the lymphokine preparations for 4 h at 37°C before aliquots of the supernatants were collected for counting in a Packard gamma counter.

LYMPHOKINES PREPARED FROM	EXPT. #	% SPECIFIC ^{51}CR RELEASED FROM L929 CELLS
C. NEOFORMANS-SENSITIZED SPLENIC CELLS + CNEF	1	2.3
	2	5.6
	3	0.7
NORMAL SPLENIC CELLS + CNEF	1	1.2
	2	1.8
	3	0.0

Table 9. Assay for macrophage migration inhibition factor (MIF) in supernatants prepared from cultures of CFA-sensitized, normal, or C. neoformans-sensitized splenic cells stimulated with CneF antigen or PPD.
Light mineral oil stimulated peritoneal macrophages from normal mice were used for the assay.

SPLENIC LYMPHOCYTES FROM ANIMALS IMMUNIZED WITH	% MIGRATION INHIBITION [1]	
	ANTIGEN FOR LYMPHOKINE PRODUCTION[2]	
	CneF	PPD
HEAT-KILLED *C. NEOFORMANS*-CFA EMULSION	63.8	53.5
CFA	9.4	75.4
NONE	0.0	0.0

[1] Percent migration inhibition = [1 - (test migration distance/control migration distance] X 100

[2] CneF = cryptococcal culture filtrate antigen
PPD = purified protein derivative

SECTION IV

DISCUSSION

The results presented in this study demonstrated that lymphokines produced from cultures of C. neoformans-sensitized splenic lymphocytes stimulated with CneF antigen or heat-killed cryptococci (HKC) were capable of inhibiting the growth of C. neoformans in an in vitro assay system. The lymphokine produced by C. neoformans-sensitized cells was not specific in its inhibitory ability since it also inhibited S. cerevisiae as well as C. neoformans. However, the lymphokine was unable to inhibit as broad a spectrum of cells as other reported lymphokines (27). Pearsall et al. (27) demonstrated that lymphokines prepared from phytohemagglutinin (PHA)-stimulated murine lymphocytes killed S. cerevisiae and C. albicans in vitro, and they reported that there was no cell clumping or mycelium formation in the lymphokine-C. albicans cultures. They also showed that a 1:2 dilution of this lymphokine was cytotoxic for peritoneal macrophage in vitro. In contrast to these results, lymphokines prepared in this study failed to inhibit the growth of C. albicans in an in vitro system, and mycelium or germ tube formation in

the lymphokine-C. albicans cultures was observed. It is obvious that the lymphokines produced from CneF antigen-stimulated C. neoformans-sensitized splenic lymphocytes and that prepared by Pearsall et al. were different effector molecules. These molecules might have acted differently on different yeast cells. Another explanation for the discrepancy of the results observed in this study and those reported by Pearsall et al. is that, possibly, in the process of germ tube formation, the organism is expressing a new set of antigenic determinants on their surface thus allowing escape from the recognition and action of the lymphokine molecules.

Murine lymphokines prepared from cultures of concanavalin A (Con A)-stimulated mouse spleen cells have been shown to enhance a variety of T-cell responses (3,10,20,30-32,34), e.g. the enhancement of T cell proliferation (31); induction of cytotoxic lymphocytes in both thymocyte populations (32) and nude mouse spleen cell cultures (10); and enhancement of cytotoxicity of sensitized lymphocytes (20). One of the aims in this study was to investigate the possibility of enhancing the effector cells ability to inhibit the growth of C. neoformans by pretreating them with lymphokines prepared from C. neoformans-sensitized splenic cells

stimulated with CneF antigen. Results of this experiment revealed that neither growth inhibitory ability of normal or sensitized splenic lymphocytes was enhanced after the pretreatment with lymphokines prepared from various sources, but instead, there was a slight reduction of growth inhibitory ability of C. neoformans-sensitized splenic lymphocytes as a result of the pretreatment. Since in the preceding report (8), CneF antigen was shown to inhibit the growth inhibitory function of C. neoformans-sensitized effector cells, the presence of CneF antigen in the lymphokine preparations might be exerting a similar effect on the effector cell functions in this study.

Another observation made in this study was that lymphokines prepared from C. neoformans-sensitized T-enriched lymphocytes exhibited a significantly ($p<0.01$) lower growth inhibitory activity on C. neoformans than those that were prepared from C. neoformans-sensitized splenic lymphocytes. It is well established that macrophages are required to be present for lymphokine production (7, 33). Since macrophages were being depleted from the non-adherent lymphocyte pool during the nylon wool column enrichment process, there may not have been a sufficient number of macrophages remaining in the T-enriched pool to function

in lymphokine production, thus a lower production of lymphokine and lower growth inhibitory activity on C. neoformans. However, at this point, when one refers back to the preceding report (8), one finds that the growth inhibitory activity of effector T-enriched lymphocytes appeared to be enhanced upon the removal of macrophages through the enrichment process, and this result seems to contradict with that just mentioned. This discrepancy could have been due to two different mechanisms participating in the growth inhibition of C. neoformans. One mechanism being a direct cellular action and the other being the lymphokine action on the pathogen. Two other observations in this and the preceding studies (8) added support to this speculation. First, in this study, CneF antigen containing 100 µg protein and carbohydrates was stimulatory to C. neoformans-sensitized splenic cells resulting in lymphokine production which in turn was inhibitory to the growth of C. neoformans; whereas, results in the preceding study showed that the same concentration of CneF antigen was suppressive to the growth inhibitory activities of the effector cell populations. If two mechanisms are involved in the overall growth inhibition, the CneF antigen seems to be stimulatory to one but inhibitory to the other.

Second, observations in this and preceding studies showed that normal unsensitized lymphocytes were able to inhibit the growth of S. cerevisiae; whereas, the supernatants obtained from these normal lymphoid cell populations stimulated with CneF antigen failed to do so. Taking all these results together, a dual-mechanism of growth inhibition of C. neoformans is proposed here. It appears that the C. neoformans-sensitized T lymphocytes can act directly on the pathogen in the absence of macrophage, but their actions can be masked physically by the soluble cryptococcal polysaccharide antigen; whereas, in the presence of macrophages, the cryptococci or soluble cryptococcal antigen probably is being engulfed, processed in the macrophages and presented to the sensitized T lymphocytes for lymphokine production. The lymphokines produced in turn exert a growth inhibitory effect on the pathogen. It is also conceivable that two different T lymphocyte subclasses could be responsible for each growth inhibition mechanism; however, further characterization of the effector T cells for direct growth inhibition and lymphokine production is required to confirm this speculation. If the hypothesis mentioned above is correct, one would expect to see a higher inhibition exhibited by the combined actions of the two mechanisms than those exhibited by either one mechanism acting alone.

Unfortunately, such results were not observed in this or the preceding studies (8). However, this is explanable when one considers that the two mechanisms may not have been acting simultaneously in the in vitro growth inhibition assays. It is possible that under certain conditions, one mechanism could have been turned on while the other one some how is being shut off. Factors such as the nature or the amount of the antigens present in the assay, whether macrophages are present in the system, the time requirement for production and action of the lymphokines on the pathogen and the presence of regulator cells in the system could be the determinants for the final expression of these two mechanisms.

In the Listeria monocytogenes-host system, immunity of the host to the Listeria infection is mediated by a population of specifically sensitized T lymphocytes that function to activate macrophages, which in turn express killing on the bacteria (26). The activation of macrophages has been shown to be mediated by soluble mediators or lymphokines produced by the specifically sensitized T lymphocytes. Whether the lymphokines produced by the C. neoformans-sensitized splenic lymphocytes were able to activate macrophages remains to be investigated.

Lim and Murphy (19) recently demonstrated that C. neoformans-sensitized lymphocytes were able to produce macrophage migration inhibition factor (MIF) upon stimulation by specific CneF antigen. Results described in this study were in full agreement with theirs. Since MIF was only produced from C. neoformans-sensitized splenic cells upon exposure to cryptococcal specific CneF antigen, MIF assay might serve as a good assay for C. neoformans-sensitized T lymphocytes.

It has been reported that MIF production occured concurrently with the production of lymphotoxin (LT) in the guinea pig system (29); however, in the present study, only MIF but not LT activities were detected in the lymphokine preparations. Gorcyca (9) showed that the lymphokines prepared from blastomycin-stimulated lymphocytes were fungicidal to Blastomyces dermatitides as well as cytotoxic to L929 mouse fibroblasts, thus, lymphotoxin activities were present. The lymphokines prepared in this study were capable of inhibiting the proliferation of yeast cells as well as inhibiting macrophage migration.

At present, it is not clear how the lymphokines exert their effects on C. neoformans; however, the importance of T cells and T-cell mediator functions in cryptococcosis

was again reaffirmed in this and the preceding studies. The *in vitro* inhibition assay system has the potential to serve as an excellent tool for further investigating the effects of lymphokine molecules on *C. neoformans* and other yeasts.

SECTION V

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SUMMARY

CBA/J mice immunized subcutaneously with heat-killed Cryptococcus neoformans-complete Freund's adjuvant (HKC-CFA) emulsions developed delayed-type hypersensitivity (DTH) responses to cryptococcal culture filtrate (CneF) antigen, as well as developed sensitized lymphoid cells in their spleens that were able to inhibit the growth of C. neoformans in vitro. The in vitro cryptococci growth inhibition assay served as an essential tool to further investigate the kinetics of the effects of the sensitized lymphoid cells on the pathogen. A close correlation between the DTH response in mice and the ability of lymphoid cells to inhibit the growth of C. neoformans was observed. Inhibition of growth of C. neoformans by sensitized splenic cells was detected by day 6 after immunization and maximum levels were attained by day 8 through 16. Inhibition of growth was highest with an effector to target cells ratios of 300:1 or greater. Kinetic studies of the effect of sensitized lymphoid cells on C. neoformans showed that inhibition of growth of C. neoformans was detectable as early as 4 h after effector and target cells were mixed. Inhibition of growth of

cryptococci increased gradually over time peaking at 24 h, but dropped significantly by 48 h. By supplementing the reaction mixtures with fresh medium or more sensitized effector lymphoid cells during incubation, the inhibition of growth of C. neoformans could be maintained through 48 h. A reduction in growth inhibitory ability was observed when splenic cells were pretreated with cryptococcal culture antigen. Characterization of the sensitized population of effector cells revealed that they were nylon wool non-adherent, Thy-1$^+$ and Ia$^+$ lymphocytes. The sensitized effector cells also appeared to be specific in their activity in that they inhibit the growth of C. neoformans but not Candida albicans nor were they able to lyse the L929 mouse fibroblasts or YAC-1 lymphoma cells in the ^{51}Cr release assay.

Results in this study showed that treatment of the C. neoformans-sensitized effector cells with Actinomycin D, an RNA and protein synthesis inhibitor completely abrogated their growth inhibition activities on C. neoformans, suggesting that certain macromolecules were required for this cellular function. Supernatant (lymphokines) collected from cultures of CneF antigen- or heat-killed cryptococci-stimulated C. neoformans-sensitized splenic lymphocytes were capable of inhibiting the growth of the pathogen in vitro. Growth inhibition of C. neoformans with lymphokines was observed at 24 h after lymphokine preparations and cryptococci were mixed, higher percent of inhibition of

the pathogen was observed at 48 h. The action of the lymphokines was shown to be a non-specific one in that they inhibited the growth of C. neoformans and S. cerevisiae but not C. albicans and they were not lytic on L929 mouse fibroblasts. Treatment of normal or C. neoformans-sensitized splenic lymphocytes with various lymphokine preparations did not enhance their growth inhibitory ability on the pathogen, suggesting that the lymphokines exert only a direct inhibiting effect on C. neoformans. Lymphokines prepared from cultures of sensitized T-enriched lymphocytes stimulated with CneF antigen exhibited a significantly lower ability to inhibit the growth of C. neoformans than those displayed by lymphokines prepared from CneF-stimulated sensitized whole splenic lymphocytes. These results suggested that macrophages were required for the production of effector lymphokine molecules.

When lymphokines prepared from C. neoformans-sensitized splenic lymphocytes stimulated with CneF antigens were assayed for lymphotoxin activities on L929 mouse fibroblasts, no lymphotoxin activity was detected with the ^{51}Cr release assay. However, the same lymphokine preparation which was capable inhibiting the growth of C. neoformans demonstrated macrophage migration inhibiting factor (MIF) activities.

A two-mechanism growth inhibition hypothesis is proposed in this study, possibly, one mechanism involves a direct cellular function while the other is through lymphokine production and function. The possibility that two different T lymphocyte subclasses could be responsible for each growth inhibition mechanism is also suggested.