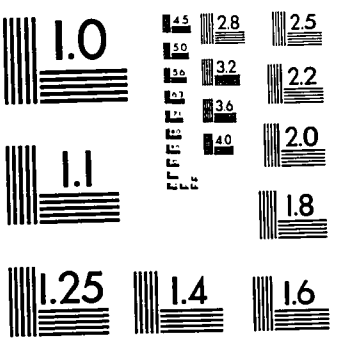

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EFFECTS OF MURINE NATURAL KILLER CELLS ON CRYPTOCOCCUS NEOFORMANS

The University of Oklahoma PH.D. 1985

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A DISSERTATION

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EFFECTS OF MURINE NATURAL KILLER CELLS ON CRYPTOCOCCUS NEOFORMANS

A DISSERTATION

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TO THE MEMORY OF MY FATHER

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GLOSSARY OF ABBREVIATIONS

ADCC, Antibody-dependent cell-mediated cytotoxicity.

Asialo GM_1, A glycosphingolipid found in high density on natural killer cells.

BACTEC system, A radiometric system which detects the metabolic activity of organisms in a culture. The system includes BACTEC instrument and the culture vials.

B-cell, Bursal or bursal equivalent-derived lymphocyte (effector cell in antibody formation).

BSA, Bovine serum albumin.

C3, The third component of complement.

C3b, A peptide fragment cleaved from C3.

CFU, Colony forming unit (a single cell which can grow as a colony on a solid medium).

C. neoformans, Cryptococcus neoformans.

CPM, Count per minute.

E:T ratio, Effector-cell to target-cell ratio.

FBS, Fetal bovine serum.

Fc receptor, A Rceptor for Fc portion of immunoglobulin molecule.

GXM, Glucuronoxylomannan.

HBSS, Hanks' balanced salt solution.

Ia antigens, I-associated antigens. Serologically defined antigens coded in the I region of H-2.

Ig, Immunoglobulin.

IgG, Immunoglobulin G.

K562, A human erythromyeloid leukemia cell line.

K cell, killer cell.

LGL, Large granular lymphocytes.

NK, Natural killer.

NRS, Normal (nonimmunized) rabbit serum.

NWN, Nylon wool nonadherent.

PBS, Phosphate buffered saline.

PBS-BSA, Phosphate buffered saline containing bovine serum albumin.

PMNL, Polymorphonuclear leukocytes.

R IgG αcrypto, IgG fraction of rabbit anti-cryptococcal serum.

SEM, Scanning electron microscopy.

T-cell, Thymus-derived lymphocyte.

Thy 1, A serologically defined T-cell associated antigen.

YAC-1, A murine moloney virus-induced lymphoma cell line.

EFFECTS OF MURINE NATURAL KILLER CELLS ON *CRYPTOCOCCUS NEOFORMANS*

CHAPTER I

INTRODUCTION

Cryptococcosis is an acute, subacute, or chronic mycosis which can appear as a pulmonary, meningeal, visceral, osseous, or mucocutaneous form of infection in man. Cryptococcosis of the central nervous system is the most commonly diagnosed form of the disease. The etiological agent is an encapsulated yeast-like organism, *Cryptococcus neoformans*, which is found in all parts of the world. Cells of *C. neoformans* vary in size from 4-20 μm in diameter and reproduce by budding at any point on the surface. The most distinct characteristic of the organism is its mucoid polysaccharide capsule which contains the antigenic determinants dividing isolates of *C. neoformans* into four serotypes; A, B, C, and D (36, 38, 123). The thickness of the capsule varies with the isolate and is also dependent on the environment in which the organism is growing (35, 84). The capsule of *C. neoformans* is considered to be a virulence factor, because acapsular mutants are known to be avirulent in experimental animals (10, 39, 40, 75). The cryptococcal capsular polysaccharide has been shown to be

anti-phagocytic (17, 76) and capable of inducing immunological unresponsiveness to cryptococci (14, 77, 90, 94-97), and these two properties of the polysaccharide capsule may contribute to the pathogenicity of the organism. The abilities of cryptococci to grow at 37°C and to produce melanin on diphenol-containing media have also been reported to be virulence factors (68, 82). The major component of the C. neoformans capsular polysaccharide is a glucuronoxylomannan (GXM) which consists of a linear mannan substituted at O-2 positions by single residues of either xylose or glucuronic acid (10, 20). The complexity of the capsular polysaccharide increases from serotype D to A to B to C (11). Serotype A and D polysaccharides are closely related and show strong cross-reactivity in immunodiffusion tests (10, 11, 20), but they are distinct from serotypes B and C in structure and antigenic characteristics (11, 37). Differences have also been discovered in the geographic distribution of serotypes. Serotypes A and D are ubiquitous in nature and have been isolated from a number of environmental sites, particularly where pigeon excreta has accumulated (5, 8, 11, 36, 109). The ecological niches of serotypes B and C are still unknown (8). Serotype A is most common in the United States and Japan (68), whereas serotype D is rare in the United States, but common in Europe. Isolates of serotypes B and C rarely cause infection except in southern California (5).

In 1966, Shadomy and Utz (119) first observed the sexual forms of C. neoformans and noted that they produced clamp connections indicating the organism should be classified as a basidiomycetes. Later, Kwon-Chung (79, 80) confirmed that the sexual forms of C. neoformans

were basidiomycete and established a new genus, Filobasidiella to include them. Studies on the sexual forms revealed further differences between serotypes A/D and B/C strains. The telemorphs, i.e. sexual forms of serotype A and D isolates are called Filobasidiella neoformans var. neoformans, and the B and C isolates are refered to as Filobasiodiella neoformans var. bacillispora (81). These two variants are found to differ morphologically, physiologically, and by DNA homology studies (2, 6, 112).

Generally it is believed that cryptococcosis is acquired by inhalation of the infectious particles (19, 36). The nature of the infectious particles present in the environment is unknown. However, desiccated yeast cells or basidiospores (50, 79, 100, 105, 126) are thought to be the likeliest candidates. Following inhalation of the infectious units, the organism may immediately enter the alveolar spaces (22, 51) or may remain in the upper respiratory tract for a period before entering the lungs (98). Primary pulmonary cryptococcal infections usually have no clinical symptoms, and most cases are self-limiting. However, when symptoms are present, they include a cough, low-grade fever, mild chest pain, weight loss, and fatigue, but these symptoms are transitory, mild, and seldom recognized as a disease entity (19, 36, 109). Pulmonary cryptococcosis has variable manifestations depending upon the immune status of the host and the severity of the disease (36, 109). In X-ray radiographs, Cryptococcus lesions may show cellular infiltrates ranging from granulomatous reactions to diffuse infiltrations, or the lesions may show no evidence of a tissue response. The absence of a tissue response and the more

diffuse infiltrates occur in immunologically compromised patients (73, 109); whereas, the granulomatous type is more frequently associated with an intact immune response. The most common type of lesions found in the immune compromised host are characterized by a locular mass of yeast cells that mechanically displace host tissue (36, 109). Furthermore, immune compromised patients are more likely to acquire disseminated disease than the patients with normal immune response (56, 72). Dissemination of cryptococci to the central nervous system and other tissues is believed to occur by the organisms gaining entrance to the blood stream (51).

The predilection of cryptococci for brain tissue is unexplained, although it has been suggested that the central nervous system (CNS) may have supporting nutrients for growth of the organism (84) or that the CNS is deficient in cryptococcal inhibitory substances and phagocytic cells (44, 67). Cryptococcal meningitis has been reported most often in either individuals with underlying disease, such as acquired immune deficiency syndrome (AIDS), malignancies, or patients undergoing immunosuppressive therapy. (13, 26, 56, 72, 117, 118, 127). Generally, individuals with deficiencies in their cell-mediated immune responses are at greater risk than normal persons (56). Whereas, humoral immune response deficiencies in the experimental animals do not associated with susceptibility to cryptococcosis (89). The duration of cryptococcal meningitis varies from a few months to 15 or 20 years (36), and before Amphotericin B therapy was employed the disease was almost always fatal. After the institution of Amphotericin B therapy the mortality dropped to 6% with a treatment failure rate of about 25%

(109). In chronic cryptococcal meningitis, remissions are common and usually followed by episodes of the disease (36). Disseminated cryptococcosis is typically diagnosed by the presence of cryptococcal antigen in body fulids (30, 49, 50, 99, 124).

Even though C. neoformans is ubiquitous in nature, and exposure to the organism is common, the occurrence of the disease is still rare. Friedman reported (42) that among over one million patients studied during the ten year period between 1971-1980 in Northern California only ten persons developed clinicaly manifested cryptococcosis, and of those ten, only two individuals had no apparent underlying immunosuppressive disease or predisposing chemotherapy. The overall incidence of disease is approximately 0.8 per one million persons per year (42). The rare incidences of the disease in normal individuals indicates that the hosts' innate and/or immune resistance mechanisms are quite adequate to resolve a primary cryptococcal infection.

Host resistance to cryptococcosis is complex and involves both innate (natural) and acquired defense mechanisms. To initiate an infection, after entering the host the organism must overcome the host's innate defensive measures, which include: mechanical, physical, chemical, and cellular barriers. Since the organism is acquired by inhalation, the upward sweeping motion of the cilia and protective mucous membranes of the respiratory tract act as mechanical barriers facilitating the removal of the organism and preventing penetration of the pathogen into the tissues (98). In addition to the above protective mechanisms, non-immunoglobulin inhibitory substances in secretions and serum (4, 67) and cationic tissue proteins (44, 45) have

been reported to function as chemical barriers against C. neoformans. If the organisms manage to escape these protective measures and infect the lung tissues, then the host's natural cellular defenses which consist of phagocytic cells, i.e. polymorphonuclear leukocytes (PMNL) and macrophages and nonphagocytic cells, i.e. natural killer (NK) cells most likely begin to eliminate the organisms.

PMNL have been reported to be capable of clearing C. neoformans from tissues of experimental animals (7, 46). However the protective role of PMNL appears to be limited or readily overwhelmed since individuals with normal functioning neutrophils have been reported to acquire cryptococcosis as readily as individual with defective PMNL function (25 ,120). Bulmer et al. (16) demonstrated that only cryptococci with small capsules were ingested by human peripheral blood neutrophils, and almost all intracellular organisms were killed by these effector cells. C. neoformans is capable of activating the alternative complement pathway (28, 29, 83) thereby generating factors which are chemotactic for PMNL. These chemotactic factors induce an influx of PMNL into the tissues (44, 45, 98). Activation of the complement pathway by cryptococci also results in the release of opsonic fragments such as C3b which bind to C. neoformans and facilitate attachment and phagocytosis of cryptococci by the neutrophils. (24, 29, 78). Several groups of investigators have shown that fresh normal serum is essential for phagocytosis of cryptococci by PMNL (24, 78), which supports the concept that complement plays a crucial role in phagocytosis. The presence of anti-cryptococcal antibody in the serum has been shown to enhance phagocytosis and

killing of C. neoformans. Miller and Kohl (87) reported that in the presence of anti-cryptococcal antibody, human peripheral blood neutrophils are effective killers of cryptococci. Their data have been supported by others showing that antibodies directed against C. neoformans can enhance phagocytosis and killing of cryptococci by neutrophils and macrophages (78, 86).

The effectiveness of macrophages in the phagocytosis and killing of cryptococci is a controversial issue (18, 25, 27, 47, 88). Bulmer and Tacker (18) showed that guinea pig alveolar macrophages phagocytized nonencapsulated cryptococci but were unable to kill the intracellular organisms. In another study, Diamond and Bennett (27), demonstrated that C. neoformans multiplied within cryptococci-activated human macrophages and killed the phagocytic cells. Their data were confirmed by others and suggest that macrophages provide a favorable place for cryptrococci growth (21, 27, 47, 70, 88). As a result of such studies, speculations have been made that the macrophages may aid in disseminating the organism from the lungs to other tissues (22). As with the PMNL, phagocytosis of C. neoformans by macrophages is enhanced by normal serum (86). Kozel et al. (78), have shown that normal serum IgG binds throughout the cryptococci capsule with low density on the surface; whereas, C3 fragments of normal serum and anti-cryptococcal antibody bind primarily on the capsular surface at high density. Therefore, the latter factors mediate binding of the opsonized cryptococci to the effector cells via the C3b and Fc receptors on the macrophages (78).

Other natural effector cells such as NK cells which are nonphagocytic have only recently been considered to function in clearance of infectious agents from tissues. NK cells comprise about 5% of the peripheral blood leukocytes and have been found in all mammalian and avian species studied (62). These natural effector cells have been detected in lymphoid organs with the exception of the thymus of normal, unstimulated animals (62). In mice NK cells are absent at birth and appear at 3 to 5 weeks of age, reach their peak in activity between 6 to 8 weeks, and decline gradually in aging mice (59, 74). NK activity varies among inbred strains of mouse, with nude or athymic mice and CBA strains having high levels of activity; BALB/c and C57BL/6 strains having intermediate activity and low activity in A strains (59, 74).

NK cells are nonadherent cells (62, 63) which express a glycosphingolipid (asialo GM_1) (71) marker and Fc receptors (60) on their surfaces but have insignificant amounts of surface immunoglobulin, Thy 1, or Ia antigens. NK cells are reactive against a broad range of target cells, from neoplastic (62, 107, 108) and virus infected cells (12, 15, 115) to allogeneic (108), and normal cells (23, 61).

Despite heterogeneity in cell surface phenotype and function (63, 125), natural killer cells appear to be quite homogenous with regard to their morphological characteristics. NK activity has been shown to be associated with large granular lymphocytes (LGL) (85, 106, 114, 121, 122) which display relatively high cytoplasmic to nuclear ratios, slightly indented to reniform eccentric nuclei, and azurophilic

cytoplasmic granules (3, 63, 85, 104, 106). The azurophilic granules vary in size and quantity and are often localized within the cytoplasmic region adjacent to the major nuclear notch (3). The cytotoxic activity of NK cells have been shown to be associated with their azurophilic granules (57, 58, 104). Killing of the tumor cell targets by NK cells is a nonphagocytic process (63-66), in which binding of NK cells to the target cells is a necessary prerequisite to killing of the target cells (41, 65, 110, 113). Binding of target cells to NK cells triggers the NK cells to release material contained within the cytoplasmic azurophilic granules into the space between the target and effector cells, resulting in killing of the target cells (57, 58, 104, 110). The granules of NK cells have been isolated by Henkart et al. (57) and have been shown to be cytolytic for a number of different target cells.

The level of NK activity of a cell population is typically measured by mixing the effector cells with ^{51}Cr-labeled target cells which are sensitive to NK cells. Murine NK cell activity is routinely assessed by the 4 h ^{51}Cr release assay against YAC-1, a mouse Moloney virus-induced lymphoma cell line (74); whereas, human NK cell activity is assessed using the K562, a human erythromyeloid leukemia cell line (63).

Natural killer cells appear to play an important role in the body's first line defense against neoplasia (62, 107, 108), and virus infection (15, 12, 63-66, 115), as well as, in regulation, differentiation, and development of normal cells (1, 23, 63, 107). NK cells have also been shown to be involved in antibody-dependent

cell-mediated cytotoxicity (ADCC) against antibody-coated target cells (101, 122). The antibody-coated target cells bind to the effector cells via NK cells' Fc receptors.

NK activity can be augmented by interferon and interferon inducers such as polyinosinic:cytidylic acid (33, 34, 48, 59, 61, 74) and Corynebacterium parvum. The latter has both the capacity to augment as well as depress the NK activity (102, 116).

Although the majority of NK cell studies have been done with tissue cell targets, recently, the possible role of NK cells against nontissue targets such as bacteria (101), fungi (69, 92), and parasites (55) has attracted some interest. In 1982 Murphy and McDaniel (92) were the first to report that NK cells were inhibitory to growth of a mycotic organism, C. neoformans. These investigators showed that murine splenic nylon wool nonadherent cells with characteristics of NK cells can inhibit the in vitro growth of C. neoformans. In a series of experiments, they determined the levels of NK reactivity of murine splenic cells simultaneously with the ability of the splenic cells to inhibit the growth of C. neoformans. These investigators varied the levels of NK activity by using strains of mice with high and low NK activity, mice of different ages, and NK augmenting and depressing agents, and they demonstrated that the level of NK reactivity of the effector cells always correlated with the ability of effector cells to inhibit the growth of cryptococci. Finally they showed that the effector cells responsible for cytolysis of YAC-1 tagets and growth inhibition of cryptococci had characteristics of NK cells in that they were esterase-negative, Thy 1-negative, immunoglobulin-negative, and

asialo GM_1-positive (92). The growth inhibitory effect of NK cells against C. neoformans is not limited to mice since rat large granular lymphocytes also have been shown to inhibit the growth of cryptococci in vitro (93).

In vivo studies by Murphy (91), using the beige mouse model reveal that NK cells are effective in early clearance of cryptococci from tissues of infected animals. The beige (bg) mutation in the C57BL/6 mouse strain results in impairment of NK cell functions, whereas other cellular activities of other cells such as promonocytes, macrophages, and cytotoxic T cells seem to be normal (111). In contrast, the heterozygous bg/+ littermates have normal levels of NK activity (111). Using bg/bg and bg/+ mice, Murphy demonstrated that one and three days after injecting 7 week old mice with 2×10^4 viable C. neoformans, the bg/+ animals had significantly lower mean numbers of cryptococci colony forming units (CFU) in their lungs and spleens than did bg/bg animals (91). Moreover, her data indicated that in vitro phagocytosis and killing of C. neoformans by macrophages and polymorphonuclear leukocytes (PMNL) from the two groups of animals were equal (98). In addition, in vivo chemotaxis of PMNL and macrophages in response to an intraperitoneal injection of cryptococci was similar for both groups of mice (98). Therefore, it was concluded that NK cells were responsible for clearance of the C. neoformans from the lungs and spleens of bg/+ mice, supporting the concept that NK cells also function in vivo against C. neoformans (91).

Even though the in vitro and in vivo growth inhibition of cryptococci by NK cells have been shown (91, 92), the nature of the

effects exerted by these nonphagocytic cells on this mycotic pathogen have not been elucidated. It was the objective of the work presented in Chapter II to first obtain highly enriched murine NK cell populations, and then to examine, in more detail, the in vitro interactions between NK cells and cryptococci. Since NK cells form conjugates with their tumor cell targets prior to the lytic event (41, 65, 110, 113), the possibility of in vitro NK cell- Cryptococcus conjugate formation was investigated. Conjugate formation can be an important step in the process of C. neoformans growth inhibition by NK cells. It is likely that binding of the cryptococci to the NK cells triggers the NK cells to release inhibitory substances into the microenviroment between the effector and the target cells. Therefore, direct contact of the NK cells with C. neoformans targets could be considered as a prerequisite for growth inhibition of cryptococci.

NK cells have Fc receptors for IgG (60), and have been shown to be involved in antibody-dependent cell-mediated cytotoxiciy (ADCC) or K cell activity against IgG coated target cells (101, 122). Therefore, it would be of interest to know whether the growth inhibitory activity of NK cells against C. neoformans could be augmented in the presence of IgG fraction of anti-cryptococcal serum via Fc receptor binding. Anti-cryptococcal antibody has been detected in early stages of infection, in patients with nondisseminated form of disease, and after successful therapy (50). Even though several investigators have attempted to demonstrate the in vivo protective role of anti-cryptococcal antibody, the results have not been conclusive (43, 52-54, 89, 103). Currently, it is believed that humoral immunity to

cryptococcosis is secondary and not essential to protective response in vivo. However, in vitro studies by Diamond (31), and Diamond and Allison (32), showed that anti-cryptococcal antibody induces killing of C. neoformans by unstimulated peripheral blood nonphagocytic leukocytes in the absence of the serum complement components. Therefore the interaction of humoral and innate cellular defense could be important factor in early stages of infection and after successful therapy when antibody appears in the patients. In Chapter III of this manuscript, the effects of anti-cryptococcal antibody on NK cell-mediated growth inhibition of cryptococci was examined.

Antibody-dependent growth inhibition of cryptococci by NK cells could be an important factor in clearance of the organisms in vivo in early stages of infection or after recovary. It is also possible that NK cells clear a primary cryptococcal infection via the opsonizing antibodies which have been shown to be present in normal human serum (78, 79).

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CHAPTER II

ABSTRACT

In Vitro Binding of Natural Killer Cells to Cryptococcus neoformans Targets

Nylon wool nonadherent splenic cells from 7-8 week old CBA mice were further fractionated on discontinous Percoll gradients. The enrichment of natural killer (NK) cells in Percoll fractions 1 and 2 was confirmed by morphological examination, immunoflourescent staining, and by assessing the cytolytic activity of each Percoll cell fraction against YAC-1 targets in the 4 h ^{51}Cr release assay. Cells isolated from each Percoll fraction were tested for growth inhibitory activity against Cryptococcus neoformans, a pathogenic yeast-like organism, using an in vitro 18 h growth inhibition assay. The results showed that NK enrichment was concomitant with the enrichment of anti-cryptococcal activity in the Percoll fractions 1 and 2. Cells from NK-rich fractions formed conjugates with the mycotic targets similar to those reported in NK-tumor systems. In addition, the percentage of effector cell- Cryptococcus conjugates was directly proportional to the level of C. neoformans growth inhibitory activity

of the effector cells employed. Scanning electron microscopy of the effector cell- Cryptococcus conjugates showed a direct contact between the effector cells and cryptococci targets. An immunolabeling method combined with scanning electron microscopy was used to demonstrate that the effector cells attached to C. neoformans were asialo GM_1-positive, therefore, had NK cell characteristics.

SECTION I

INTRODUCTION

Cryptococcus neoformans is a ubiquitous, encapsulated yeast-like organism which causes infections in man ranging from asymtomatic pulmonary infections to fatal disseminated meningitis. The most severe cryptococcal disease usually occurs in immunocompromised individuals (3, 26). Since C. neoformans is present in nature, many people are exposed to it, but only a small number actually acquire the disease. This implies that there must be an active natural surveillance mechanism present in the host against this organism.

The murine model has provided insights into the interaction of resistance mechanisms of the host against cryptococci, and using such models, both natural and acquired host defenses have been described. Macrophages and polymorphonuclear leukocytes have been reported as providing innate host defense against C.neoformans (1, 4, 27). Recently, data generated by Murphy and McDaniel (20) indicated that natural killer (NK) cells, are potentially the third means of natural cellular resistance against this organism. Their data for the first time, provided substantial indirect evidence that unstimulated murine splenic cells with characteristics of NK cells could effectively

inhibit the in vitro growth of C. neoformans (20). It was shown that cytotoxicity of the various murine splenic effector cell populations against YAC-1 targets directly correlated with the effector cells' abilities to inhibit cryptococci growth. When adherent cells were removed from the effector cell pools by passage through nylon wool columns, both NK activity against YAC-1 targets and growth inhibitory activity against C. neoformans increased. The effector cells were similar to NK cells in that they were nylon wool nonadherent without immunoglobulin or significant amounts of Thy 1 or Ia antigens on their surfaces, but expressed an asialo GM_1 surface marker (20).

Although the growth inhibition of C. neoformans by NK cells or NK-like cells has been demonstrated, the nature of the effector cell-Cryptococcus target interactions is, as yet unknown. Therefore, we have begun studies directed toward elucidating the mechanisms by which NK cells affect C. neoformans targets. The NK-tumor target model appeared to be a useful guide for directing these investigations. In that model, NK cells kill their targets through a nonphagocytic means, by forming conjugates with their tumor targets in vitro (6, 9, 24, 25). Binding of the effector cells to the targets is a pre-lytic event in NK-tumor systems (9, 24). So one interest we had was whether or not effector cell- C. neoformans conjugates could be observed in our system. In this report, we present data that show NK enriched cell populations formed conjugates with C. neoformans targets. Furthermore, the numbers of effector cell- C. neoformans conjugates were directly proportional to the levels of growth inhibitory activity of the effector cell populations. Finally, using immunolabeling combined with

scanning electron microscopy, we demonstrated that effector cells which were attached to cryptococci were asialo GM_1 positive, a property characteristic of NK cells (11, 13). Considering: (a) the cell populations being used were highly enriched for NK cells; (b) asialo GM_1 is found in high density on NK cells (13); and (c) dilution of anti-asialo GM_1, under the conditions being used, did not label the cells having low density asialo GM_1, then, the data presented provides strong evidence that NK cells were the cells forming conjugates with C. neoformans.

SECTION II

MATERIALS AND METHODS

Mice. Inbred CBA/J mice purchased from the Jackson laboratories, Bar Harbor, Maine, were used between 6-8 weeks of age for this investigation.

Tumor cell target. YAC-1, a Moloney virus-induced lymphoma obtained originally from Ronald Herberman, National Cancer Institute, was the cell line used routinely in the 4 hr ^{51}Cr release assay to assess NK activity. YAC-1 cells were maintained in culture in RPMI 1640 medium (Grand Island Biological Co., Grand Island, NY.) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 µg/ml streptomycin and 100 U/ml penicillin.

Mycotic target. *Cryptococcus neoformans* isolate 184, an A serotype employed throughout this study was previously described by Murphy and Cozad (19). The organisms were maintained on modified Sabouraud agar slants and were harvested from slants after 72 h incubation at room temprature.

Preparation of the effector cells. Effector cells were prepared as previously described by Murphy and McDaniel (20). Briefly, mice were sacrificed by cervical dislocation. Spleens were pooled and

single cell suspensions were prepared in Hanks' balanced salt solution (HBSS). Erythrocytes were removed by treating for 2 min. with ammonium-chloride Tris-buffered solution, a treatment which did not significantly affect NK cell activity. The cells were washed twice in RPMI 1640 medium containing 5% FBS before being passed through nylon wool columns. The NWN cell populations contained an average of 95% viable cells, with only 2% immunoglobulin-positive cells and 1% esterase-positive cells. To further enrich for NK cells, NWN cells were separated into five fractions on discontinuous Percoll gradients consisting of 70 to 30% Percoll (Pharmacia. Uppsala, Sweden) by using the method of Luini et al. (17). Each cell fraction was collected, washed twice in HBSS, suspended in RPMI 1640 medium containing 10% FBS, and used in the experiments. The cell viability of each cell fraction was determined by trypan blue dye exclusion.

Cytotoxicity assay against YAC-1 targets. The ^{51}Cr release assay described by Murphy and McDaniel (20) was used to determined the levels of NK cell reactivity in the cell fractions obtained from Percoll gradients. Briefly, YAC-1 cells were labeled for 1 h at 37°C with 100 µCi of radioactive sodium chromate (Amersham Corp., Arlington Heights, IL.) and then were used as targets in a 4 h assay. The ratio of effector cells to target cells 25:1 was used throughout this study. The percentage of ^{51}Cr released into the supernatant was calculated by using the following formula:

% specific release = [(CPM experimental - CPM spontaneous)/CPM maximum] X 100.

Experimental wells contained effector and target cells, spontaneous

release wells contained ^{51}Cr-labeled target cells and medium, and maximum ^{51}Cr release wells contained labeled target cells mixed with 2 N HCL.

Immunofluorescent microscopy. Each Percoll fraction was treated separately with goat anti-Thy 1 serum (20), rabbit anti-mouse immunoglobulin (20), or rabbit anti-asialo GM_1 (Wako Chemicals USA, Inc., Dallas, Tex.); this was followed by treatment with rabbit anti-goat serum for the anti-Thy 1-treated cells or goat anti-rabbit serum in the case of anti-mouse immunoglobulin-treated cells or anti-asialo GM_1-treated cells. Each of the secondary antisera had been conjugated to fluorescein isothiocyanide (Cappel Laboratories, Westchester, Pa.). For controls, cells were treated with tissue culture medium in place of goat anti-Thy 1 antibody or normal rabbit serum to replace rabbit anti-mouse immunoglobulin serum or rabbit anti-asialo GM_1 antibody, and then appropriate secondary antiserum was applied. Next, the cells were examined for the percentage of fluorescent cells after treatment with each antiserum to indicate the percent Thy 1, immunoglobulin, and asialo GM_1 surface markers. A total of 400 cells were counted in each case by using an Olympus model BH-2 fluorescent microscope. In some experiments, adherent cell fractions obtained from nylon wool columns were also stained with the same concentration of anti-asialo GM_1 and the secondary antibody as a control.

Nonspecific esterase staining. Each Percoll cell fraction was stained for nonspecific esterase by the method of Koski et al. (14) to assess macrophage contamination.

Giemsa staining. Cytocentrifuged slide preparations of each Percoll cell fraction were stained with 3% aqueous Giemsa solution and examined by using a 100 X objective on an Olympus microscope.

C. neoformans growth inhibition assay. The growth inhibition assay which we used has been described previously (20). Briefly, 0.1 ml portions of each effector cell population (10^6 cells per ml) were mixed with 0.1 ml portions of C. neoformans target cells (2 X 10^4 cells per ml) in RPMI 1640 medium containing 10% FBS and antibiotics (ratio of effector cells to target cells, 50:1) in duplicate in 96-well flat-bottom microtiter plates. Control wells contained the target cells and tissue culture medium. The plates were incubated for 18 h at 37°C in an atmosphere of 5% CO_2; then the contents of each well were serially diluted, and 1 ml portions of each final dilution were plated in duplicate onto modified Sabouraud dextrose agar plates. The number of C. neoformans CFU was determined after 3 days of incubation at room temperature, and the percentage of growth inhibition was calculated as follows: % of growth inhibition = [(mean CFU of control - mean CFU of experimental culture)/mean CFU control] X 100.

Enumeration of effector cell-C. neoformans conjugates. Samples of effector cells obtained from different Percoll fractions or NWN cells were incubated with C. neoformans target cells at a ratio of effector cells to target cells of 2:1 for 18 h at 37°C in an atmosphere containing 5% CO_2. Then the samples were stained with 0.25% alcian blue in serum-free RPMI 1640 medium (pH 6.4) for light microscopy. The percentage of effector-target conjugates in each

fraction was calculated after counting 400 effector cells.

Preparation of antibody-latex conjugates (immunolatex). Covalent binding of goat anti-rabbit antibodies (Cappel) to 0.24 μm latex sphere (Polysciences Inc., Warrington) was done by using a modification of the method of Gamliel et al. (7). While being stirred at room temperature, 0.2 ml of 2.5% solid monodispersed latex spheres and 0.05 ml of diluted antiserum containing 250 μg of protein per ml were added to 4.65 ml of 0.01 M phosphate buffer (pH 9.4). The final volume was brought to 5.0 ml by adding 0.1 ml of 1% glutaraldehyde. The reaction mixture was stirred for 2 h, quenched with 0.1 M glycine, and dialysed against phosphate buffer (pH 9.4) for 24 h at 4°C. The conjugated latex preparation was centrifuged at 17,400 X g for 25 min, and the pellet was suspended in phosphate-buffered saline (PBS) (pH 9.4). The large aggregates were removed by centrifugation at 1,940 X g for 20 min before the conjugated latex spheres were used for indirect labeling of cells.

Cell labeling and specimen preparation for SEM. Effector cells obtained from Percoll fractions 1 and 2 were adjusted to a concentration of 10^7 cells per ml, and 0.1 ml of this preparation was incubated with 0.1 ml of C. neoformans at a concentration of 5 X 10^6 cells per ml (ratio of effector cells to target cells, 2:1). For controls, 0.1 ml of the C. neoformans target cells was incubated in the presence of an equal volume of tissue culture medium for 18 h at 37°C. After incubation, the effector cell-target cell mixtures or target cells alone were fixed in 1% glutaraldehyde in PBS (pH 7.0) at room temperature. Excess glutaraldehyde was removed by incubating the

cells with 0.1 M glycine in PBS (pH 7.0) for 30 min, followed by two washes with PBS (pH 7.0) containing 0.1% bovine serum albumin. Next, the cells were treated with 0.05 ml of a 1:40 dilution of rabbit anti-asialo GM_1 or normal rabbit serum as a control for 30 min at room temperature. After two washes with phosphate-buffered saline containing 0.1% bovine serum albumin (PBS-BSA) (pH 7.0) and a final wash with PBS-BSA (pH 9.4), the cells were treated for 30 min at room temperature with 0.1 ml of monodispersed latex spheres conjugated to goat anti-rabbit antiserum. The unbound latex spheres were removed by three washes with PBS-BSA (pH 9.4) and low-speed centrifugation. The labeled cells were stored in cacodylate buffer-0.1 M sucrose plus 1% glutaraldehyde overnight at $4^{\circ}C$. Then the cells were mounted on poly-L-lysine (Sigma Chemical Co., St. Louis, Mo.) coated cover glasses (Carolina Biological Supply. Burlington, N.C.), treated with 1% osmium tetroxide at $4^{\circ}C$ for 1 h, and washed with cacodylate buffer. The cells were dehydrated with a series of increasing concentrations of ethanol in water and subjected to critical point drying from liquid CO_2. The cover glasses were mounted on specimen stubs, and this was followed by sputter coating with gold-palladium. Samples were examined with an Etec Autoscan microscope.

SECTION III

RESULTS

Effect of Percoll fractionation of NWN splenic cells on C. neoformans growth inhibitory activity. Effector-target conjugates can be more frequently observed if highly purified populations of NK cells are used; therefore after obtaining the nylon wool nonadherent (NWN) splenic cell fraction, we further enriched for NK cells by fractionation of the NWN cells on 5-step discontinuous Percoll gradients. To establish that NK enrichment was being achieved by Percoll fractionation, the morphology, phenotype, and function of the splenic NWN cells and the cells from each Percoll fraction were determined. Light microscope examination of Giemsa stained cytocentrifuged preparations of the cells showed that the morphological characteristic of the cells in each fraction were consistant with the report of Luini et al. (17), low density cells from fractions 1 and 2 contained about 35% large granular lymphocyte (LGL), plus some large agranular lymphocytes (LAL) as described by Kumagai et al. (16) and Itoh et al. (10); fractions 3 and 4 contained mainly small lymphocytes, and fraction 5 consisted of cell debris and erythrocyte ghosts. The phenotypes of the various cell fractions were determined by surface

marker analysis using immunofluorescent staining. As indicated in Table 1, approximately 40% of the cells in fraction 1 and 2 stained with anti-asialo GM_1, a marker found in high density on NK cells (11, 13). Although asialo GM_1 has been reported to be found in low density on macrophages and polymorphonuclear leukocytes of other species (9, 13), the dilution of anti-asialo GM_1 antibody used in this study, in an indirect immunofluorescence assay did not label the adherent fraction of murine splenic cells. Fractions 1 and 2 had 21% and 17%, respectively, Thy 1-positive cells, but virtually no immunoglobulin-positive cells. Finding Thy 1^+ cells in fractions 1 and 2 was not unexpected since Thy 1 is found on some, but not all, NK cells (18). Fraction 3, on the other hand, consisted predominantly of small lymphocytes with 54% of the cells having Thy 1 antigen on them, an insignificant percentage of immunoglobulin-positive cells, and only 6% asialo GM_1-positive cells, indicating that fraction 3 was the T cell enriched population. Fraction 4 cells were morphologically and phenotypically similar to cells from fraction 3 with approximately 30% Thy 1-positive cells. Fraction 5 cells did not have any of the above markers. All fractions were free of significant macrophage contamination as indicated by the 0-1% esterase-positive cells (Table 1). The NK functional activity of the NWN and each Percoll cell fraction was determined with a 4 h ^{51}Cr release assay using YAC-1 tumor cells, and a reduced effector-target ratio of 25:1 was employed due to insufficient yields from certain of the cell fractions (Fig. 1). The cells from fractions 1 and 2 demonstrated approximately 77% of the total measurable YAC-1 cytotoxicity. These observations confirmed that

Percoll fractions 1 and 2 were enriched for NK cells, whereas, fractions 3, 4, and 5 had low to insignificant activities against YAC-1 targets indicating reduced numbers of NK cells.

Once we established that splenic cells with NK morphology, surface phenotype and functional activity against YAC-1 targets were isolated in Percoll fractions 1 and 2, then, the growth inhibitory activities of these NK-rich fractions, as well as that of the NWN cells and the NK-deficient fractions, i.e. 3, 4, and 5 were tested against C. neoformans using an effector-target ratio of 50:1 which was one tenth that used in previous studies (20). The results shown in Fig. 1. demonstrated that almost 80% of the total measurable growth inhibitory activity against C. neoformans was present in the NK-rich fractions. Furthermore, the anti-cryptococcal activities of fractions 1 and 2 were approximately three to four times higher than the activity of the NWN cell population when tested in identical assays. Fraction 3 had low growth inhibitory activity, and fractions 4 and 5 did not show significant levels of anti-cryptococcal activity.

C. neoformans-binding cells. Since NK cells form conjugates with their tumor cell targets prior to the lytic event (9, 24), the possibility of effector- Cryptococcus conjugate formation in the in vitro growth inhibition system was investigated by light microscope examination of alcian blue-stained preparations of effector cell- C. neoformans mixtures. Alcian blue served as a differential stain, with cryptococcal cells staining under the conditions used and the effector cells remaining unstained. To assay cryptococci binding capacity of the different effector cell populations, NWN and Percoll cell fractions

1 through 5 were used against C. neoformans at an E:T ratio of 2:1, to give sufficient numbers of conjugates to be observed microscopically. The percentage of cryptococcal binding cells, i.e. % conjugates, for each of the effector-target cell mixtures are shown in Fig. 1. The numbers of effector- C. neoformans conjugates were directly related to the NK activities of the effector cell populations (correlation coefficient = 0.96). For example, the NK-rich fractions, i.e. fractions 1 and 2 contained higher numbers of cryptococcal-binding cells than did the NWN cell population from which the fractions were isolated. In direct contrast, the NK-deficient populations, fractions 3, 4, and 5, which had low percentages of LGL and asialo GM_1 positive cells, had reduced numbers of conjugates when compared to the percentage of conjugates in the unfractionated NWN population (Fig. 1).

SEM studies of effector cell-C. neoformans conjugates. To gain a higher level of resolution and obtain a more detailed view of the effector-target cell interactions and morphology, samples of the effector-cryptococci mixtures, effector cells alone, or C. neoformans cells alone were processed for SEM after an incubation period of 18 h, using identical conditions of preparation for all samples. Since the cell recovery from fraction 1 was very low, approximately 1-3% of total input (Table 1), fractions 1 and 2 were pooled and used against C.neoformans for these studies.

In the scanning electron microscope, the effector cells appeared as generally homogenous cell populations in terms of size, morphology, and density of surface projections. Almost all effector cells displayed a uniform, and rather dense array of microvilli, and delicate

ruffles on their spherical surfaces (Fig. 2-4). On the other hand, C. neoformans cells were variable, both in size and surface morphology. Heterogeneity was expected for the cryptococci cells because they vary in size between 4-20 μm and budding forms are frequent. Cryptococcal cells appeared spherical to oval-shaped with relatively smooth surfaces having occassional fragments of a lacy material which was most likely capsular remnants. The capsular material was apparently partially removed from the cells during the preparation for SEM studies. In the SEM images of effector cell- C. neoformans conjugates, effector cells usually had a broad contact area with the cryptococcal targets, and in virtually all observed cases, appendages were seen connecting the effector cells to the targets (Fig. 2-4, 22, Murphy, Current Topics in Medical Mycology, Vol. I, in press). Once we had established visualization of the effector cell- C. neoformans conjugates with SEM, we wanted to demonstrate that the effector cells attached to C. neoformans cells had NK-associated surface markers by an indirect immunoelectron microscopy method. This included treating the cells with rabbit anti-asialo GM_1 as the primary antibody, followed by goat anti-rabbit antibody conjugated to latex spheres as the secondary antibody system. In all the observed cases, the lymphocytes attached to cryptococci were labeled with immunolatex spheres (Fig. 3). In control experiments where the cells were first treated with normal rabbit serum in place of rabbit anti-asialo GM_1, followed by goat anti-rabbit conjugated latex spheres, the effector cells attached to C. neoformans targets remained free of latex spheres (Fig. 4), indicating the effector cells had been specifically labeled by this method. C.

neoformans cells neither associated with the anti-asialo GM_1, nor with normal rabbit serum; therefore, they remained free of latex spheres in both cases (Fig. 5). In the SEM preparations of the effector- C. neoformans cell mixtures, both budding and non-budding yeast cells were seen in direct contact with the effector cells. Although C. neoformans and effector cells could be easily differentiated with the electron microscope, we selected for publication only the conjugates which had budding C. neoformans cells, so that it would be absolutely clear to those viewing only a minimal number of photomicrographs which cells were the effectors and which cells were the targets.

Phagocytosis of C. neoformans by the effector cells or the tendency toward phagocytosis of the cryptococcal targets was not observed during the SEM studies of the effector- C. neoformans conjugates (Fig. 2-4, 22, Murphy, Current Topics in Medical Mycology, Vol. I, in press). This might be expected since, the effector cells collected from the low density fractions, i.e. fractions 1 and 2, were virtually free of phagocytic cells such as macrophages and polymorphonuclear leukocytes (Table 1). These observations indicate that growth inhibition of cryptococci was by a nonphagocytic mechanism, and they strengthen the view that NK cells, not phagocytic cells, were responsible for C. neoformans growth inhibition in our system.

Table 1. Characteristics of Percoll fractionated nylon wool nonadherent murine splenic cells.

Percoll Fractions	Cells Recovered X 10^6	% Recovered of Input	% LGL	% Asialo GM_1^+	% Thy 1^+	% Mouse Ig^+	% Non-specific Esterase Positive
NWN	-	-	6	9	75	2	1
1	0.7	3	41	44	21	0	0
2	4.2	21	32	36	17	0	0
3	10.7	53	5	6	54	1	1
4	1.8	9	1	3	29	0	0
5	0.3	1	0	0	2	0	0

Figure 1. Comparison of activities of cells from the 5 different Percoll fractions in ^{51}Cr release assay against YAC-1 targets and the cryptococci growth inhibition assay. E:T ratios of 25:1 and 50:1 were used in the ^{51}Cr release and growth inhibition assay, respectively. The percentages of effector cell- *C. neoformans* conjugates were calculated as follows:

% conjugates = (Number of effector cells bound to cryptococci/Total number of effector cells) X 100

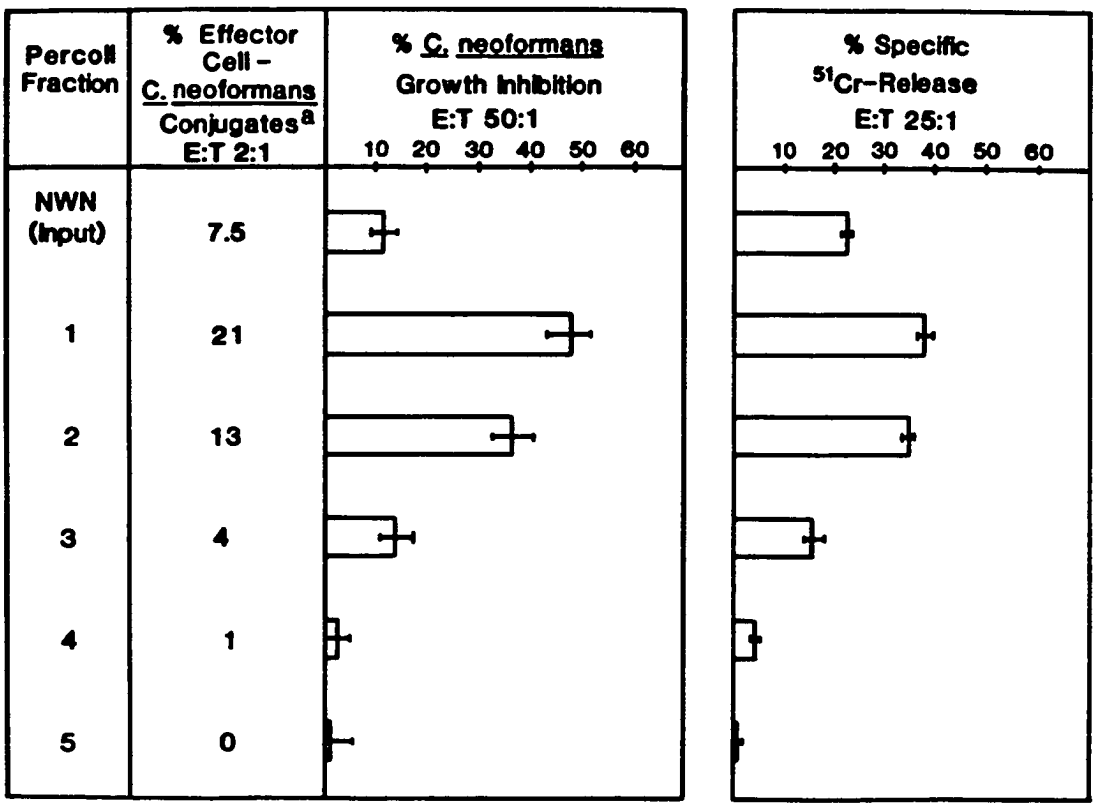
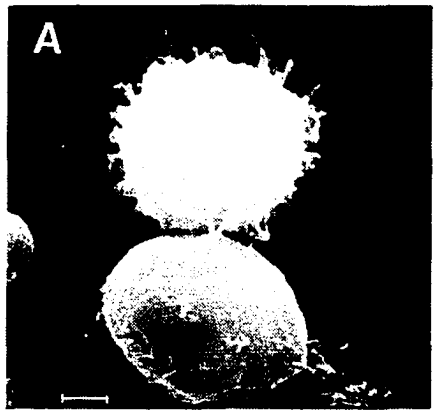

Percoll Fraction
% Effector Cell – C. neoformans Conjugates[a] E:T 2:1
% C. neoformans Growth Inhibition E:T 50:1
10 20 30 40 50 60
% Specific ^{51}Cr-Release E:T 25:1
10 20 30 40 50 60
NWN (Input)
7.5
1
21
2
13
3
4
4
1
5
0

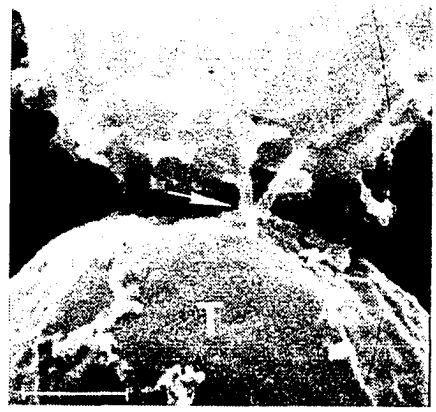
Figure 2. (A) Scanning electron micrograph of effector cell- C. neoformans conjugates. (B) Higher magnification of the effector cell-target cell contact area in (A). The arrow indicates appendages of the effector cell (E) extended toward the cryptococcal target cell (T).

Figure 3. Scanning electron micrograph of effector- C. neoformans conjugates stained with rabbit anti-asialo GM_1, followed by goat anti-rabbit antibody-conjugated latex spheres. The arrows indicate the latex spheres on the effector cell.

Figure 4. Scanning electron micrograph of effector- C. neoformans conjugates treated with normal rabbit serum, followed by goat anti-rabbit antibody-conjugated latex spheres in a control experiment. The arrow indicates the effector cell.

Figure 5. Representative scanning electron micrograph of C. neoformans treated with anti-asialo GM_1, followed by goat anti-rabbit antibody-conjugated latex spheres. Bars = 1.0 μm.





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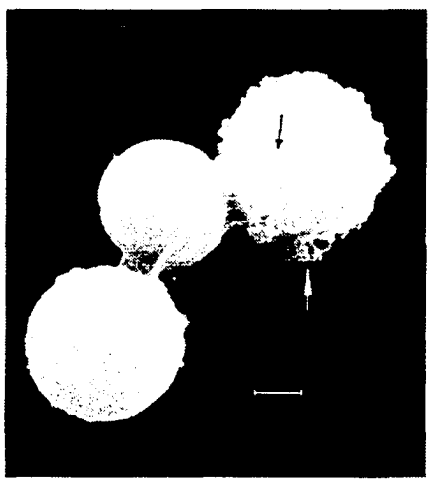

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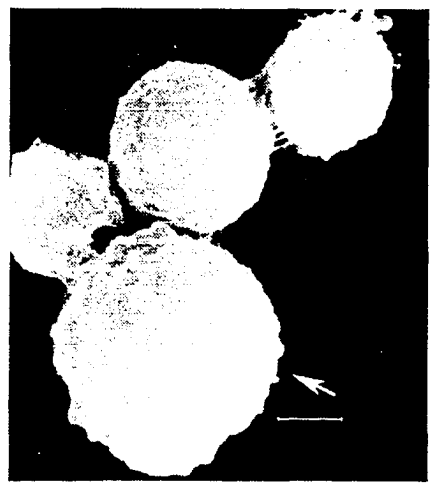

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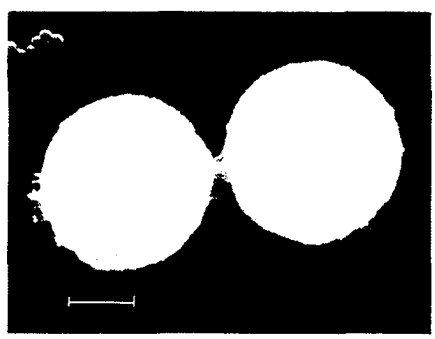

[5]

SECTION IV

DISCUSSION

Direct evidence is offered in this paper showing the effector cells collected from NK-rich Percoll gradient cell fractions 1 and 2 were capable of binding to C. neoformans targets and forming effector-target conjugates similar to those reported by others in NK-tumor systems (6, 24, 25). Furthermore, the numbers of effector cell- C. neoformans conjugates were directly proportional to the levels of NK activity of the effector cell populations employed.

In past studies (20), it was shown that cytotoxicity of YAC-1 targets induced by the various murine splenic effector cell populations directly correlated with the effector cells' growth inhibitory activities against C. neoformans. When adherent cells were removed from the effector cells by nylon wool passage, both NK activity against YAC-1 target and growth inhibitory activity against cryptococci increased. In present study, when we further enriched for NK cells by fractionation of the NWN splenic cells through 5-step discontinuous Percoll gradients, the NK activity against YAC-1 targets as well as growth inhibitory activity against cryptococci was enhanced 2-5 fold in the NK-rich Percoll fractions 1 and 2. Furthermore with NK-rich

effector cell populations, an E:T ratio one-tenth of that used in previous investigations could be used for the assay, and a substantial level of cryptococci growth inhibition was achieved with this significantly lower ratio. Percoll fractions 1 and 2, which were virtually free of macrophages, as shown by their esterase-negative characteristics, inhibited the growth of cryptococci considerably better than NWN cells, which had 1% macrophages when used in identical assays. These results eliminate the possibility that contaminating macrophages were involved in inhibiting C. neoformans growth. Furthermore, phagocytosis, which is generally a prerequisite to macrophage killing was not observed at any time when Percoll fractions 1 and 2 effector cells were associated with cryptococci. The effector cells had a broad contact area with C. neoformans targets, but they had neither pseudopodial projections nor did they showed any tendencies to engulf the organism (Fig 2 A and B) Instead, the appendages of the effector cells extended toward the targets and made a perpendicular contact with the cryptococcal cell surface (Fig 2 B). Similar appendages have been observed in scanning electron microscopy by us (unpublished data) as well as others (2) between NK cells and target cells which are recognized by NK cells, i.e. YAC-1 and K562 cells. These observations, strengthened the concept that C. neoformans growth inhibition was not the result of a phagocytic mechanism. In contrast the inhibitory effects on the cryptococci targets are exerted, most likely, after the direct contact is established between the effector and the target cells, since conjugates can be detected earlier than growth inhibition (unpublished data). It is possible that soluble

inhibitory substances (factors) are secreted by the effector cells into the microenvironment between the two cells or into the medium as has been suggested in the NK-tumor cytolytic models (2, 5, 28). The inhibitory factors could be associated with the azurophilic granules seen in NK cells (8, 23).

In the SEM studies of the effector- C. neoformans conjugates, effector cells in direct contact with cryptococci, specifically bound anti-asialo GM_1 antibody. The rabbit anti-asialo GM_1 antibody used in the immunoscanning electron microscopy experiments has been shown to be relatively specific for NK cells in many laboratories (11, 12, 13). In addition, it has been shown previously (20) that treatment of NWN cells with anti-asialo GM_1 and complement significantly reduced both NK activity against YAC-1 targets and growth inhibitory ability against C. neoformans. Although asialo GM_1 has been reported to be found in low density on macrophages and polymorphonuclear leukocytes of other species (9, 13), the dilution of anti-asialo GM_1 antibody used in this study, in an indirect assay did not label the adherent fraction of murine splenic cells. Therefore, we concluded that the effector cells in direct association with C. neoformans were NK cells.

Observations made during the SEM studies suggested that the NK cells were likely to recognize some structures on the cryptococci cell wall other than the capsule. This was proposed because cryptococci or effector cell- C. neoformans mixtures which were subjected to additional washing steps during the immunolabeling procedures had less lacy capsular material clinging to the cryptococci surfaces than did

the cells which were prepared for studies not involving immunolabeling. Other investigators have also reported the destruction of capsular material during routine EM specimen preparation (15). Despite the loss of the capsular material, effector cell appendages were firmly attached to the cryptococci and did not dissociate during immunolabeling preparation for electron microscopy. These appendages were in association with the smooth surface of the organism rather than with the lacy capsular material (Fig 2 B). Other data from our laboratory also support the idea that the effector cells do not associate with the cryptococci capsule. For example, growth inhibition of C. neoformans by the effector cells was independent of the size or serotype of the cryptococci capsules (20). Furthermore, in preliminary experiments, when C. neoformans culture filtrates which contained capsular polysaccharide (21) were added to effector- C. neoformans cell mixtures at the initiation of the growth inhibition assay, cryptococci growth inhibition was not affected (Murphy, unpublished data); suggesting that capsular components could not block binding of the effector cells to the cryptococci. Further work must be done to establish the nature of the effector cell attachment site on the C. neoformans targets.

Our data establish that the effectors against C. neoformans fit the characteristics defined for NK cells. The effector cells are nylon wool nonadherent, nonphagocytic, low density, LGL, immunoglobulin-negative, free of or have low density Thy 1 antigen, and bear asialo GM_1. In this study we demonstrated that NK cells make direct contact with cryptococci targets without phagocytosis, indicating the growth inhibition of the cryptococci is an extracellular

event. Furthermore, we showed the percentage of the conjugates was proportional to the cryptococci growth inhibition, suggesting association of effectors with the cryptococci targets may be essential to inhibition of the growth. Further studies are being directed toward elucidating the entire mechanism of C. neoformans growth inhibition by NK cells.

SECTION V

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CHAPTER III

ABSTRACT

Antibody-Dependent Cell-Mediated Growth Inhibition of Cryptococcus neoformans by Natural Killer Cells

Previous data from this laboratory indicate that normal murine nylon wool nonadherent splenic cells, with characteristics of NK cells, effectively inhibit the in vitro growth of *Cryptococcus neoformans*, a yeast-like pathogen. NK cells have been shown to be involved in antibody-dependent cell-mediated cytotoxicity (ADCC) against IgG-coated tumor cells and xenogenic erythrocytes. Therefore, we were interested in assessing the effects of the IgG fraction of rabbit anti-cryptococcal serum on the NK cell-mediated inhibition of *C. neoformans* growth. The conventional method of determining the numbers of colony forming units (CFU), used previously for assessment of viable cryptococci at the end of the growth inhibition assay, did not give accurate measurements of the numbers of viable cells due to minor clumping of the organisms in the presence of anti-cryptococcal antibody. Therefore, the BACTEC radiometric system was evaluated for determining the viability of cryptococci at the end of the growth

inhibition assay. We showed that the BACTEC growth index readings reflected the numbers of viable cryptococci initially inoculated into the culture vials, and that the growth index readings were not affected by clumping of C. neoformans, the presence of nonviable organisms, or the presence of the effector cells. After establishing that the BACTEC system provided an appropriate index of the numbers of viable cryptococci at the end of the growth inhibition assay, that method was employed in subsequent studies to determine the effects of anti-cryptococcal antibody on NK cell-mediated growth inhibition of cryptococci. Our results showed that the anti-cryptococcal antibody significantly augmented the ability of NK cells to inhibit the growth of C. neoformans, when compared to normal rabbit serum or tissue culture medium. Furthermore, the antibody alone did not have an adverse effect on cryptococci, confirming that the reduced growth indices obtained from test wells which contained antibody, NK cells and cryptococci were due to the effects of the NK cells. Maximum anti-cryptococcal activity of the NK cells was observed in the presence of 16 μg/ml IgG; however, significant augmentation in anti-cryptococcal activity was seen with antibody concentrations as low as 3 μg/ml. Using different populations of murine splenic cells which had varying degrees of NK cell activities, we were able to show that NK cell activities, as determined by ^{51}Cr release from YAC-1 targets, directly correlated with the antibody-dependent cell-mediated growth inhibition against cryptococci, suggesting NK cells were the effector cells. Furthermore, in every case the antibody-dependent activity of NK cells against C. neoformans was higher than the spontaneous activity

of the NK cells against the organism, emphasizing that NK cell activity against cryptococci can be augmented by specific antibody. In addition, as NK cell numbers were enriched by Percoll fractionation of NWN splenic cells, antibody-dependent and spontaneous growth inhibitory activities of the effector cells were concomitantly augmented, again indicating NK cells were the effector cells in antibody-dependent growth inhibition of cryptococci. With NK-enriched effector cell populations, the antibody-dependent activity was higher than the spontaneous activity against C. neoformans. Antibody-dependent anti-cryptococcal activity of NK cells could be detected as early as 4 h after mixing the effector cells with the cryptococci and antibody. Based on the results from these in vitro studies, we propose that NK cells have the potential to play a role in antibody-dependent, as well as, spontaneous clearance of C. neoformans in vivo.

SECTION I

INTRODUCTION

Antibody-dependent cell-mediated cytotoxicity (ADCC) against Cryptococcus neoformans was first described by Diamond (5). Later Diamond and Allison (6) reported that a population of nonphagocytic leukocytes from human peripheral blood was responsible for the ADCC against this yeast-like pathogen. This report appeared before natural killer (NK) or killer (K) cells had been recognized as a distinct cell population. However, based on our current knowledge of these nonphagocytic cell types, it seems reasonable to hypothesize that NK or K cells were the effector cells in their experiments.

More recently murine NK cells have been shown to inhibit the in vitro growth of C. neoformans in the absence of anti-cryptococcal antibody (22). Since NK cells have Fc receptors for IgG (9, 10) and have been shown to function in ADCC against IgG-coated tumor cell targets (2, 10, 15, 24), xenogenic erythrocytes (29, 30), and bacteria (23), it is possible that these same cells could also inhibit the growth of cryptococci in an antibody dependent fashion. The name K cell has generally been used for nonphagocytic, large granular lymphocytes (LGL) functioning as effector cells in ADCC (10). NK and K

cells copurify as a small subpopulation of lymphoid cells and have similar morphological and phenotypic characterstics (10, 15, 28). Therefore, NK and K activities appear to be two manifestations of the same cell population, and it is the presence or absence of the antibody that determines which function is expressed (2, 10).

The primary objective of this work has been to determine whether or not the growth inhibitory activity of NK cells against C. neoformans was augmented in the presence of anti-cryptococcal antibody. In previous studies, using NK cells as effector cells, inhibition of C. neoformans growth was assessed by determining the numbers of cryptococcal colony forming units (CFU) present after incubating the organism with NK effector cells for 18 h (22). Adding anti-cryptococcal antibody to the growth inhibition assay caused minor clumping of the cryptococci, such that the number of CFU was not a reliable index of the number of viable organisms. So to circumvent this problem, we developed an alternative procedure for measuring the numbers of viable cryptococci at the end of the growth inhibition assay. This procedure included inoculating BACTEC vials containing a radiolabeled carbon source with growth inhibition assay mixtures, then measuring with a BACTEC instrument the amount of $^{14}CO_2$ evolved by the cryptococci after a 17-22 h incubation period. After determining that the BACTEC growth index measurements correlated with the number of viable C. neoformans in the initial inoculum, we proceeded to use this method to study the effects of anti-cryptococcal antibody on NK/K cell-mediated growth inhibition of cryptococci. We found that the effector cell populations with high levels of NK cell

activity against C. neoformans had augmented reactivity against IgG-coated cryptococci. Our data suggest that anti-cryptococcal antibody plays an accessory role in the inhibitory activity of NK cells against C. neoformans. and potentially could enhance the ability of NK cells to clear cryptococci from tissues of infected animals.

SECTION II

MATERIALS AND METHODS

Mice. CBA/J mice and C57BL/6 bg/bg and bg/+ littermates were used in this investigation. CBA/J mice were purchased from Jackson Laboratories, Bar Harbor, ME. The original breeding pairs of C57BL/6 bg/bg and bg/+ mice were obtained from Jackson Laboratories and were bred in the University of Oklahoma animal facilities. All animals were used at 7-8 weeks of age.

Organisms. Cryptococcus neoformans isolate 184 employed throughout this study was previously described by Murphy and Cozad (21). The organism was maintained on modified Sabouraud agar slants and was harvested after 72 h incubation at room temperature.

Preparation of the effector cells. Effector cells were prepared as previously described by Murphy and McDaniel (22). Briefly, monodispersed cells from the spleens of normal mice were suspended in Hanks' balanced salt solution (HBSS), and erythrocytes were removed by treating for 2 min with ammonium-chloride Tris buffered solution. The cells were washed twice in HBSS and resuspended in RPMI 1640 medium (Grand Island Biological Co., Grand Island, NY.) supplemented with 5% heat-inactivated fetal bovine serum (FBS), 100 µg/ml streptomycin and

100 U/ml penicillin before being passed through nylon wool columns (NWC). The nylon wool nonadherent (NWN) cells contained an average of 95% vaiable cells, 1-2% immunoglobulin positive cells and 1% esterase positive cells. To enrich for large granular lymphocytes (LGL), splenic NWN cells were fractionated on 5-step discontinuous Percoll gradients according to the method of Luini et al. (17). Then, the NK cell-rich fractions 1 and 2, which contained 35% to 40% LGL, were collected and used as effector cells in designated experiments.

Chromium release assay. The NK activities of the effector cells were determined by the standard 4 h ^{51}Cr release assay against YAC-1 targets as described by Murphy and McDaniel (22). Briefly, YAC-1 cells were labeled for 1 h at 37°C with 100 μCi radioactive sodium chromate (Amersham Crop. Arlington Heights, IL.), and were used as targets. An effector cell to target cell ratio of 50:1 (E:T = 50:1) was employed. The percentage of ^{51}Cr released was calculated using the following formula:

% specific release = [(CPM Experimental - CPM Spontaneous)/CPM Maximum] X 100.

Experimental wells contained effector and target cells, spontaneous release wells contained ^{51}Cr labeled target cells and medium, and maximum release wells contained ^{51}Cr labeled target cells mixed with 2N HCl.

Anti-cryptococcal antibody. The purified IgG fraction (1.6 mg/ml) of rabbit anti-cryptococcal antibody (R IgG α crypto), described elsewhere (13), was generously provided by Dr. Thomas Kozel, University of Nevada, Reno, NV. Normal rabbit serum (NRS) was used as a control.

Both R IgG α crypto and NRS were filtered through 0.22 μm Milex-GV low binding Millipore filters and were heated at 56°C for 30 min before use.

Assay of antibody-dependent cell-mediated growth inhibition of cryptococci. One tenth milliliter of each effector cell population (1 X 10^7 or 1 X 10^6 cells/ml) was mixed with 0.1 ml of *C. neoformans* targets (2 X 10^4 cells/ml) in duplicate in RPMI 1640 plus 10% FBS and antibiotics to give an E:T ratio of 500:1 or 50:1, respectively. Control wells contained the target cells alone or the effector cells alone. To each well was added 0.05 ml of the appropriate dilution of R IgG α crypto, NRS, or medium. The final dilution of R IgG α crypto used was 1:100 unless otherwise stated. For controls, the same dilution of NRS was employed. After mixing the effector and target cells with the antibody or NRS in microtiter plates, the plates were incubated at 37°C in 5% CO_2 for 18 h or as indicated in particular experiments. After mixing the contents of the wells thoroughly, 0.1 ml from each well was either serially diluted and plated on modified Sabouraud dextrose agar or directly inoculated into duplicate BACTEC 6B aerobic culture vials containing enriched tryptic soy broth with a radiolabeled carbon source (Johnston Laboratories, Towson, MD.). The culture plates were incubated at room temperature for 3 days, then the numbers of *C. neoformans* colony forming units (CFU) were counted. The BACTEC culture vials were incubated on a reciprocal shaker at 200 rpm at room temperature. Growth indices were read when the control vials, which contained the cryptococci target cells alone, showed slight visible turbidity and a

positive growth index reading in the range of 30 to 120 which represented the linear portion of the graph (Fig. 6). The BACTEC model 301 (Johnston Laboratories, Cockeysville, MD.) was used to read the growth indices of the vials. The percentage of cryptococcal growth inhibition was calculated with the following formula:

% growth inhibition = [(Control growth index - Test growth index)/Control growth index] X 100.

BACTEC system as a means for assessment of viability of cryptococci. The principle of the BACTEC system is based upon measuring the amount of $^{14}CO_2$ in the atmosphere of the head gas above the culture medium in the sealed glass vials after sufficient growth of the organism has occurred. The organisms metabolize the ^{14}C-labeled substrate in the culture medium resulting in the production of $^{14}CO_2$. The level of $^{14}CO_2$ in the head gas is measured by injecting a pair of sterile needles into the rubber septum of the vials and aspirating the head gas into an ionization chamber (3). In the gas filled ionization chamber, ion pairs are formed causing a short pulse of electric current to be produced between the anode and cathode which is proportional to the level of ionization in the chamber. The level of ionization is proportional to the amount of $^{14}CO_2$ formed by the organisms. In the BACTEC system, the electric current generated in the ionization chamber is amplified and read on an arbitrary linear scale as the growth index. After the growth index reading is made, the vial is flushed with sterile analytical 5% CO_2 and 95% dry air, so that subsequent growth index readings on the vial reflect that amount of $^{14}CO_2$ generated

since the last reading.

In order to standardize the BACTEC system for these in vitro assays and to establish that a linear relationship exists between the growth index and the numbers of viable and metabolically active cryptococci in the inoculum, preliminary studies were performed as follows. Different numbers of viable C. neoformans cells, based on the numbers of CFU, or combinations of viable and heat-killed or formalin-killed cryptococci cells, based on hemocytometer counts of the latter, were injected into the BACTEC 6B culture vials to give a total of 10^4 cells per vial. All the BACTEC vials were read immediately after inoculation to make sure the amount of $^{14}CO_2$ did not exceed the background level, i.e. growth index readings of 8-12. Then, the vials were incubated at room temperature with constant agitation and the growth indices were read at 17 and 22 h after inoculation.

To confirm that the $^{14}CO_2$ was only produced by actively respiring organisms, levels of radioactivity in the supernatants, as well as the washed cryptococcal cells, were determined after incubation the BACTEC vials. Briefly, the protocol included mixing the vials and removing 10 ml of the culture fluid from each vial, centrifuging the culture fluids, reserving 0.1 ml of each supernatant for scintillation counting, and washing the cell pellets three times. After the cells were washed, they were resuspended in 10 ml of saline, and 0.1 ml of each cell suspension was counted along with the corresponding culture supernatant medium using a Beckman LS 100C liquid scintillation counter.

Statistical analysis. Means, standard error of the means, and unpaired Student's t tests were calculated using an Apple II Plus computer. Correlation coefficient was calculated by linear regression analysis.

SECTION III

RESULTS

Assay of antibody-dependent cell-mediated growth inhibition of C. neoformans. When NWN splenic cells were incubated for 18 h with C. neoformans along with R IgGα crypto, the numbers of cryptococci CFU cultured from the assay mixtures were extremely low and variable (1-3 X 10^3/ ml). Moreover, the numbers of cryptococcal CFU from control wells which contained cryptococci and R IgGα crypto were also very low (2-3 X 10^3/ml) in comparison to the numbers of CFU cultured from wells containing NRS or tissue culture medium and cryptococci (85 X 10^3/ml). Microscopic examination of the contents of the wells containing the various mixtures of effector and target cells with or without antibody revealed that in the wells containing R IgGα crypto, the C. neoformans cells were in small clumps; whereas, in the wells with NRS or medium, the cryptococci were dispersed as single or occasional budding cells. The clumping was most likely responsible for the reduced numbers of CFU cultured from wells containing R IgG α crypto. By devising another method for assessing the level of viable cryptococci present at the end of the incubation period, we hoped to alleviate the problem introduced by the clumping of C. neoformans. The

method employed was to inoculate BACTEC 6B vials with 0.1 ml of the contents of the wells from the growth inhibition assay. For comparison, in the first series of experiments, the microtiter well contents were also diluted and plated as before. After an appropriate incubation period, the growth index of each BACTEC vial was read and compared with the CFU counts from corresponding microtiter wells. The results showed that despite clumping of cryptococci in the presence of the antibody, growth indices of the vials containing antibody-treated C. neoformans alone were similar to the vials having cryptococci treated with NRS or medium. Plate counts again showed greatly reduced numbers of CFU from wells containing R IgG α crypto. These data indicated that clumping of the organism did not adversely affect the BACTEC measurements and that antibody alone did not inhibit the growth of C. neoformans. Since the BACTEC system seemed to eliminate the problem introduced by clumping, it appeared to be a useful method for comparing numbers of viable cryptococci in the various microtiter wells at the end of the growth inhibition assay. However, before shifting to the use of the BACTEC system, it was necessary to confirm that the BACTEC growth index readings were accurate reflections of the numbers of viable C. neoformans in the initial BACTEC vial inoculum.

Standardization of the BACTEC system To demonstrate the relationship between the BACTEC readings and the numbers of viable cryptococci, different numbers of viable C. neoformans cells were inoculated into individual BACTEC vials either alone or in combination with heat-killed C. neoformans cells. The numbers of viable C. neoformans initially inoculated into BACTEC vials were determined by

plate counts. After incubating the vials for 17 and 22 h on a shaker at room temperature, the growth indices of the vials were read. Then the numbers of CFU in the initial inoculum were compared to the resulting growth index readings from the BACTEC. The results are shown in Fig. 6. The data indicated that there was a linear relationship between the numbers of viable C. neoformans in the original inoculum and the BACTEC growth indices (correlation coefficient = 0.99), providing the growth indices were read in the range of 30 to 120. Furthermore, the presence of killed cells did not affect the growth indices, because the growth indices of the vials with equal numbers of viable cryptococci were the same regardless of the presence or absence of the killed cells. Moreover, no differences were observed when formalin-killed C. neoformans cells were used in place of heat-killed cryptococci in this assay. The growth indices of the vials containing only nonviable organisms were always at background level.

To confirm that $^{14}CO_2$ in the head gas of the culture vials was only produced by viable organisms, 10 ml samples were withdrawn from the BACTEC vials which had been inoculated 20 h previously with various numbers of viable C. neoformans. The cryptococci cells were pelleted, washed, and resuspended in 10 ml of saline. Then, 0.1 ml of each sample of washed cells and the corresponding growth medium supernatant were counted separately in a liquid scintillation counter. The results are shown in Fig. 7. There was an inverse relationship between the numbers of scintillation counts in the cell pellets and the numbers of scintillation counts in the growth medium as the BACTEC growth indices increased. These data indicate that the cryptococcal

cells incorporated ^{14}C, thereby reducing ^{14}C in the medium as the turbidity of the culture medium increased. These changes were concomitant with the organisms releasing $^{14}CO_2$ into the head gas of the vial. Taken together, the data confirmed that the BACTEC growth index readings, within the linear range of 30 to 120 (Fig. 6), are directly proportional to the numbers of viable cryptococcal cells present in the inoculum.

NK cells as effector cells of antibody-dependent cell-mediated growth inhibition of cryptococci. Satisfied that the BACTEC method provided a reliable index of viable cryptococci, experiments were repeated to demonstrate the effects of R IgG α crypto on NK cell-mediated growth inhibition of cryptococci. Data from one representive experiment are shown in Table 2. As with earlier studies, the BACTEC readings for control wells, i.e. R IgG α crypto, NRS, or medium plus target cells were essentially all the same; whereas, when assayed with the CFU count method the control wells containing R IgG α crypto and cryptococci had greatly reduced numbers of CFU in comparison to the other controls. Since the BACTEC data were the most reliable, calculations of the % C. neoformans growth inhibition were made using that data (Table 2). As indicated by the 49% growth inhibition, the effector cells in the presence of the R IgG α crypto inhibit the growth of cryptococci more effectively than in the presence of NRS or medium which showed only 25 and 22% growth inhibition, respectively. When mean growth indices of the test wells containing R IgG α crypto, effector cells and target cells were compared with those of the control wells (cryptococci and R IgG α crypto), the wells

containing R IgG α crypto, effector cells and cryptococci had significantly lower readings ($p < 0.02$). Furthermore, test wells containing effector cells and R IgG α crypto had significantly lower growth index readings than the test wells containing effector cells and NRS or medium ($p < 0.01$). Effector cells alone did not contribute to the BACTEC growth index reading as indicated by the fact that the readings were within the background range of 8 to 12 (Table 2).

The optimal concentration of R IgG α crypto for antibody-dependent cell-mediated growth inhibition of cryptococci was determined. For this assay, various concentrations of antibody, ranging from 3 to 32 μg/ml of protein, were added to the effector cell-target cell mixture in which NWN cells were used as effector cells. As shown on Fig. 3, 16 μg/ml of R IgG α crypto gave the maximum ADCC effects against C. neoformans; therefore, this concentration was used in subsequent experiments.

Effects of various levels of NK activity on antibody-dependent cell-mediated growth inhibition of cryptococci. To determine if the antibody-dependent growth inhibition of C. neoformans correlated with the levels of the NK activity, various effector cells having high, intermediate, or low NK activities were compared. For this purpose, the anti-cryptococcal activities of NWN cells from CBA/J mice, known to have high NK activity, C57BL/6 bg/+ mice, having intermediate NK activity, and bg/bg mice with low NK activity were assessed against C. neoformans in the presence and absence of R IgG α crypto . Simultaneously, the NK activity of each population of effector cells was determined using the 4 h ^{51}Cr release assay against YAC-1

targets. Data shown in Table 3 indicate that the antibody-dependent activity of the various effector cell populations against C. neoformans correlated with the levels of NK activity of the cell population. For example, NWN splenic effector cells from CBA/J mice demonstrated the highest degree of antibody-dependent anti-cryptococcal activity and the highest level of NK activity against YAC-1 targets, followed by NWN cells from bg/+ mice with intermediate activities, then cells from bg/bg mice with very low activities. In each case, the anti-cryptococcal activities of the effector cells were higher in the presence of R IgG α crypto than in its absence.

Effects of NK cell enrichment on antibody-dependent cell-mediated growth inhibition of C. neoformans. To further establish that the antibody-dependent anti-cryptococcal activity was associated with the NK cells, NWN splenic cells from CBA/J mice were further enriched for NK cells by Percoll fractionation, and the LGL-rich fractions 1 and 2 (35-40% LGL) were collected and used as effector cells in the C. neoformans antibody-dependent growth inhibition assay. Table 4 shows that NK-enrichment was concomitant with the enrichment of the effector cells responsible for antibody-dependent growth inhibition of cryptococci. In the presence of R IgG α crypto, LGL-rich effector cell populations were almost twice as effective in inhibiting the growth of C. neoformans as the unfractionated NWN cells when used in identical assays ($p < 0.005$) (Table 4).

Kinetics of NK cell-mediated antibody-dependent growth inhibition of C. neoformans. Diamond and Allison (6) showed that ADCC with human mixed leukocyte populations killed C. neoformans within 4 h in

vitro; whereas, Miller et al. (19), using human polymorphonuclear leukocytes as effector cells in ADCC assays, noted maximum killing of cryptococci by 8 h. In our experiments, it was obvious that murine LGL-rich effector cells were able to inhibit the growth of cryptococci by an antibody-dependent mechanism within 18 h. To assess how early after mixing the LGL-rich effector cell with C. neoformans target cells in the presence of R IgG α crypto a measureable effect could be seen on the targets, assays for viability of C. neoformans were performed after 4, 8, or 16 h of incubation. As indicated in Table 5 LGL-rich effector cells inhibited the growth of cryptococci targets by 53% after 4 h, when compared to control cryptococci incubated with R IgG α crypto alone. The pecentage of antibody-dependent growth inhibition of cryptococci increased slightly after 8 h, reaching a maximum of 64% by 16 h.

Figure 6. Correlation between the number of viable C. neoformans present in the initial inoculum and the resulting BACTEC growth index read after the designated incubation time.
(Correlation coefficient = 0.99).

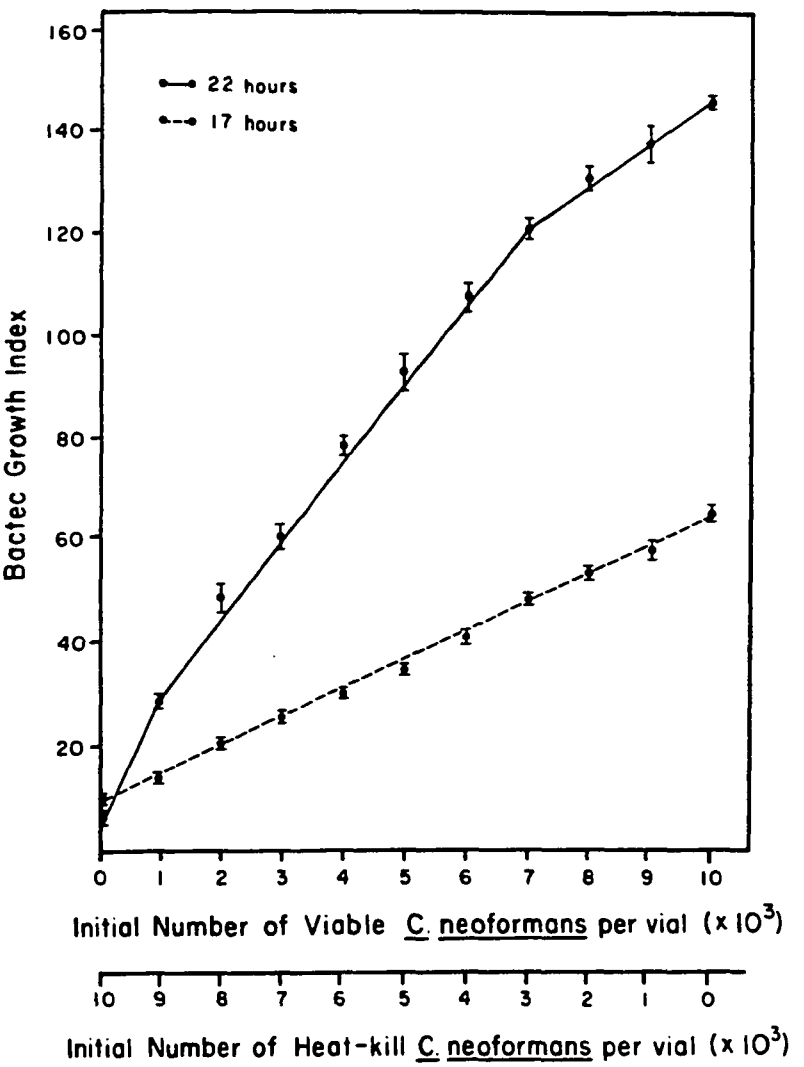
160
140
120
100
80
60
40
20
22 hours
17 hours
Bactec Growth Index
0 1 2 3 4 5 6 7 8 9 10
Initial Number of Viable C. neoformans per vial (x 10³)
10 9 8 7 6 5 4 3 2 1 0
Initial Number of Heat-kill C. neoformans per vial (x 10³)

Figure 7. Correlation between the BACTEC growth index, metabolic activity, and growth of C. neoformans cultured in BACTEC 6B aerobic vials.

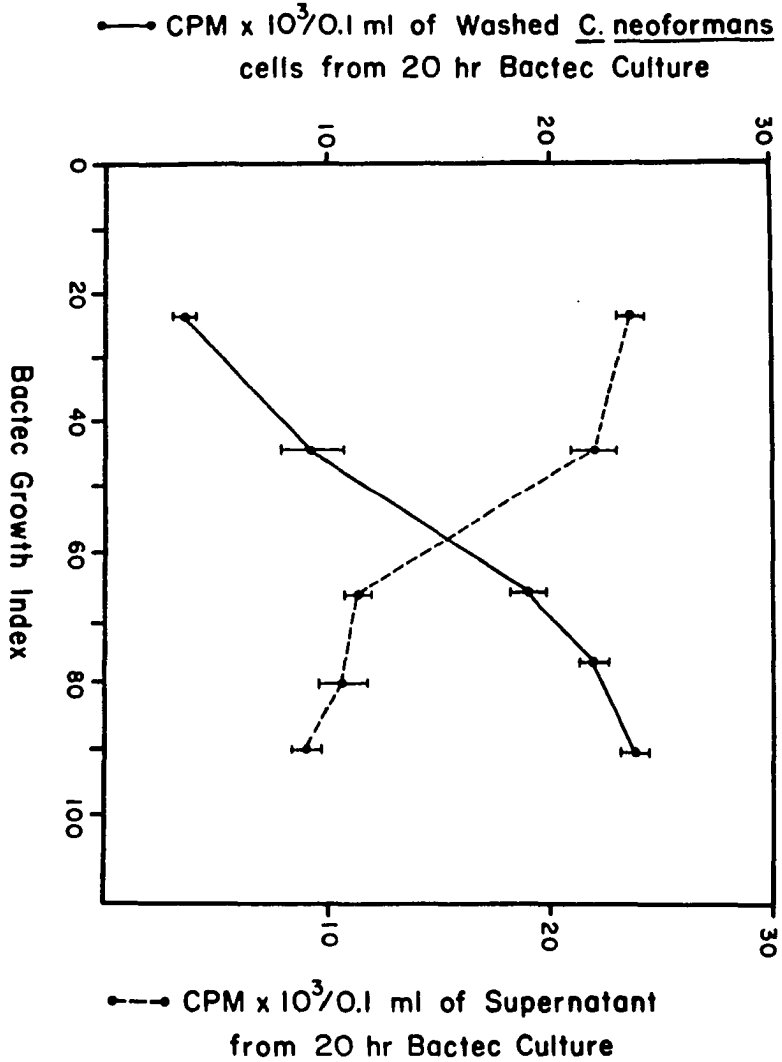
CPM x 10³/0.1 ml of Washed C. neoformans cells from 20 hr Bactec Culture
Bactec Growth Index
0
20
40
60
80
100
10
20
30
CPM x 10³/0.1 ml of Supernatant from 20 hr Bactec Culture

Figure 8. Effect of various concentrations of R IgG α crypto on cell-mediated growth inhibition of C. neoformans.

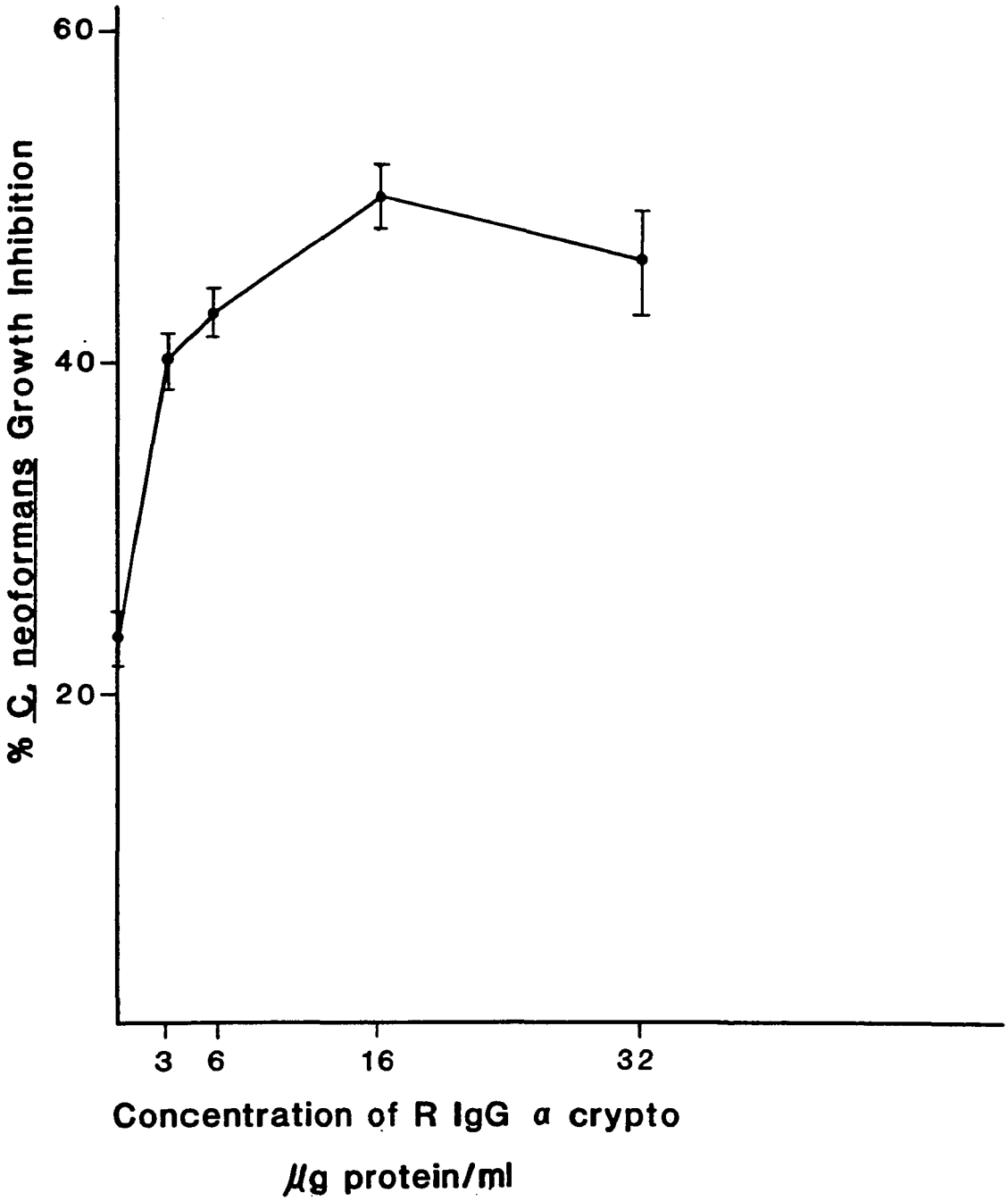
60
40
20
% C. neoformans Growth Inhibition
3
6
16
32
Concentration of R IgG α crypto
μg protein/ml

Table 2. Comparison of BACTEC growth index readings with numbers of cryptococci CFU determined after incubating the organism with murine nylon wool nonadherent splenic cells for 18 h in the presence or absence of the specific antibody.

Assay Mixture	Serum Added	CFU X 10^3[a]	BACTEC Growth Index[a]	% *C. neoformans* Growth Inhibition[a,b]
Effector Cells + *C. neoformans*[c]	R IgG α crypto[d]	2 ± 1	35 ± 1	49 ± 1
	NRS[e]	62 ± 1	48 ± 1	25 ± 1
	Medium	59 ± 1	52 ± 1	22 ± 2
C. neoformans alone	R IgG α crypto	3 ± 0	69 ± 4	
	NRS	83 ± 4	65 ± 1	
	Medium	84 ± 5	67 ± 2	
Effector Cells alone	R IgG α crypto	NG[f]	10 ± 1	
	NRS	NG	9 ± 0	
	Medium	NG	10 ± 1	

[a]Mean of eight samples ± standard error of the mean.

[b]Calculated from BACTEC growth indices.

[c]Effector-cell to target-cell ratio was 500:1.

[d]R IgG α crypto = IgG fraction or rabbit anti-cryptococcal serum (16 μg/ml).

[e]NRS = Normal (nonimmunized) rabbit serum diluted 1:100.

[f]NG = No growth.

Table 3. Comparison of antibody-dependent anti-cryptococcal activity of splenic cells from different strains of mice.

Source of Effector Cells[a]	% ^{51}Cr released from YAC-1[b] E:T = 50:1	Serum Added	BACTEC Growth Index[b]	% *C. neoformans* Growth Inhibition[b] E:T = 50:1
CBA/J	35.6 ± 1.0	R IgG αcrypto[c]	49 ± 1	52 ± 1
		NRS[d]	70 ± 2	29 ± 2
		Medium	78 ± 1	25 ± 1
C57BL/6 bg/+	19.2 ± 1.8	R IgG αcrypto	63 ± 3	38 ± 2
		NRS	82 ± 2	18 ± 1
		Medium	88 ± 1	16 ± 1
C57BL/6 bg/bg	5.0 ± 1.3	R IgG αcrypto	91 ± 2	11 ± 2[e]
		NRS	94 ± 1	4 ± 1
		Medium	101 ± 3	4 ± 1
None		R IgG αcrypto	102 ± 4	
		NRS	99 ± 1	
		Medium	105 ± 0	

[a]Effector cells were nylon wool nonadherent splenic cells from 7-8 week old mice.

[b]Mean of four samples ± standard error of the mean.

[c]R IgG α crypto = IgG fraction of rabbit anti-cryptococcal serum (16 μg/ml).

[d]NRS = Normal (nonimmunized) rabbit serum diluted 1:100.

[e]$p < 0.005$ compared to antibody-dependent anti-cryptococcal activity of splenic cells from bg/+ or CBA/J mice.

Table 4. Effect of NK cell-enrichment on antibody-dependent cell-mediated growth inhibitory activity against C. neoformans.

Effector Cells	% ^{51}Cr released[a] from YAC-1 E:T = 50:1	Serum Added	BACTEC Growth[a] Index	% C. neoformans[a] Growth Inhibition E:T = 50:1	Compared to corresponding LGL assays p <
NWN[b]	35.6 ± 1.0	R IgG α crypto[d]	45 ± 1	38 ± 1	0.005
		NRS[e]	65 ± 3	14 ± 4	0.05
		Medium	64 ± 1	18 ± 1	0.02
LGL[c]	53.2 ± 2.8	R IgG α crypto	23 ± 1	68 ± 1	
		NRS	44 ± 1	41 ± 1	
		Medium	47 ± 2	40 ± 3	
None		R IgG α crypto	73 ± 3		
		NRS	76 ± 5		
		Medium	78 ± 3		

[a]Mean of four samples + standard error of the mean.

[b]Nylon wool nonadherent CBA/J splenic cells.

[c]Large granular lymphocytes-enriched splenic cells from CBA/J mice.

[d]R IgG α crypto = IgG fraction of rabbit anti-cryptococcal serum (16 µg/ml).

[e]NRS = Normal (nonimmunized) rabbit serum diluted 1:100.

Table 5. Kinetics of NK cell-mediated antibody-dependent growth inhibition of cryptococci.

Length of Assay Hours	Assay Mixture	Serum Added	BACTEC Growth Index[a]	% *C. neoformans* Growth Inhibition[a]
4	LGL ± *C. neoformans*[b]	R IgG α crypto[c]	43 ± 3	53 ± 3
		NRS[d]	83 ± 2	15 ± 2
	C. neoformans alone	R IgG α crypto	91 ± 4	-
		NRS	98 ± 1	-
8	LGL ± *C. neoformans*	R IgG α crypto	38 ± 1	57 ± 1
		NRS	62 ± 2	25 ± 2
	C. neoformans alone	R IgG α crypto	89 ± 2	-
		NRS	83 ± 1	-
16	LGL ± *C. neoformans*	R IgG α crypto	24 ± 1	64 ± 2
		NRS	34 ± 2	43 ± 3
	C. neoformans alone	R IgG α crypto	66 ± 2	-
		NRS	59 ± 4	-

[a]Mean of four samples ± standard error of the mean.

[b]LGL: *C. neoformans* ratio = 50:1.

[c]R IgG α crypto = IgG fraction of rabbit anti-cryptococcal serum (16 μg/ml).

[d]NRS = Normal (nonimmunized) rabbit serum diluted 1:100.

SECTION IV

DISCUSSION

Data previously reported from this laboratory indicate that normal murine nylon wool nonadherent splenic cells with the characteristics of NK cells can effectively inhibit the in vitro growth of C. neoformans (22). In the present study, we examined the antibody-dependent growth inhibitory abilities of these effector cells against cryptococci. Preliminary experiments showed that minor clumping of cryptococcal cells occurred in the presence of the antibody. Therefore the conventional CFU count method, which was previously used for assessment of the number of viable C. neoformans at the end of the growth inhibition assay (22), did not give accurate measurements. Thus, the BACTEC system was examined for suitability as an alternative method for determining the viability of cryptococci at the end of the growth inhibition assay.

We found that the BACTEC growth index readings, over the linear range of 30 to 120 correlated with the numbers of viable C. neoformans in the initial inoculum, and that the readings were not affected by the presence of nonviable organisms in the inoculum (Fig. 6). In addition, depletion of ^{14}C in the culture vial media paralleled the

incorporation of radioactivity into the growing cryptococcal cells and the release of $^{14}CO_2$ into the head gas which was read as the growth index (Fig. 7). The BACTEC growth index readings were not affected by the clumping of cryptococcal cells in the assay, because growth indices of the vials inoculated with the C. neoformans treated with antibody, normal serum, or medium were similar (Table 2). Moreover, the growth indices of the control vials containing effector cells alone were always at the background level (Table 2) indicating that the effector cells transferred with the cryptococci into the BACTEC vials did not contribute to the growth index readings. In repeated experiments, the growth indices were reproducible. In addition, the culture vials were free of microbial contamination, thus the indices reflected only cryptococcal metabolic activity. Taken together these results indicate that the radiometric method, in contrast to the CFU count method, is a reliable estimate of viable C. neoformans in an assay, when single cell suspensions cannot be made. Since the BACTEC radiometric system overcomes the clumping limitation, it could possibly be used for viability measurements of other mycotic pathogens where clumping is a problem once the appropriate culture media and optimal conditions for their growth are determined.

Using the BACTEC radiometric assay, we demonstrated that the growth inhibitory activity of murine splenic NK cells was augmented in the presence of the R IgG α crypto when compared to the normal serum. R IgG α crypto alone did not inhibit the growth of cryptococci, i.e. growth indices of the control vials inoculated with antibody treated cryptococci were similar to the growth indices of vials inoculated with

cryptococci treated with NRS, or medium (Table 2). Thus, we concluded that inhibition of growth of C. neoformans was augmented by an antibody-dependent cell-mediated mechanism.

Increasing the concentration of the antibody in the assay from 3 μg/ml to 16 μg/ml resulted in an increase in the growth inhibition of cryptococci (Fig. 8), presumably due to the optimal coating of the target cells with higher concentrations of the antibody. However, at concentrations higher than 16 μg/ml, the percent growth inhibition of cryptococci did not continue to increase (Fig. 8).

The antibody-dependent anti-cryptococcal activities of the various murine splenic cells were directly proportional to their NK activities against YAC-1 targets (Table 3). For example CBA/J mice, having a high level of NK reactivity against YAC-1, showed a high level of cryptococcal antibody-dependent growth inhibition. Whereas, bg/+ splenic cells with intermediate NK activity also had an intermediate level of ADCC activity against cryptococci, and bg/bg mice demonstrated both low NK activity against YAC-1 and low growth inhibitory activity against C. neoformans. When NWN cells were enriched for LGL by Percoll fractionation, both antibody-dependent and NK cell mediated growth inhibitory activities of the effector cells against cryptococci were enhanced (Table 4). The LGL-rich effector cells inhibited the growth of C. neoformans in the presence of antibody significantly better than NWN cells ($P < 0.005$). The LGL-rich effector cells inhibited cryptococci growth by 53% as early as 4 h after mixing the effector and target cells (Table 5). These findings are in accordance with the previous reports by other investigators that the effector cells with

distinct LGL morphology and natural killer function are also capable of ADCC or K cell activity against IgG coated target cells (2, 15, 24, 23, 29, 30).

It has been reported that most LGL have Fc receptors on their surfaces (9, 10), a situation which facilitates binding of antibody-coated target cells to the LGL. Therefore, enhanced cryptococcal growth inhibitory effects in the presence of R IgG α crypto could be due to the rapid and more stable binding of the effector cells to the targets by the means of the antibody bridge.

The fact that enhancement of antibody-dependent anti-cryptococcal activity was concomitant with the enrichment of LGL and that splenic cells from bg/bg mice with a selective defect in NK/K activity (26, 27), showed impairment in antibody-dependent anti-cryptococcal activity suggests that the same effector cells might be involved in both NK and K activity against C. neoformans.

The in vivo relevance of antibody-dependent cell-mediated growth inhibition of cryptococci by NK/K cells and its role(s) in host defense against cryptococcal infection is not clear at this point. Anti-cryptococcal antibodies are generally not detected in patients with disseminated crptococcosis (1, 7, 16, 25); but when antibodies are present they are associated with improved prognosis (4). Circulating antibodies to C. neoformans have not always been found to be protective in experimental cryptococcosis. Graybill et al. (8) demonstrated that survival of mice challenged with C. neoformans increases if cryptococci are precoated with rabbit anti-cryptococcal antibody or inoculated into a site that can immediately interact with passively transferred

antibody. In vitro studies suggest that specific antibodies enhance phagocytosis of cryptococci by macrophages and PMNL (11, 12, 14, 18). Miller et al. (19) reported ADCC against C. neoformans using human polymorphonuclear leukocytes (PMNL) as effector cells. Also, anti-cryptococcal antibodies have been shown to be essential in the in vitro killing of cryptococci by human peripheral blood nonphagocytic lymphocytes. The data presented here suggest that murine splenic LGL or NK cells are capable of inhibiting the in vitro growth of C. neoformans by an antibody-dependent mechanism as well as by an antibody-independent mechanism. It is likely that in vivo the various effector cells function together to achieve clearance of the cryptococci and clearance of the organism by the effector cells is augmented in the presence of specific antibody. Kozel and Gotschlich (14) and Mitchell and Friedman (20) independently demonstrated that normal human serum contains a cryptococcal reactive IgG which augments phagocytosis of C. neoformans by PMNL and macrophages. Thus one would expect killing of cryptococci by these effector cells to be enhanced in normal serum. Cryptococcal reactive IgG in normal serum may also play a role in antibody-dependent NK cell-mediated clearance of C. neoformans in vivo.

SECTION V

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CHAPTER IV

SUMMARY

Previous data generated by Murphy and McDaniel indicate that normal murine nylon wool nonadherent splenic cells, with characteristics of natural killer (NK) cells, effectively inhibit the in vitro growth of Cryptococcus neoformans, a yeast-like pathogen. To further study the in vitro interactions of NK cells with C. neoformans, an enriched population of NK cells was required. Therefore nylon wool nonadherent cells from spleens of 7-8 week old mice were further fractionated on discontinuous Percoll gradients. The enrichment of NK cells in Percoll fractions 1 and 2 was confirmed by morphological examination, immunofluorescent staining, and by assessing the cytolytic activity of each Percoll cell fraction against YAC-1 targets in the 4 h ^{51}Cr release assay. Cells isolated from each Percoll fraction were tested for growth inhibitory activity against C. neoformans, using an in vitro 18 h growth inhibition assay. The results showed that NK enrichment was concomitant with the enrichment of anti-cryptococcal activity in the Percoll fractions 1 and 2. Cells from NK-rich fractions formed conjugates with the mycotic targets similar to those reported in the NK-tumor system. In addition, the percentage of

effector cell- Cryptococcus conjugates was directly proportional to the level of C. neoformans growth inhibitory activity of the effector cells employed. Scanning electron microscopy of the effector cell-Cryptococcus conjugates showed a direct contact between the effector cells and cryptococcal targets. An immunolabeling method combined with scanning electron microscopy was used to demonstrate that the effector cells attached to C. neoformans were asialo GM_1 positive and, therefore, had NK cell characteristics.

NK cells have Fc receptors on their surfaces and have been shown to be involved in antibody-dependent cell-mediated cytotoxicity (ADCC) against IgG-coated target cells. Thus it was of interest to see whether or not cryptococcal growth inhibitory activity of NK cells could be augmented in the presence of anti-cryptococcal antibody. To examine this hypothesis the cryptococcal growth inhibitory ability of NK cells was tested in the presence of the purified IgG fraction of rabbit anti-cryptococcal serum. A conventional colony forming unit (CFU) count method, previously used for assessment of viable cryptococci at the end of the growth inhibition assay, did not give an accurate measurement of viable cells due to minor clumping of the organism in the presence of the antibody. Therefore, a BACTEC radiometric system was used for determining viability of cryptococci at the end of the growth inhibition assay. BACTEC growth index readings reflect the amount of $^{14}CO_2$ evolved by cryptococci in the culture vials as the organisms grow. The growth index readings were not affected by the clumping of C. neoformans, the presence of nonviable organisms, or presence of the effector cells. Using the

BACTEC system, it was shown that antibody alone was not cytotoxic to cryptococci. However, the growth inhibitory activity of the NK cells against C. neoformans was significantly augmented in the presence of rabbit anti-cryptococcal antibody, when compared to normal rabbit serum or medium. Furthermore, the antibody-dependent growth inhibitory activity of the various effector cells against C. neoformans was directly proportional to their levels of NK activity against YAC-1 target cells. In addition, enrichment of NK cells by Percoll fractionation was concomitant with enhancement of antibody-dependent growth inhibitory activity against C. neoformans. The data indicate: i) the BACTEC system can be used as an alternative to CFU counts when clumping of cryptococci is a problem. ii) The in vitro growth inhibitory activity of NK cells against cryptococci is significantly augmented in the presence of the antibody presumably due to Fc-receptor binding. iii) The effector cells involved in spontaneous and antibody-dependent growth inhibition of cryptococci are either NK cells or copurify and coexist in the same population with NK cells.

Future investigation on the in vitro effects of NK cells on C. neoformans could possibly focus on the effects of the purified cytoplasmic granules of NK cells on this pathogen. It is also of interest to define the binding site/s of NK cells on the cryptococci capsule or cell wall and to elucidate the mechanisms by which NK cells recognize the mycotic target. Experiments could be done to examine how early conjugates are formed after mixing the NK cells with C. neoformans; how early after conjugation a measurable effect can be detected on cryptococcal targets; and whether or not conjugate

formation is a prerequisite for growth inhibition of cryptococci.

Further study on antibody-dependent growth inhibition of cryptococci by NK cells could focus on the mechanism/s by which antibody enhances the growth inhibitory ability of the effector cells and the role of NK cell Fc receptors in this process.