Interleukin-2 Activated Anti-Fungal Activity of Lymphocytes

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INTERLEUKIN-2 ACTIVATED ANTI-FUNGAL ACTIVITY OF LYMPHOCYTES

by

David W. A. Beno

A Dissertation Submitted to the Faculty of the Graduate School of Loyola University of Chicago in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

January

1991
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VITA

David Beno was born on June 15, 1962 in Madison, Wisconsin.

He graduated from Illinois College, Jacksonville, Illinois, as president of his class with majors in biology and chemistry.

He entered the graduate program in 1984, and has conducted both his masters and doctoral research under the direction of Herbert L. Mathews.
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<tr>
<td>A</td>
<td>angstrom</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>activated</td>
<td>splenocytes incubated with rIL-2</td>
</tr>
<tr>
<td>C'</td>
<td>complement</td>
</tr>
<tr>
<td>C9</td>
<td>complement component 9</td>
</tr>
<tr>
<td>Day 0 splenocytes</td>
<td>splenocytes directly from animal</td>
</tr>
<tr>
<td>Day x (x = 1-9)</td>
<td>splenocytes incubated x days with rIL-2</td>
</tr>
<tr>
<td>DTH</td>
<td>delayed type hypersensitivity</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>dpm</td>
<td>disintegrations per minute</td>
</tr>
<tr>
<td>FITC RAMIg or GAMIg</td>
<td>fluorescein conjugated rabbit or goat anti-Ig</td>
</tr>
<tr>
<td>fraction 1, fract. 1 or interface 1</td>
<td>cells which did not enter 50% concentration of Percoll</td>
</tr>
<tr>
<td>fraction 2, fract. 2 or interface 2</td>
<td>cells which did not enter 60% concentration of Percoll</td>
</tr>
<tr>
<td>fraction 3, fract. 3 or interface 3</td>
<td>cells which did not enter 70% concentration of Percoll</td>
</tr>
<tr>
<td>h</td>
<td>hour(s)</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's balanced salt</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
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<tr>
<td>GG</td>
<td>glucan</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte-monocyte colony stimulating factor</td>
</tr>
<tr>
<td>GMP</td>
<td>glucan mannoprotein</td>
</tr>
<tr>
<td>IFN gamma</td>
<td>interferon gamma</td>
</tr>
<tr>
<td>IL-1</td>
<td>interleukin-1</td>
</tr>
<tr>
<td>IL-2</td>
<td>interleukin-2</td>
</tr>
<tr>
<td>IL-2R</td>
<td>interleukin-2 receptor</td>
</tr>
<tr>
<td>IL-4</td>
<td>interleukin-4</td>
</tr>
<tr>
<td>IU</td>
<td>inhibitory units for <em>C. albicans</em></td>
</tr>
<tr>
<td>i. v.</td>
<td>intravenous</td>
</tr>
<tr>
<td>kD</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LAK</td>
<td>lymphokine activated killer</td>
</tr>
<tr>
<td>LGL</td>
<td>large granular lymphocyte</td>
</tr>
<tr>
<td>LU</td>
<td>lytic units for tumor cells</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>min</td>
<td>minute(s)</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PE-avidin</td>
<td>phycoerythrin conjugated avidin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
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<tr>
<td>PMN</td>
<td>polymorphonuclear leukocyte</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethysulfononylfluoride or p-α-toulenesulfononyl fluoride</td>
</tr>
<tr>
<td>rIL-2</td>
<td>recombinant interleukin-2</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>SCID</td>
<td>severe combined deficiency syndrome</td>
</tr>
<tr>
<td>S. D.</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SDA</td>
<td>Sabouraud's dextrose agar</td>
</tr>
<tr>
<td>selected</td>
<td>population positively selected for expression of specific surface marker</td>
</tr>
<tr>
<td>TAME</td>
<td>p-α-toulenesulfononyl-L-arginine methyl ester or N&quot;p-tosyl-L-arginine</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>unactivated</td>
<td>splenocytes incubated without rIL-2</td>
</tr>
<tr>
<td>unselected</td>
<td>population negatively selected for expression of specific surface marker by depletion of undesired population</td>
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Introduction

Candida albicans is an opportunistic pathogen with a wide range of infectious capabilities. Infections vary from localized to systemic, and are potentially fatal. The immune response to C. albicans varies directly with the type of C. albicans infection. Localized infections are controlled by polymorphonuclear leukocytes (PMNs) and later by macrophages (Smith, 1985). Deep seated infections are contained by PMNs as well (Wilton and Lehner, 1981). However, multiple cell populations have been implicated in the immune response to systemic fungal infection, including T lymphocytes (Mahanty et al., 1988). The evidence for absolute involvement of any one effector population is inconclusive. Animal models with genetic defects in various lymphoid populations have failed to elucidate a precise anti-C. albicans mechanism (Mahanty et al., 1988).

Therefore, it is possible that alternative cell populations, activated by the immune response to C. albicans, may be responsible for and contribute to the actual immunological and protective response to C. albicans.
Immune Response to C. albicans.

Host defense against infection with *C. albicans* is mediated by both phagocytic cells and aspects of classical cell-mediated immunity (Mahanty et al., 1988). Other immune cell populations have also been assessed for their role in host defense activity against *C. albicans* including B lymphocytes (Smith, 1985) and natural killer (NK) cells (Zunino and Hudig, 1988). However, the relative contribution of each cell population in protection against specific forms of candidiasis remains to be elucidated.

A. Role of phagocytic cells

Invasive infections of *C. albicans* are the result of hyphal forms which are too large to be ingested by phagocytic cells but have been shown to be damaged by them via extracellular mechanisms (Diamond and Haudenschild, 1981; Hurtrel et al., 1980; Diamond and Krzesicki, 1978). The proposed anti-fungal mechanisms for these cells are the production of reactive oxygen intermediates such as hydrogen peroxide, super oxide anion, hydroxyl radical, and singlet oxygen (Nathan et al., 1983). Oxygen-independent killing of macrophages against a variety of targets has been attributed to a number of secreted substances including arginase, cytolytic protease, thymidine, complement cleavage products, macrophages cytotoxin, lysosomal cationic proteins, and tumor necrosis factor (Smith, 1985; Cleary and Marciano-
As a consequence, these cells have been implicated as the primary means by which invasive infections of *C. albicans* are limited (Ashman and Papadimitriou, 1987). Further evidence for the important role of phagocytic cells comes from studies in which T lymphocyte-deficient nude mice were found to be relatively more resistant to infection than normal animals due to increased macrophage activity (Rodgers and Balish, 1980). Despite the decrease in T lymphocyte activity, nude mice possess an enhanced NK activity and a normal level of lymphokine activated killer (LAK) cell activity (Hasui et al., 1989). Therefore, the resistance to infection in nude mice may not be an exclusive consequence of increased macrophage activation. It is possible that alternative cell populations, such as NK and LAK, may serve to mediate host protection against the fungus in the nude mouse as well as in the normal host environment.

B. Role of T cells

Thymus-dependent, cell-mediated immunity provides protection against *C. albicans* as evidenced by enhanced susceptibility to candidiasis in thymectomized mice (Mahanty et al., 1988). Further evidence is displayed by patients with chronic mucocutaneous candidiasis (Wilton and Lehner, 1981), malignancies (Bodey, 1986), and immunodeficiency syndromes (Smith, 1985), who often display defective delayed-type hypersensitivity (DTH) reactions to *C. albicans*.
antigen. T lymphocytes from a majority of these patients show no responsiveness to *C. albicans* antigen in vitro (Odds, 1979).

In addition to the DTH response, Cenci et al. (1989) have suggested an important role for inflammatory L3T4+ lymphocytes as a cofactor of phagocytic cell activation. The loss of L3T4+ lymphocytes significantly reduced host resistance to *C. albicans* as judged by DTH responsiveness, lymphokine production, and survival (Cenci et al., 1989). Cenci et al. (1989) have postulated that immune L3T4+ T lymphocytes contribute to protective immunity against systemic *C. albicans* infection through the secretion of cytokines which activate cells with candidacidal activity. However a portion of animals possess the capacity to survive following L3T4+ lymphocyte depletion. The authors suggest alternative mechanisms may provide protection under conditions for which the activity of this T lymphocyte subset is reduced.

**C. Role of B cells**

A role for B lymphocytes in protection against fungal infection is not established (Hector et al., 1982). Although some host protection can be transferred with immune serum, B lymphocytes and their products play a minor role in the protective host response to *C. albicans* (Piccolella et al., 1980).
D. Role of natural killer cells

Natural killer (NK) cells have been shown to interact with certain fungi. In vitro, cells with both functional and phenotypic characteristics of NK have been shown to bind to and inhibit the growth of Cryptococcus neoformans as well as to inhibit the growth of Paracoccidiodes brasiliensis (Nabavi and Murphy, 1985; Murphy and McDaniel, 1982; Jimenez and Murphy, 1984). In vivo, NK have been shown to mediate growth inhibition of Cryptococcus neoformans (Hidore and Murphy, 1986). Other investigators have failed to demonstrate human NK anti-fungal activity against C. albicans (Djeu and Blanchard, 1987). Those investigators found that enriched human LGL possessed no innate activity against C. albicans. The LGL population could not be activated by low levels of IL-2 to inhibit C. albicans growth although cytotoxicity for the human NK susceptible tumor cell line K562 was enhanced. Activated LGL supernate contained the capacity to enhance PMN function against C. albicans. These data suggest that this LGL population did not mediate direct anti-C. albicans activity but rather functioned in an indirect manner by inducing the release of TNF which then activated PMN anti-C. albicans activity (Djeu et al., 1988).

NK cells do not appear to have a major role in resistance to murine C. albicans infections (Vecchiarelli et al., 1985). Isolated splenic NK cells show no direct anti-
C. albicans inhibitory activity which suggests that NK may not mediate destruction of yeast species other than cryptococci (Zunino and Hudig, 1988).

Severe combined immunodeficient (SCID) mice have no detectable levels of immunoglobulins in their serum and their mature T and B lymphocytes are markedly reduced in number or even absent (Hasui et al., 1989). Mice homozygous for SCID gene possess normal macrophage activity and an expanded pool of natural killer cells with normal functional activity (Lauzon et al., 1986). Comparisons of homozygous SCID to heterozygous SCID (phenotypically normal) BALB/c mice show no difference in the recovery of C. albicans from organs following infection. This observation suggests that a non-T, non-B lymphocyte may mediate anti-fungal activity (Mahanty et al., 1988). The exact role of T lymphocytes is unclear as both athymic nude mice and T lymphocyte deficient SCID mice both possess normal or enhanced activity against C. albicans which is in contrast to studies demonstrating a T lymphocyte requirement for immunity. Whether the protection is mediated by activated macrophages or by an activated lymphocyte population is unknown. Both nude mice and SCID mice possess normal LAK activity (Hasui et al., 1989).

Infection with Candida and C. albicans.

A. Virulence of Candida and C. albicans
Although antigenic variation of *C. albicans* and variation in virulence have been described (Ausiello *et al.*, 1989), it is changes in the host which contribute most to susceptibility to infection with *C. albicans* (Wilton and Lehner, 1981; Meunier, 1989). Numerous species of candida are pathogenic, including *C. albicans*, *Candida tropicalis*, *Candida krusei*, and *Candida parapsilosis*. In general, *C. albicans* is the species most associated with invasive candidiasis following fungemia (Meunier, 1989). Invasive or systemic candidiasis has a mortality rate of over 70% in immunosuppressed patients and is difficult to both diagnose and treat (Matthews and Burnie, 1989).

Systemic candidiasis is the most serious form of candidiasis. It is recognizable by invasive infection involving the parenchyma of two or more visceral organs including heart, kidney, spleen, lung, and brain among others. Histologically, the lesions in systemic candidiasis resemble small abscesses composed of a mixture of PMNs and mononuclear cells (Luna and Tortoledo, 1985).

**B. Candidiasis in immunosuppressed patients**

A variety of immunocompromised patients are susceptible to *Candida* infections. A number of factors contribute to the increasing frequency of fungal infections in these patients. In patients with cancer undergoing combination chemotherapy, the patient becomes debilitated, malnourished
and host defense mechanisms are impaired. The patients become highly susceptible to infection and resistant microorganisms, such as fungi, can cause superinfection (Bodey, 1966). Fungal infections are especially prevalent among children with acute lymphocytic leukemia who experience prolonged periods of neutropenia due to the disease or its therapeutic treatment (Gold, 1984). Infections caused by Candida species are the most common fungal diseases in immunocompromised patients (Hawkins and Armstrong, 1984). Fungi cause 21% of the fatal infections in patients with acute leukemia, 13% in patients with lymphoma, and 6% in patients with solid tumors (Bodey, 1986). Candida spp. account for a majority of serious fungal infections with Candida albicans the most frequent pathogen (Ashman et al., 1990).

Other prime candidates for life-threatening invasive candidal infections are those with defects in normal immune function, in particular patients with chronic granulomatous disease. Persons with profound defects in cell-mediated immunity, such as transplant recipients, patients with acquired immunodeficiency syndrome, and patients receiving chronic steroid therapy are also at significant risk of invasive candidal infection. Finally, therapeutic maneuvers, such as antibiotics and steroids, that compromise normal mucocutaneous barriers to infection will enhance C. albicans colonization and predispose patients to systemic
candidiasis (Meunier, 1989).

**Binding of C. albicans to Host Tissue.**

A primary concern of the host immune response to *C. albicans* is the mechanism by which the fungus attaches to host tissues. An understanding of the mechanisms which permit *C. albicans* to colonize host tissue may allow a further understanding of the immune recognition of *C. albicans*. The cell surface of this organism has been investigated because of its perceived role in virulence. The cell wall is composed of several layers and includes the major antigens of the yeast cell. The cell undergoes significant compositional changes during conversion of yeast form to the hyphal form (Reiss et al., 1986). This phenomenon is associated with the invasiveness of the organism.

Adherence of *C. albicans* to the mucosal epithelial cells is an important initial step in the process of colonization and invasion. Hydrophobic interactions of the cell wall with the endothelium are believed to be important in *C. albicans* binding to target tissues (Hazen, 1989). Apical growth and elongation as a compartmentalized tube allows for invasion of epithelial cells by direct penetration of the epithelial cell membrane (Braun and Calderone, 1978).
Despite the utilization of hydrophobic interactions, a
more detailed mechanism for binding of *C. albicans* to other
cell populations is unknown. Bouchara et al. (1990) have
shown that *C. albicans* in the hyphal form, possess germ tube
specific receptors for laminin which mediate its attachment
to basement membrane. Laminin is a multifunctional
glycoprotein which promotes the adhesion of various
eucaryotic cell types in vitro. Specifically, laminin
receptors have been found on cells that normally interact
with basement membrane (Yannariello-Brown et al., 1988), as
well as on cells that extravasate, such as tumor cells,
granulocytes, lymphocytes and macrophages. Basement
membranes are normally unexposed in vivo, with the exception
of the endothelia of kidney glomeruli to which candidal
infections seed thus serving as a source of dissemination in
systemic candidiasis.

The location of the laminin receptor on *C. albicans* has
been specified by electron microscopy. The receptor is
connected to the outer fibrillar layer of the hyphal cell
wall. The receptor is not found on the yeast form nor on
the mother cell of the hyphae. The binding of the hyphae
was saturable and specific. Two types of receptors were
found. The first had a molecular mass of 68 kilodalton (kD)
which is similar to laminin receptors previously described
on different cell types including tumor cells and
endothelial cells (Rao et al., 1983; Yannariello-Brown et
The second receptor was a doublet of 60 and 62 kD, which may correspond to a degradation product of the first as found in tumor cell lines (Graf et al., 1987).

The doublet may also be related to previously described \textit{C. albicans} proteins. Calderone et al. (1988) found that by further separating 70 and 62 kD proteins isolated from \textit{C. albicans} cell wall on high-performance liquid chromatography that complement component C3d receptor could be isolated at 60 kD. The complement component C3d fragment has also been found to bind exclusively to the hyphal form of \textit{C. albicans} (Eigentler et al., 1989). Another possible explanation is that the 68 and 60/62 doublet are related to other adhesion molecules. Tronchin et al. (1988) isolated four proteins which are involved in the adherence of \textit{C. albicans} hyphae to plastic. The two major proteins involved in binding were 68 and 60 kD. Either similar proteins or functions of the same proteins are involved.

Bouchara et al. (1990) found that the same components of 68 and 60 kD which bound laminin also bound another common basement membrane protein, fibrinogen, and to some extend the complement component C3d. Fibrinogen, greatly reduced the binding of laminin to \textit{C. albicans}, suggesting that binding of fibrinogen leads to a stearic hindrance of the laminin receptors or that binding of these two host proteins to hyphae may be by the same receptor (Bouchara et al., 1990). Fibronectin, bovine serum albumin,
transferrin, and multiple oligosaccharides did not bind the laminin receptor indicating a degree of specificity. However, the capacity of the receptor to bind unrelated proteins suggests multiple biological activities for the receptor. The molecular mechanism of recognition of these unrelated host proteins by the same C. albicans receptors remains unclear. These data do suggest that attachment of C. albicans hyphae to tissue and cell surfaces may correlate with the presence of an extracellular matrix component which mediates binding to the hyphal surface.

Due to the role phagocytic cells play in the clearance of C. albicans, multiple studies have assessed the mechanism by which these effector populations bind the fungus. The state of the phagocytic cell, the presence or absence of serum components, and the physiological state of C. albicans are contributing factors to cellular recognition and binding (Houston and Douglas, 1989). The best characterized interaction is that of rat alveolar macrophages which bind C. albicans through a mannose/N-acetylglucosamine receptor. This receptor is demonstrable on human peripheral blood mononuclear cells (PBMC) which were cultured in vitro for three days without lymphokines. Fresh PBMC however, do not possess the receptor (Stahl et al., 1980).

Consideration must be given to the physiological state of C. albicans during the binding process. Growth in enriched media or within the host increases the production
of the outer fibril layer. An increase in the fibril layer will increase both the adherence and virulence of *C. albicans*. Phagocytic cells appear to recognize *C. albicans* by lectin-carbohydrate interactions which promote strong attachment following increased fibril production (Houston and Douglas, 1989). Lectin-like interactions mediate attachment of *C. albicans* to vaginal and buccal cells via mannoprotein fibrils on the outer cell envelope (Critchley and Douglas, 1987). The fibrils also promote binding to PMNs as well as alveolar and peritoneal macrophages by a related mechanism (Warr, 1980; Kolotila et al., 1987). Furthermore, macrophage binding to yeast depends on a lectin-like macrophage receptor specific for glycoproteins terminating in mannose or fucose residues (Stahl and Gordon, 1982).

The binding is not believed to be mediated by specific receptors but rather by a variety of factors such as the activation state of the cell in combination with the state of the host and the time course of the infection (Houston and Douglas, 1989). Most likely, the activated phagocyte recognizes a component of the cell wall through either lectin-type interactions, or sugar residues before activating its extracellular cytotoxic machinery. A similar recognition mechanism is possible for other non-specific effector populations.

Hidore and Murphy (1989) evaluated the binding of NK
cells to \textit{Cryptococcus neoformans}. They found both similarities and differences in the physical and kinetic binding and growth inhibition of cryptococci compared with binding and lysis of tumor targets. Electron microscopy studies demonstrated that in contrast to the broad area of interdigitating membrane contact observed in NK cell-tumor cell conjugates, the contact area of NK cell-cryptococci conjugates is much smaller, does not appear to interdigitate, and is less intimate (Murphy, 1989). They found binding to be Mg$^{2+}$ dependent. Growth inhibition was Ca$^{2+}$ and temperature dependent for both fungal and tumor targets. Murphy therefore suggests a similar recognition and effector mechanism for cryptococci and tumor cells. The structural differences between the fungal and tumor cell target would still permit intimate cell to cell interaction. The investigation of Murphy (1989) did not identify the mechanism by which NK recognizes, binds, and mediates its effect on \textit{C. neoformans}. Despite the binding and cytotoxic activity demonstrated against the cryptococci, NK cells do not possess anti-\textit{C. albicans} activity (Vecchiarelli \textit{et al.}, 1985). However, Zunino and Hudig (1988) found that \textit{C. albicans} inhibited the binding of NK cells to K562 tumor targets.

\textbf{Immunoregulation of C. albicans Infection.}

A. Suppression of the immune response
During infection, *C. albicans* itself may both up-regulate and down-regulate the immune system. Neutrophil function was shown by Diamond and Haudenschild (1981) to be impaired by substances liberated from hyphae of *C. albicans*. Normal T-lymphocyte functions may also be impaired during the course of *C. albicans* infections. The lack of T-cell reactivity to *C. albicans* antigens, a characteristic of patients with chronic mucocutaneous candidiasis, was noted by Valdimarsson et al. (1973) to be occasionally reversed when the infection was controlled, thus suggesting the fungal infection induced the abnormality in lymphocyte function. Evidence of immunosuppression includes a decrease in the *in vivo* response to the common skin test antigens and suppressed *in vitro* proliferative responses to *C. albicans* antigens (Cuff et al. 1989). Stobo et al. (1976) found generalized abnormalities in T-cell recognition of fungal antigens in infected patients and showed that the abnormality was due to the generation of suppressor T lymphocytes. Cuff et al., (1989) have described the suppressor cell to be Thy-1+, Lyt-1+, Lyt-2−, and L3T4+. Others have found the suppressor population to be CD8+ or even a B lymphocyte population (Domer, 1989). The mediator of suppression appears to reside in the cell wall polysaccharide, mannann, although its exact makeup is unknown (Podzorski et al., 1990). The observed immunosuppression has been proposed to be due to factors released during the
yeast to hyphae transition of *C. albicans* (Cockayne and Odds, 1984).

B. Activation of the immune response

In murine models, it has been shown that inoculation of live or inactivated *C. albicans* cells or purified components of the *C. albicans* cell wall, can modulate the activity of the immune system. Components of other microorganisms have been shown to activate MHC-unrestricted cell cytotoxicity (Tarkkanen *et al.*, 1986; Bonavida *et al.*, 1986). *C. albicans* induces strong anti-tumor responses in mice infected with heat-killed yeast and hyphae from *C. albicans* (Kohoshis *et al.*, 1978; Marconi *et al.*, 1985). These responses are also induced by inoculating mice with either of the two primary components of the cell wall of *C. albicans*, mannoprotein (GMP) or glucan (GG). NK and macrophage effectors are activated following administration of either component. GMP also induces lymphoproliferation and production of gamma interferon as well as the generation of cell-mediated cytotoxicity in PBMC (Ausiello *et al.*, 1988). GMP induced lytic effectors are similar to IL-2 induced lytic effectors in potency, target specificity, and type of precursor cells. These data suggest that cytokines produced in response to the infection are involved in nonspecific anti-microbial resistance induced by *C. albicans* (Vecchiarelli *et al.*, 1989).
Variability in the Murine Immune Response to C. albicans.

The C57Bl/6 mouse is highly resistant to challenge with C. albicans. Other inbred murine strains are much more susceptible to the fungus (Marquis et al., 1986). The differential susceptibility between murine strains is not solely due to differences in phagocytosis and/or splenic clearance of the microorganism. Various murine strains such as C57Bl/6 (high resistance), BALB/c (intermediate resistance), and CBA/H (low resistance) show no correlation between survival following C. albicans challenge and either of those biological activities (Ashman and Papadimitrou, 1987). SCID mice, deficient in T and B lymphocytes, possess macrophage activity which is not significantly different from corresponding inbred strains (Hasui et al., 1989). Nude mice and mice which have been thymectomized, lethally irradiated, and bone marrow reconstituted show no difference in macrophage activity or clearance of C. albicans when compared to normal mice (Ashman and Papadimitrou, 1987).

A correlation does exist between the production of cytokines and the development of resistance to the fungus within murine strains. Neta and Salvin (1983) reported that the release of lymphokines from mice following sensitization with cell walls of C. albicans was under genetic control. They found the capacity of a murine strain to release cytokines in vivo parallels the capacity of that strain to
resist i.v. infection with *C. albicans*. *C. albicans* infected strains of mice which released high levels of IL-1 and IFN gamma (high responder strains, such as C57Bl/6) were also noted to have lower tissue numbers of *C. albicans* than did those strains of mice which released low levels of cytokines (low responder strains, such as C3H).

Due to the relevance of cytokine release in the differentiation and activation of microbicidal splenocytes, Vecchiarelli *et al.* (1989) studied cytokine production in the unprimed murine response to *C. albicans*. Using hybrid BALB/c x DBA/2 mice (two intermediate responder strains), they evaluated the pattern and kinetics of selected cytokine production in *C. albicans* infected animals. GM-CSF, TNF alpha, IFN gamma, and IL-1 were measured in the sera and spleen cell supernates of infected mice. High levels of GM-CSF, TNF alpha, IFN gamma, and IL-1 were found six hours after infection. The high cytokine levels persisted for many days. A correlation existed between the ability of *C. albicans* to induce anti-microbial protection *in vivo* and cytokine production *in vitro*. These data coupled with that of Neta and Salvin (1983), suggest an involvement of cytokines in the host protective response to *C. albicans* in the mouse.

Further evidence for the involvement of cytokines in *C. albicans* infection is demonstrated by *C. albicans* induction of TNF production by human monocytes and LGLs *in vitro* (Djeu
et al., 1988). TNF was detected as early as eight hours after incubation with C. albicans. Maximal levels of TNF occurred between 24 h and 48 h. No priming signal was required. Only partial inhibition of NK activity and TNF induction was produced by the elimination of CD16+ (Fc receptor) or NKH1+ in the LGL population. Furthermore, antibody and complement depletion of cells positive for Leu 7, a phenotypic marker of some NK cells, had little effect on C. albicans induction of TNF by NK cells. These data suggest a population of LGL besides NK release TNF upon stimulation with C. albicans.

TNF functions as an anti-tumor agent. However, TNF is primarily regarded as a key product of the mononuclear cell response to microbial antigen involved in septic shock. TNF has long been known to activate phagocytic cells to enhance anti-microbial activity and therefore may play a role in microbial immunity (Philip and Epstein, 1986). Djeu et al. (1988) have postulated that TNF production is a normal response of monocytes and LGL to stimulation by fungi such as C. albicans. Release of TNF assists in activation of PMN and other effector functions to control C. albicans growth (Djeu et al., 1988). The effect of TNF resulting from C. albicans infection on other effector populations has not been studied.

The release of IFN gamma in response to C. albicans infection is believed to activate macrophages and other
cellular populations which when activated by these cytokines acquire increased anti-fungal activity. The inhibitory activity of macrophages activated with IFN gamma is well documented (Nathan et al., 1983). Another result of IFN gamma release may be activation of MHC-unrestricted lymphocytes. IFN gamma has previously been demonstrated to produce a synergistic effect with low doses of IL-2 in the activation of MHC-unrestricted lymphocytes (Limb et al., 1989). TNF also synergizes with low doses of IL-2 to induce activation of lymphocytes with MHC-unrestricted cytotoxic activity. This activity requires the presence of IL-2 (Blay et al., 1989). Therefore, IFN gamma and TNF can cooperate in the activation of lymphocytes to enhanced cytotoxic activity.

Human IFN gamma directly inhibits *C. albicans* germ tube formation (Kalo-Klein and Witkin, 1990). The mechanism is unclear. However, the authors speculate that specific receptors exist for IFN gamma and that the interaction of IFN with the *C. albicans* yeast form inhibits CAMP production and therefore hyphal formation (Kalo-Klein and Witkin, 1990). By inhibiting yeast to hyphal transition, IFN gamma may directly alter the pathogenic potential of *C. albicans*.

Besides host production of IFN gamma and TNF, an essential part of the immune response to microbial infection is the production and release of IL-2. Ausiello et al. (1988) exposed human PBMC *in vitro* to *C. albicans* and at
daily intervals the release of IFN gamma and IL-2 as well as 
cytotoxicity for the tumor cell target, K562, was 
determined. They found that IFN gamma and IL-2 were 
produced first, after which significant anti-K562 
cytotoxicity was generated. Although IFN gamma production 
peaked prior to IL-2 production, the generation of cytotoxic 
activity for the tumor target correlated with maximum IL-2 
production following seven to ten days of culture.

Disturbances in the production of IL-2 and/or defective 
responses to IL-2 may often be the cause of lowered immune 
responsiveness (Gillis, 1983). Dowjat et al. (1985) 
demonstrated inter-strain differences in the proliferative 
response of normal spleen cells to IL-2. The differential 
response appeared to be genetically determined by multiple 
dominant or codominant genes. The genetic determinants most 
likely control differences in the composition of T 
lymphocyte populations residing in the spleen, or in the 
density of IL-2 receptors on splenocytes. The proliferative 
response of splenocytes from C57Bl/6 mice to IL-2 were among 
the highest of the strains examined (high responding 
strains), while C3H splenocytes possessed the lowest 
response to IL-2 (low responder strains) (Dowjat et al., 
1985). Differences in the kinetics of cytokine production 
were noted between the two types of responders. The 
cytokine production of low responder strains peaked prior to 
the cytokine production of high responder strains (Zhou et
Furthermore, high responder spleen cells contained two to four times more IL-2R transcript than low responding strains and possessed a higher frequency of IL-2R^+ cells in the spleen (Zhou et al., 1989a).

It is interesting to note that the IL-2 highly responsive strain, C57Bl/6 (Dowjat et al., 1985), was highly resistant to *C. albicans* infections (Marquis et al., 1986). The ability of this high responder strain to stimulate higher levels of cytokine production for extended lengths of time implies a possible role for this cytokine in the differential anti-fungal response. The increased levels of IFN gamma, TNF and IL-2 suggest a higher potential for activation of MHC-unrestricted populations. This increase may be responsible for the observed increase in survival following *C. albicans* infection in high responder strains of mice.

Cytokine Induction of MHC-Unrestricted Lymphocytes in Response to *C. albicans*.

The cell wall of *C. albicans* is a powerful biological agent, with a range of potential effects not dissimilar from those induced in normal PBMC by IL-2 (Vecchiarelli et al., 1989). The microorganism is able to stimulate non-specific lymphocyte proliferation both *in vivo* and *in vitro* (Ausiello et al., 1989). *C. albicans* possesses the ability to initiate a cascade of events which allows generation of
potent cytotoxic effectors that are active against tumor cell targets in vitro and in vivo (Cassone et al., 1987). The activation of these lymphocytes is through lymphokine production which is induced by the \textit{C. albicans} cell wall (Ausiello et al., 1988).

Cassone et al., (1987) found \textit{C. albicans} cell wall components to induce anti-YAC-1 activity of peritoneal effectors. At least two cell wall fractions, the glucomannan protein (GMP) and the insoluble glucan (GG), induced the appearance of peritoneal immunoeffectors (55% LGL cells, 30% macrophages, and 10% PMNs) which were similar to those induced by the intact cells. The activity of the cell wall fractions did not differ in the intensity of the elicited effect, but rather in the kinetics of cytotoxicity and its duration. The cytotoxic potential generated by the GMP was detected as early as the activity induced by intact \textit{C. albicans}, but lasted a shorter period of time. The effector response of GG peaked later than the response to intact \textit{C. albicans}. These findings indicate multiple compounds within the cell wall of \textit{C. albicans} induce anti-tumor activity with varying effects. The immune response to \textit{C. albicans} may be regulated by the breakdown products of the cell wall either through immunological degradation or release of cell wall components by \textit{C. albicans} (Cassone et al., 1987).

Studies evaluating the induction of cell-mediated
cytotoxicity in cultured human PBMC by GMP, have shown GMP induces a strong, LAK type, MHC-unrestricted cytotoxic response, similar to that induced with IL-2 (Ausiello et al., 1989). Analysis of surface phenotype coupled with complement-mediated depletion experiments, indicated a strong similarity between GMP and IL-2 activated cytotoxic effectors. Depletion of CD16\(^+\) (Fc receptor) and CD38\(^+\) (p45) lymphocytes substantially decreased the cytotoxic activities of the induced population against tumor targets (Ausiello et al., 1989).

The above studies demonstrate that the cell wall of \textit{C. albicans} is a powerful antigen-complex which has the capacity to serve as an immunomodulator. The \textit{in vivo} relevance, if any, of \textit{C. albicans} antigen induced activated lymphocytes in immune surveillance against \textit{C. albicans}, tumors, or other microorganisms is presently unknown. The ability of the host to respond to infection with \textit{C. albicans} by producing lymphocytes with MHC-unrestricted cytotoxic activity would indicate such a response is possible.

**IL-2 Induction of MHC-Unrestricted Lymphocytes.**

A. LAK cell phenomenon

IL-2 is a lymphokine produced by helper T cells that stimulates the growth and differentiation of lymphocytes (Herberman and Holden, 1978). The incubation of normal lymphoid cells with IL-2 leads to the generation of a MHC-
unrestricted cytotoxic lymphocyte population termed lymphokine-activated killer (LAK) cells (Timonen et al., 1981). Several reports have shown that LAK cells are effective in some patients with advanced cancer when combined with IL-2 treatments (Richards, 1989).

A significant amount of literature has addressed the functional capabilities of this cell population. The term LAK has been referred to as both an activity as well as a cell population. No agreement upon a defined function associated with the term LAK has been reached. Rather, any function mediated by a lymphokine activated killer cell has been described as LAK activity. IL-2 is an inducer of LAK activity. However, the term lymphokine within lymphokine activated killer cell indicates that any cellular product of lymphocytes which induces this activity may result in LAK activity. Furthermore, the wide range of activities mediated by this population may not be intrinsic to the entire population. It has therefore been argued that LAK activity is a function of multiple cell populations rather than a single cell population mediating several functional activities (Reynolds and Ortaldo, 1987; Lotzova and Herberman, 1987). The precise relationship of these multiple cell types or functional activities is unknown.

B. Characterization of LAK

Incubation of mouse spleen cells in vitro with IL-2
results in the generation of a cell population capable of lysing a wide variety of tumor cells. IL-2 alone is fully capable of generating LAK activity in the absence of other added lymphokines (Yang et al., 1986). Other cytokines including TNF, IFN gamma and IL-4 also provide a degree of activation for LAK activity which when coupled with low levels of IL-2 provides a level of activity similar to that obtained with high doses of IL-2 alone (Blay et al., 1990).

Lafreniere et al., (1986) have shown that IL-2 generated murine LAK cells stimulated in vitro remain cytotoxic for 11 days. However, the stimulated cells failed to proliferate following the first cycle of expansion which concluded at day 8. Early studies of LAK phenotype in C57Bl/6 mice demonstrated the expression of ASGM-1 and Thy-1.2 (Merluzzi et al., 1986; Merluzzi, 1985). The majority of splenic LAK effectors reside in the Thy-1\(^+\), Lyt-2\(^+\) population (Yang et al., 1986; Mule et al., 1987). More recent studies have demonstrated the effector phenotype of 9 day LAK cells to be Thy-1\(^+\), Lyt-2\(^+\), and ASGM-1\(^+\) cells. (Mule et al., 1989). Furthermore, it has been shown that 5-day IL-2 induced effectors consist of two mutually exclusive subsets bearing either the NK 1.1 or Lyt-2 phenotypic marker (Kalland et al., 1987).

LAK Target Cell Recognition.

A model for LAK cell binding by a variety of cell
surface proteins common to T cells and NK cells has been proposed (Thiele and Lipsky, 1989). Antibodies to CD18/CD11a (LFA-1), CD2 (LFA-2) and CD45 have all been shown to interfere with LAK activity for tumor cells (Schmidt et al., 1985; Thiele and Lipsky, 1988). It has also been noted by a number of investigators that no single member of this group of cell-surface proteins can be implicated in the lysis of all susceptible targets by a given population of effector cells (Thiele and Lipsky, 1989). Thus, when LAK cells have been examined, lysis of some susceptible targets is not affected by binding of the same antibody to the same effector cell population (Pawelec et al., 1985; Thiele and Lipsky, 1988). The variability with which anti-CD45, anti-CD2 or anti-CD18 antibodies inhibit killing, and the variability with which these agents interfere with cytotoxicity, suggests that no unique cell surface receptor is likely to explain the recognition and triggering of lysis of all susceptible targets by a population of LAK effector cells. When multiple effector target cell combinations have been examined, variation in inhibition of lysis of antibodies to these cell surface proteins has been found to be primarily dependent on the target cell employed. Furthermore, an inverse relationship between the degree of inhibition induced between antibodies to CD18/CD11a and antibodies to CD2 has been noted. Lysis of targets most resistant to inhibition by anti-CD18/CD11a are relatively
susceptible to inhibition by anti-CD2. The inverse is also true; target cells resistant to anti-CD2 inhibition are susceptible to inhibition by anti-CD18/CD11a (Thiele and Lipsky, 1988). These experiments suggest that the apparently promiscuous nature of target cell killing exhibited by LAK cells appears related to the presence of multiple sets of non-polymorphic receptor molecules which, in varying combination, can interact with all or virtually all, of a heterogeneous panel of tumor cell targets (Thiele and Lipsky, 1989).

Evidence for the role of laminin-like molecules as structures involved in tumor cell recognition by LAK was provided by several findings. First of all, the structure is expressed only on lymphocytes mediating non-MHC restricted cytotoxicity. Secondly, affinity purified antibodies to laminin block target cell killing. Finally, anti-laminin antibodies modulate the levels of IL-2 induced proliferation of LAK (Schwarz and Hiserodt, 1988). The potential role of laminin-like molecules as receptors for LAK in mediating anti-tumor activity was emphasized by expression of a laminin matrix on tumor cells of mice, rats, and humans but not on normal cells. Therefore, the interaction with laminin may serve as a mechanism for recognition of cells that express altered growth or differentiation properties.
Mechanisms of Cytotoxic Activity for Tumor Cells.

Binding of tumor cell targets by effector cells is required for cell mediated cytotoxicity. The binding process can occur through any of the complex mechanisms previously discussed or through antibody-antigen mediated contact. Any process which brings these two cell populations together possesses the potential to permit the cytotoxic process.

The mechanisms of cytotoxicity can be separated into distinct steps. The first two are recognition and binding, followed by the post binding cytotoxic effect which leads to the lysis of the target cell.

A. Recognition

The recognition of the target cells by MHC-unrestricted lymphocytes is unclear. No singular receptor has been identified. It is presumed that LAK cells possess membrane structures or receptors that mediate the recognition process by which they distinguish targets (Edwards et al., 1989). Recognition of tumor cells by non-antigen specific lymphocytes has also been attributed to either recognition of precise proteins or non-specific lectin like molecules (Edwards et al., 1989). Glycosylated proteins, glycolipids, sialic acid, and amino sugars inhibit the interaction of tumor cells and nonspecific effectors (Axberg, 1988; Yagita et al., 1989). Subtle molecular changes in surface exposed
carbohydrates of target cells alter their sensitivity to lysis by nonspecific effectors (Axberg, 1988). Furthermore, simple sugars such as mannose block the cytotoxic activity of natural cytotoxic cells against solid tumors in vitro (Stutman et al., 1980). Several other possible target molecules have been proposed to explain cell recognition including transferrin (Alarcon and Fresno, 1985), laminin (Hiserodt et al., 1985), fibrinogen (Schwarz and Hiserodt, 1988), and lectin-like recognition structures (Stutman et al., 1980).

As discussed in a previous section, antigen-independent pathways of effector-target cell binding mediate the interaction of CD2 on effector cells and LFA-3 (CD58) on target cells, LFA-1 (CD11a/CD18) on effector cells and ICAM-1 (CD54) on target cells, and CD45 on effector cells (Thiele and Lipsky, 1989). The relative contribution of these pathways to cell binding is variable among combinations of effector and target cells. Nitta et al. (1989) have isolated another minor adhesive molecule on NK and LAK cells, CD56. However no definitive receptor has been isolated and a combination of receptors may provide a complex recognition scheme for these cells.

B. Binding

In order to exert their cytotoxic activity, cells must bind the target, undergo an activation process leading to
release of lytic factors and, finally, establish suitable spatial conditions in order for these factors to reach the target and to exert their function (Edwards et al., 1989). Thus an intimate relationship between effector and target cell is necessary.

Recent evidence suggests that the target cell may participate actively in the mechanism leading to its own lysis. Results of the interaction between cells stimulated with low levels of IL-2 and both NK-resistant and NK-sensitive tumor targets have revealed slightly different mechanisms of cytotoxicity. LAK and K562 interactions were characterized by a broad cell to cell contact whereas tumor cell targets susceptible only to LAK cells did not undergo extensive surface structure redistribution and the cell to cell contact was established to a lesser extent. Therefore, it is conceivable that the susceptibility of the target cell is also related to the ability of the target cell to redistribute its surface structures, probably through a rearrangement of cytoskeletal components. These changes might produce enhancement of the lytic effect by establishing the appropriate micro-environment for cytotoxic factors released by the effector cell (Arancia et al., 1989).

C. Cytotoxic granules

A current model postulates that stored cytotoxins in
cytoplasmic granules are released onto target surfaces by Ca^{2+}-dependent degranulation after appropriate stimulation by the target cell (Hayes et al., 1989). These granules can be isolated from many killer cell types and contain a pore-forming cytotoxin called either perforin, cytolysin, or pore-forming protein (Zanovello et al., 1989). For example, studies by Young (1989) have shown spleen cells cultured in vitro in the presence of IL-2 and other T-cell growth factors acquire perforin-containing granules (Young, 1989). Various molecules including TNF, serine esterases, proteoglycans, and lysozymes have been isolated from cytotoxic granules (Kamada et al., 1989). The subcellular location of the protease and serine esterase activity appears to be in the outer cytoplasmic membrane and within the intracellular granules (Lavie and Zucker-Franklin, 1989).

D. Release of granules

A simple and attractive model to explain lymphocyte mediated killing has been proposed (Young, 1989). Once the effector cell is bound, the microtubule-organizing center and the Golgi apparatus to which the granules are attached, are repositioned to face the contact area with the target cell (Zanovello et al., 1989). This rearrangement serves to direct the cytoplasmic granules toward the contact site and to allow their content to be secreted by exocytosis into the
narrow space between effector and target cell (Arancia et al., 1989). Upon release of the granules into the directed space, perforin binds and polymerizes into cylinders with an internal diameter of 160 Å. These cylinders orient in the plane of the target cell membrane to form pores analogous to the polymerized C5 to C9 components of the membrane attack complex of the complement system. Pore formation leads to rapid lysis.

An alternative model of cytotoxicity is the internal disintegration model in which the LAK cell induces an autolytic mechanism in the target. This model, originally proposed by Russell (1983), derives from the observation that target cell DNA is released from the nucleus before lysis and during the cytotoxic reaction. The DNA of some, but not all target cells undergoes fragmentation into repeat units of 150-180 base pairs during attack. Whether the endonuclease activity is directly introduced from outside or is endogenously activated from inside the target cell during the lethal event is unknown. Isolated granules have been demonstrated to mediate target cell DNA release (Hayes et al., 1989). This activity is mediated by a serine protease located in the secretory granules of the effector cell.

Proteolytic enzymes (proteases and serine esterases), have long been implicated as having a major role in the lytic phase of the cytotoxicity reaction (Lavie et al., 1985). Besides a role in the release of DNA from target
cells, proteases are involved in models of granule release. A variety of such enzymes are distributed within different compartments of the effector cell. It is clear that all enzymes involved in the cytotoxic response are not active at all times during the cytotoxic response. Furthermore, all enzymes are not active against all target cells with which they come in contact, rather a degree of selectivity exists. Complex cellular mechanisms triggered by the binding of the target cell via the receptor are involved along with proteolysis. This may explain the selective nature of the lysis which is not indiscriminately active against all cells, but is dependent on target cell binding by the specific receptor followed by triggering of a specific cytotoxicity mechanism. Therefore upon recognition of a given target cell, any or all of several proteases which induce the cytotoxic mechanism may be activated. A model has been suggested by which surface-associated proteolytic enzymes that are concealed from the external environment during the resting phase become exposed during cytolysis and participate in the initial phase of target cell damage (Lavie et al. 1985).

While protease inhibitors diminish cytotoxicity against tumor cells, they are only active during the lytic process (Lavie et al., 1985). Chelating agents for Ca\(^{2+}\) but not Mg\(^{2+}\), have been shown to block the exposure of the surface proteolytic enzymes (Young et al., 1986). Furthermore,
conjugate formation is not inhibited by addition of protease inhibitors. It is believed that the activity of the proteolytic enzymes occurs at a stage which precedes the calcium-dependent effector phase but which follows the magnesium-dependent cell adhesion phase (Rodgers et al. 1988). The accessibility of these proteolytic enzymes to inhibition declines first to large molecular weight inhibitors and within a few minutes to smaller molecular weight inhibitors. Following a ten minute incubation, addition of protease inhibitors fails to inhibit the cytotoxic response indicating the lytic process is completed within a concealed and localized microenvironment.

Upon contact and recognition of a target cell, the effector cell initiates a series of events by which proteases, contained within granules, are brought to the effector cell surface and released into the microenvironment. This process mediates the cytotoxic activity of the effector cell (Lavie et al. 1985; Hayes et al., 1989).

Relevance of Cytokine Activated Lymphocytes in C. albicans infection.

The physiological role of cytokine activated lymphocytes in C. albicans infection is at present uncertain. This phenomenon may be an artifact of in vitro activation protocols. However, cells with MHC-unrestricted cytotoxic activity have been shown to be present in fluids
isolated from inflammatory sites (Goto and Zvaifler, 1985), and at low frequency in the peripheral blood of normal individuals (Lanier et al., 1987). Therefore, the possibility that cytokine activated lymphocytes play a role in non-specific effector functions involved in host resistance to neoplasia or various infectious diseases, must be considered. Cytokine activated lymphocytes may possess relevant anti-C. albicans activity during the immune response. C. albicans possesses the ability to increase cytokine production in the host. The cytokines which are produced in response to C. albicans have been shown to activate MHC-unrestricted, cytotoxic lymphocytes (Vecchiarelli et al., 1989). Furthermore, MHC-unrestricted cytotoxic lymphocytes can be isolated from mice either inoculated with C. albicans or its cell wall constituents (Cassone et al., 1987; Ausiello et al., 1988). It is unfortunate that these investigations failed to evaluate the anti-C. albicans activity of these cellular populations.

This dissertation will evaluate rIL-2 activated, lymphocyte-mediated, anti-C. albicans growth inhibitory activity, in vitro.

The major objectives of this investigation were: 1) to determine whether such a population can be activated to mediate anti-C. albicans activity, 2) to characterize the cell population through phenotyping and functional activity, and 3) to provide a basis for assessment of IL-2 activated
lymphocytes as a protective anti- \textit{C. albicans} effector population.
MATERIALS AND METHODS

Mice
C57Bl/6 female mice, ages 6 to 7 weeks, were obtained from Jackson Laboratory, Bar Harbor, ME. Experimental mice were 6 to 12 weeks of age when used in experiments.

Tumor Cell Lines
The cell line P815, a methylcholanthrene-induced mastocytoma in DBA/2 mice (Plaut et al., 1973) was obtained from the American Type Culture Collection (ATCC-TIB-64), Rockville, MD. YAC-1, a Moloney virus-induced lymphoma in A/Sn mice (Sjogren and Marbrook, 1965) was obtained from Dr. J. Clancy, Loyola University Medical Center, Maywood, IL. These tumor cell lines were used for assessment of tumor cytotoxic activity. Cells were maintained in suspension cultures in vitro in Corning 25 cm² tissue culture flasks (Corning Glass Works, Corning, NY) in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; low LPS; Gibco Laboratories), 100 units/ml penicillin, 100 µg/ml streptomycin (Whittaker M. A. Bioproducts, Walkersville, MD), 0.1 mM non-essential amino acids and 2 mM l-glutamine (Gibco Laboratories, Grand
Island, NY). This medium was used throughout, except where noted, and referred to as culture medium.

**Fungal Culture**

*Candida albicans* American Type Culture Collection (ATCC) 58716, was obtained from Dr. T. Hashimoto, Loyola University Medical Center, Maywood, IL, and used throughout this investigation. Cultures were stored at 25°C on Sabouraud's dextrose agar (SDA) (Becton Dickinson and Co., Cockeysville, MD). Cells used for experimentation were cultured overnight at 37°C on SDA, collected as isolated colonies, and washed once in Hank's balanced salts solution (HBSS). Cultures of yeast form with greater than 15% budding *C. albicans* were discarded. *C. albicans* were inoculated into RPMI 1640 medium prepared as described above except supplemented with 1% FBS not 10% FBS. This medium is referred to as assay medium. *C. albicans* hyphal forms were obtained by incubation at 37°C in assay medium. Inoculum of $1 \times 10^5$ yeast cells/ml yielded approximately 100% hyphal fragments ranging in length from 30-50 µm when incubated for 2 h at 37°C.

**Recombinant IL-2**

Purified recombinant human IL-2 was obtained from Hoffmann LaRoche, Inc., Nutley, NJ. The recombinant IL-2 (rIL-2) was diluted to desired concentrations in culture
protease Inhibitors

TAME (p-α-toluenesulfonyl-L-arginine methyl ester or Nα-p-tosyl-L-arginine), aprotinin (trasylol), and PMSF (phenylmethanesulfonylfluoride or p-α-toulenesulfonyl fluoride) were purchased from Sigma Chemical Co. (St. Louis, MO). TAME, a serine protease inhibitor, specific for arginine binding sites, was diluted to a final experimental concentration of 10 µM. Aprotinin, a trypsin inhibitor, was used at a final experimental concentration of 10% of the initial commercial solution, and PMSF, a serine esterase inhibitor, was dissolved in ethyl alcohol prior to final experimental concentration of 300 µg/ml in RPMI 1640. Addition of these reagents to target cells showed no alteration in [3H] uridine uptake by these cells compared to untreated C. albicans. The reagents did not affect effector cell viability as judged by trypan blue exclusion.

IL-2 Activation of Lymphocytes

Spleens from untreated mice were aseptically removed. Single cell suspensions were prepared by dissociating the spleen through a 60-gauge wire mesh with the hub of a syringe. Spleen cells were washed once in HBSS prior to placement in culture medium containing 5 X 10⁻⁵ M 2-mercaptoethanol (2-ME) at a concentration of 2.5 x 10⁶ medium.
cells/ml with 1500 U/ml rIL-2 in Falcon, Multiwell plates (Becton Dickinson and Co., Oxnard, CA). The cells were harvested following incubation at 37°C, overlaid onto Lymphocyte Separation Medium (Litton Bionetics, Kensington, MD) and centrifuged at 1000 X g for 20 min. The cells at the interface were washed twice with HBSS prior to assessment of cytocidal or growth inhibitory activity.

**Percoll Separations**

Freshly isolated splenocytes, harvested as above, were subjected to discontinuous Percoll gradients consisting of 50 to 70% Percoll (Sigma Chemical, St. Louis, MO) at 1500 x g for 30 min. Each cell fraction was collected, washed twice in HBSS and resuspended in assay media prior to experimentation. Day 7 cell fractions were designated as fraction 1, representing the population banding above the 50% gradient; fraction 2, representing the population banding between the 50% and the 60% gradient; and fraction 3, representing the population banding below the 60% gradient.

**Supernate Collection**

Day 7 cultured cells were harvested by centrifugation, and separated from culture medium. Following separation, culture media from normal cells as well as from those co-cultured with *C. albicans* at 25:1 effector : target ratios
were concentrated 75 fold with a Diaflo ultrafiltration membrane (Amicon Corp., Danvers, MA) concentration device with a UM10 filter. 12 to 200 µl of concentrated supernate were analyzed for inhibitory and cytotoxic activity.

preparation of Cell Sonicates

Effector populations were resuspended in polypropylene 12 x 75 mm tubes at 5 x 10^6 cells/ml in 1 ml. Populations were subjected to sonication by the Fisher Sonic Dismembrator Model 300 (Farmingdale, NY) at 35% capacity for 15 seconds while on ice. Sonicated cells were < 1% viable as judged by trypan blue exclusion. Preparations of sonicates including membranes and cytosols were treated as equivalent cell populations at 5 x 10^6 /ml for analysis of inhibitory or cytotoxic activity.

Kinetic Analysis of Cell Recovery and Proliferation

Freshly isolated spleen cells (collected as above) were cultured in culture media and harvested (as above) sequentially from day 1 to day 9 of culture. The total number of recoverable cells divided by the original number of cells placed in culture, multiplied by 100 was the percent recovery. Lymphoid cells were plated in 96 well flat-bottomed microtiter plates (Corning 25861, Corning, NY) at 5 x 10^5 cells/well. Cells were cultured with or without
300 U/well of rIL-2. Cells were cultured from 1 to 9 days after which they were pulsed with 1 µCi/well of $[^{3}\text{H}]$ thymidine (ICN Radiochemicals, Irvine, CA). After 6 h of incubation, cells were harvested on the PhD harvester (Cambridge Technology, Cambridge, MA) and associate radioactivity determined with a Beckman LS5801 β liquid scintillation counter (Irving, CA). Proliferation was defined as the incorporation of $[^{3}\text{H}]$ thymidine in the experimental wells containing rIL-2 (300 U/ml) divided by the incorporation of $[^{3}\text{H}]$ thymidine in the control wells without rIL-2.

**Flow Cytometric Analysis**

Antibodies used to analyze the phenotype of cell populations included monoclonal rat anti-mouse Lyt 2.2 (clone 53-6.7; biotin conjugated), monoclonal rat anti-mouse Thy 1.2 (clone 30H12; biotin-conjugated) and monoclonal rat anti-mouse L3T4 (clone GK 1.5; phycoerythrin-conjugated), all from Becton-Dickinson (Mountain View, CA). Monoclonal anti-NK 1.1 (clone PK136; mouse IgG) was obtained from ATCC (Rockville, MD). Rabbit anti-Asialo GM-1 (ASGM-1) was obtained from Wako Chemicals USA, Inc. (Dallas, TX), and was used with goat anti-rabbit Ig fluorescein conjugate (Boehringer Mannheim Biochemicals; Indianapolis, IN). Fluorescein conjugated goat anti-mouse IgG from Zymed Laboratories Inc. (South San Francisco, CA) was utilized to
detect surface IgG. Streptavidin-phycoerythrin was obtained from Vector Laboratories (Burlingame, CA). Effector populations were prepared for phenotyping by washing 1.5 x $10^6$ cells per sample once with cold phosphate buffered saline (PBS). The cells were incubated with the primary antibody for 30 min. on ice. Following two washes in cold PBS, the cells were incubated for an additional 30 min. on ice with secondary antibody, protected from light. Control samples consisted of cells incubated only with the secondary antibodies. The cells were washed twice in cold PBS, and then fixed with 0.1 ml of 1% paraformaldehyde in saline and stored at 4°C. The samples were washed twice and resuspended in PBS prior to flow cytometric analysis (FACS Analyzer ISA, Becton-Dickenson, Mountain View, CA).

Complement-Mediated Depletion Analysis

Antibodies used to deplete cell populations included monoclonal anti-Lyt 2.2 (clone AD4(15); mouse IgM) from Accurate Chemical and Scientific Corp. (Westbury, NY), anti-Thy-1 from Hybritech Inc. (San Diego, CA), monoclonal anti-NK 1.1 (clone PK136; mouse IgG) obtained from ATCC (Rockville, MD), and rabbit anti-ASGM-1 obtained from Wako Chemicals USA, Inc. (Dallas, TX). Viable lymphoid cells were obtained with a Lymphocyte Separation Medium gradient as above and washed twice with HBSS. Effector populations were depleted of cells bearing specific antibodies by
incubating $1 \times 10^7$ cells with saturating levels of antibody for 1 h at 4°C. The cells were washed 3 times with HBSS with 1% FBS to remove excess antibody and then 100 µl of Low Tox M rabbit complement (Accurate Chemical and Scientific Corp., Westbury, NY) was added to the cell pellet. Following a 1 h of incubation at 37°C, cells were washed, obtained with a Lymphocyte Separation Medium gradient as above, and washed twice prior to assessment of inhibitory or cytotoxic activity. Effectiveness of the depletion was assessed by flow cytometric analysis and number of recovered cells from treated populations compared to untreated populations.

Immunomagnetic Selection

Antibodies used for immunomagnetic selection included monoclonal anti-Lyt 2.2 (clone 19/178; mouse IgG) obtained from Dr. Erlich Hammerling, Sloan-Kettering Institute New York, NY, monoclonal anti-NK 1.1 (clone PK136; mouse IgG) obtained from ATCC (Rockville, MD), and magnetic goat anti-mouse IgG obtained from Advanced Magnetics (Cambridge, MA). Viable lymphoid cells were obtained with a Lymphocyte Separation Medium as above and washed twice with HBSS. $1 \times 10^7$ day 7 cells were treated with saturating levels of antibody for 1 h at 4°C. The cells were washed 3 times with HBSS with 1% FBS to remove excess antibody and then 1 ml of magnetic antibody was added to the cell pellet. Following 1
h of incubation at 4°C, antibody sensitized populations were removed with a magnet. Separated populations were activated with 1500 U/ml rIL-2 18 h at 37°C, 5% CO₂. Effector populations were washed, centrifuged over a Lymphocyte Separation Medium gradient as above, and washed twice prior to assessment of inhibitory or cytotoxic activity.

**Tumor Cell Cytotoxicity Assay**

Tumor cells maintained in vitro were washed once in culture medium, pelleted by centrifugation at 500 x g for 10 min and resuspended in approximately 0.1 ml of culture medium. 100 µCi of [⁵¹Cr] chromate (New England Nuclear, Boston, MA) was added to 1 x 10⁷ cells in a final volume of 0.2 ml. The cells were incubated at 37°C with 5% CO₂ for 1 h with agitation every 10 min. The cells were washed 4 times in HBSS, resuspended to 5 x 10⁵ cells/ml in culture medium and 0.01 ml (5 x 10³ cells) were aliquoted to each well of a 96 well, round bottom assay plate (Corning Glass Works, Corning, NY). Radiolabelled YAC-1 or P815 cells were cultured for 4 h with lymphoid cells. Following 4 h of incubation, the supernates were removed using a Skatron harvesting press (Skatron Inc., Sterling, VA) and associated radioactivity determined with a 4/600 ME PLUS automatic gamma counter. Maximum release of radioactivity was obtained by adding 0.05% Nonident P-40 (Sigma Chemical Co., St. Louis, MO). Results are expressed as %
% cytotoxicity = 
\[
\frac{\{(\text{experimental DPM}) - (\text{minimum DPM})\}}{\{(\text{maximum DPM}) - (\text{minimum DPM})\}} \times 100.
\]
All experimental means were calculated from triplicate values. Lytic units (LU), were calculated by the method of Pross et al. (1981), and represent the number of effector cells per $1 \times 10^7$ effectors required to achieve 20% lysis of the target. Lytic units were calculated by a program written by David Coggins (NCI, Fredrick, MD).

**C. albicans Inhibition**

Fungal cells used for experimentation were collected from isolated, overnight SDA colonies, and washed once in HBSS. Yeast form cells were resuspended to $2 \times 10^5$ / ml in assay media. 1 $\times 10^4$ cells were then added to individual wells of 96 well plates (Corning 25861, Corning, NY). *C. albicans* hyphal forms were obtained by incubation at 37°C in 5% CO$_2$ for 2 h. Various numbers of effector cells were added at ratios of 50:1 to 6:1. After 3 h incubation at 37°C in 5% CO$_2$, effector cells were lysed and removed by washing with water using a PhD cell harvester (Cambridge Technology, Cambridge, MA). 0.05 ml of RPMI 1640 with 1% FBS and 1 µCi per well of [³H] uridine (ICN Radiochemicals, Irvine, CA) was added to individual wells. Following 1 h incubation at 37°C, 5%CO$_2$, 25 U lyticase (Sigma Chemical, St. Louis, MO) in 50 µl HBSS was added to individual wells for
Cells were then harvested with a PhD harvester and associated radioactivity determined with a Beckman LS5801 β liquid scintillation counter. Growth Inhibition of \textit{C. albicans} = \frac{[\text{DPM } \textit{C. albicans} \text{ control}) - (\text{DPM (effector and } \textit{C. albicans} - \text{ effector control})]}{\text{DPM } \textit{C. albicans} \text{ control}} \times 100. \text{ All cultures were prepared at least in triplicate and the mean inhibition of those values determined. Inhibitory units (IU), a modification of the lytic unit method of Pross et al. (1981), were calculated as the number of effector cells per } 1 \times 10^7 \text{ effectors required to achieve } 20\% \text{ inhibition of the target. Inhibitory units were calculated as DPM } \textit{C. albicans} \text{ alone} - \text{ DPM experimental sample} = \text{ inhibited counts. Inhibited counts were determined by a modification of a program written by David Coggins (NCI, Frederick, MD) and presented as inhibitory units.}

\textbf{Cold target Inhibition}

Tumor cytotoxicity and \textit{C. albicans} inhibitory assays were conducted at 40:1 effector : target ratio. Additionally, cultured tumor targets (P815 and YAC-1) and heat inactivated (5 minutes at 100°C) \textit{C. albicans} hyphae were utilized as cold targets. Cold target inhibitors were washed twice in HBSS prior to co-incubation with effector and target cells at concentrations from } 1 \times 10^4 \text{ to } 5 \times 10^6 \text{ additional cold targets per well. Naive thymocytes were utilized as unrelated control cell populations. Thymocytes
were added to each well such that the final concentration of cold targets and thymocytes of each well totalled $5 \times 10^6$ cells per well. Tumor culture supernates were collected both from overnight and 3 day tissue culture flasks as well as from cultures of $5 \times 10^6$ cells in 0.1 ml from a 96 well plate following a 4 h culture period. *C. albicans* supernates were collected following heat inactivation. The supernates were assessed for inhibition of cytotoxicity and *C. albicans* inhibition.

**Statistics**

Comparisons of mean LU and IU values were performed using the t test for small-samples concerning difference between two means (Freund, 1979).
RESULTS

Activation of Anti-fungal and Anti-tumor Activity of splenocytes with rIL-2

A. Anti-fungal and anti-tumor activity of splenocytes

The purpose of initial experiments was to determine the inherent anti-fungal and anti-tumor activity of murine splenocytes. Splenocytes possessed no growth inhibitory activity against \textit{C. albicans} as judged by inhibition of $[^3\text{H}]$ uridine uptake (Figure 1A). Neither was there activity against the NK-resistant cell line, P815, as judged by $[^{51}\text{Cr}]$ release (Figure 1C). Low levels of activity were observed for the NK sensitive target, YAC-1, as judged by $[^{51}\text{Cr}]$ release (Figure 1B). This activity could be enriched by discontinuous Percoll density gradient selection. Splenocytes from interface 1 (cells which did not enter a 50% concentration of Percoll) showed enhanced activity against the YAC-1 tumor cell line when compared to unfractionated splenocytes ($p<0.05$) (Figure 2B).

Unfractionated splenocytes possessed 5.2\% large granular lymphocytes compared to splenocytes from interface 1 which possessed 38.3\% large granular lymphocytes as judged by Wright-Geimsa staining of cytospin preparations. No
Figure 1.

*C. albicans* growth inhibition and tumor cell cytotoxicity of day 0 splenocytes. A. *C. albicans* growth inhibition was assessed by the incorporation of [3H] uridine. Data are presented as % inhibition and inhibitory units per 10^7 cells at 20% inhibition (IU). B. YAC-1 cytotoxicity is expressed as % cytotoxicity as judged by [51Cr] release and by lytic units per 10^7 cells at 20% cytotoxicity (LU). C. P815 cytotoxicity is expressed as % cytotoxicity as judged by [51Cr] release and by lytic units per 10^7 cells at 20% cytotoxicity (LU). Data are expressed as the mean ± S. D. for 3 independent experiments.
Figure 1.
Figure 2.

C. albicans growth inhibition and tumor cell cytotoxicity of percoll fractionated day 0 splenocytes. Day 0 splenocytes were fractionated by discontinuous percoll density gradient separation. Cells from each interface were harvested and washed: Fraction 1, 50% gradient (Fract. 1); fraction 2, 60% gradient (Fract. 2); and fraction 3, 70% gradient (Fract. 3). A. C. albicans growth inhibition was assessed by the incorporation of [3H] uridine. Data are presented as % inhibition and inhibitory units per 10^7 cells at 20% inhibition (IU). B. YAC-1 cytotoxicity is expressed as % cytotoxicity as judged by [51Cr] release and by lytic units per 10^7 cells at 20% cytotoxicity (LU). C. P815 cytotoxicity is expressed as % cytotoxicity as judged by [51Cr] release and by lytic units per 10^7 cells at 20% cytotoxicity (LU). *, (p<0.05) Significant difference between fraction 1 activity and unfractionated splenocyte activity. Non-significant comparisons are not labelled, all significant comparisons are labelled. Data are expressed as the mean ± S. D. for 3 independent experiments.
Figure 2.

A. C. albicans
- UNFRAC. 0.1 ± 0.05 IU
- FRACT. 1 0.2 ± 0.05 IU
- FRACT. 2 0.2 ± 0.05 IU
- FRACT. 3 0.1 ± 0.05 IU

B. YAC-1
- UNFRAC. 15.0 ± 2.4 IU
- FRACT. 1 42.8 ± 8.6 IU
- FRACT. 2 1.5 ± 0.6 IU
- FRACT. 3 0.2 ± 0.1 IU

C. P815
- UNFRAC. 0.0 ± 0.0 LU
- FRACT. 1 0.0 ± 0.0 LU
- FRACT. 2 0.0 ± 0.0 LU
- FRACT. 3 0.0 ± 0.0 LU
increase in anti-YAC-1 tumor cell activity was noted for the other Percoll separated splenocytes. No enrichment of activity was noted for any of the other Percoll separated cell populations against either C. albicans (Figure 2A) or p815 (Figure 2C).

These data demonstrate no anti-C. albicans or anti-P815 activity in either splenocytes or Percoll separated populations at the effector to target ratios used in these experiments. The anti-YAC-1 activity of normal splenocytes was enhanced in low density Percoll fractions, suggesting that resident splenic NK do not mediate anti-C. albicans activity.

B. Activation of anti-fungal and anti-tumor activity in splenocytes with rIL-2

Previous studies have shown that activation of lymph node cells by rIL-2 augments their anti-fungal activity (Beno and Mathews, 1990) and anti-tumor activity (Herberman and Holden, 1978). To evaluate the effect of this cytokine on splenocytes, such cell populations were activated with rIL-2 and assayed for their capacity to mediate anti-C. albicans and anti-tumor activities (Figure 3).

Following 1 day activation by rIL-2, no increase in either C. albicans growth inhibition (Figure 3A) or anti-P815 cytotoxic activity (Figure 3C) was noted for these splenocytes when compared to the activity of day 0
splenocytes or splenocytes cultured for 1 day without rIL-2 (unactivated) (p>0.05). However, the increase in anti-YAC-1 cytotoxic activity (Figure 3B) was significant when compared to day 0 splenocytes or unactivated day 1 splenocytes (p<0.05). Following 3 days activation with rIL-2, C. albicans growth inhibition (Figure 4A) was increased significantly when compared to day 0 splenocyte activity or the activity of splenocytes cultured for 3 days without rIL-2 (unactivated day 3) (p<0.05). Anti-P815 cytotoxic activity of splenocytes (Figure 4C) was increased significantly at day 3 of culture (p<0.05). Splenocytes cultured without rIL-2 showed no (p>0.05) anti-P815 activity. Anti-YAC-1 activity (Figure 4B) was enhanced significantly from day 0 to day 3 of incubation with rIL-2 (p<0.05). No change in anti-YAC-1 activity was observed for splenocytes cultured 3 days without rIL-2 (p>0.05) when compared to day 0 splenocytes. Following activation with rIL-2 for 7 days, C. albicans growth inhibition and antitumor cytotoxic activity was increased significantly over day 0 splenocytes (p<0.05) (Figure 5). No cells were recovered from splenocytes cultured without rIL-2 for more than 3 days. The C. albicans growth inhibitory activity of splenocytes cultured for 7 days with rIL-2 was increased significantly (p<0.05) when compared to 3 day activated splenocyte populations for all targets examined (Figures 4 and 5). Day 0 splenocyte activity against all targets
C. albicans growth inhibition and tumor cell cytotoxicity of
day 1 rIL-2 activated populations. A. C. albicans growth
inhibition was assessed by the incorporation of [3H]
uridine. Data are presented as % inhibition and inhibitory
units per 10^7 cells at 20% inhibition (IU). B. YAC-1
cytotoxicity is expressed as % cytotoxicity as judged by
[^51]Cr release and by lytic units per 10^7 cells at 20%
cytotoxicity (LU). C. P815 cytotoxicity is expressed as %
cytotoxicity as judged by[^51]Cr release and by lytic units
per 10^7 cells at 20% cytotoxicity (LU). *, (p<0.05)
Significant difference between activity of day 1 rIL-2
activated population compared to day 0 splenocyte activity.
**, (p<0.05) Significant difference between activity of day
1 rIL-2 activated populations compared to the activity of
day 1 cultured without rIL-2. Data are expressed as the
mean ± S. D. for 4 individual experiments.
Figure 3.
C. albicans growth inhibition and tumor cell cytotoxicity of day 3 rIL-2 activated populations. A. C. albicans growth inhibition was assessed by the incorporation of [3H] uridine. Data are presented as % inhibition and inhibitory units per 10^7 cells at 20% inhibition (IU). B. YAC-1 cytotoxicity is expressed as % cytotoxicity as judged by [51Cr] release and by lytic units per 10^7 cells at 20% cytotoxicity (LU). C. P815 cytotoxicity is expressed as % cytotoxicity as judged by [51Cr] release and by lytic units per 10^7 cells at 20% cytotoxicity (LU). *, (p<0.05) Significant difference between activity of day 3 rIL-2 activated population compared to day 0 splenocyte activity. **, (p<0.05) Significant difference between activity of either day 3 rIL-2 activated populations compared to the activity of day 3 splenocytes cultured without rIL-2. Data are expressed as the mean ± S. D. for 4 individual experiments.
Figure 4.
Figure 5.

*C. albicans* growth inhibition and tumor cell cytotoxicity of day 7 rIL-2 activated populations. A. *C. albicans* growth inhibition was assessed by the incorporation of $[^3H]$ uridine. Data are presented as % inhibition and inhibitory units per $10^7$ cells at 20% inhibition (IU). B. YAC-1 cytotoxicity is expressed as % cytotoxicity as judged by $[^51Cr]$ release and by lytic units per $10^7$ cells at 20% cytotoxicity (LU). C. P815 cytotoxicity is expressed as % cytotoxicity as judged by $[^51Cr]$ release and by lytic units per $10^7$ cells at 20% cytotoxicity (LU). *, (p<0.05) Significant difference between activity of day 7 rIL-2 activated population compared to day 0 splenocyte activity. Data are expressed as the mean ± S. D. for 4 individual experiments.
A. C. albicans

B. YAC-1

C. P815

Figure 5.
displayed in Figures 3, 4, and 5 are taken from Figure 1 and used to demonstrate comparative activities. The activity of day 0 splenocytes was compared twice in parallel with the previous studies of rIL-2 activated lymphocytes at days 1, 3, and 7. No differences in activity were observed between these cell populations and the day 0 splenocytes presented above.

These data demonstrate a concurrent increase over time in anti-fungal and anti-tumor activities when splenocytes were cultured with rIL-2 at days 1, 3, and 7. Furthermore, despite the presence of enhanced NK activity at day 1, no anti-fungal activity was observed suggesting NK do not mediate anti-\textit{C. albicans} activity. The induction of anti-P815 activity at days 3 and 7 does correlate with the ability of the population to mediate anti-\textit{C. albicans} activity. These results suggest activation to P815 tumor cytotoxic activity is associated with cell population activation to anti-fungal activity.

C. Kinetic analysis of lymphoproliferation, cell recovery, anti-fungal activity, and anti-tumor activity of splenocytes following activation with rIL-2.

To establish the time course for maximal functional activity as well as maximal cell recovery, splenocyte populations were analyzed for anti-tumor lytic activity, anti-\textit{C. albicans} growth inhibitory activity, cell recovery,
and lymphoproliferation during 9 consecutive days of activation with rIL-2 (1500 U/ml).

In separate experiments from those described in Figures 1-5, splenocytes acquired anti-tumor activity against both YAC-1 and P815 cell lines with peak activity occurring at day 7 of culture with 1500 U/ml rIL-2 (Figure 6). A significant increase in anti-YAC-1 activity was noted as early as day 1 when compared to day 0 splenocytes (p<0.05). Anti-P815 activity was not significantly increased over the day 0 splenocyte population until day 3 in culture and demonstrated peak activity at day 7 (p<0.05). C. albicans growth inhibition peaked at day 7 of culture with significant growth inhibition appearing by day 3 when compared to the activity of day 0 splenocytes (p<0.05). The activity against all targets increased until day 7 at which time the activity against all targets declined numerically, but not significantly from day 7 (p>0.05) (Figure 6). Maximal cell recovery coincided with maximal anti-fungal activity, on day 7 (Figures 6 and 7). Maximal proliferation of splenocytes was on day 4 of activation, 3 days prior to maximal cell recovery (Figure 7). Splenocytes cultured without rIL-2 showed no proliferative capacity and cell viability decreased from 75.3% ± 8.0% at day 1 to 8.8% ± 9.2% by day 3 of culture. Without rIL-2 no viable lymphocytes were recoverable after 3 days in culture.

These data expand on previous experiments within this
Figure 6.

Kinetic analysis of anti-tumor and anti-C. albicans activity of splenocyte populations activated with rIL-2. Splenocyte populations were cultured for 0 to 9 days with 1500 U/ml rIL-2 and assessed at daily intervals for anti-tumor activity against YAC-1 and P815 and anti-C. albicans inhibitory activity. C. albicans growth inhibition was assessed by the incorporation of $[^3H]$ uridine. Data are presented as inhibitory units per $10^7$ cells at 20% inhibition (IU). YAC-1 and P815 cytotoxicity is expressed as lytic units per $10^7$ cells at 20% cytotoxicity (LU) as judged by $[^51]Cr$ release. Data are expressed as the mean ± S. D. for 3 independent experiments.
Figure 6.
Figure 7.

Kinetics of proliferation and cell recovery from cultures of rIL-2 activated populations. Splenocyte populations were assessed for proliferation following culture with 1500 U/ml rIL-2 at the days indicated. The percent of recovered cells during the activation with rIL-2 is also expressed. Data are expressed as the mean ± S. D. for 3 independent experiments.
Figure 7.
study to demonstrate maximal anti-tumor activity, anti-
fungal activity, and cell recoverability occur at day 7 of
culture with rIL-2. Due to these observations, the day 7
rIL-2 activated population was selected for further
characterization.

Susceptibility of Various Strains of Candida to rIL-2
Activated Population

To examine if the anti-fungal effect of the splenocyte
population was limited to C. albicans strain (ATCC #58716)
utilized in this study, various clinical isolates of C.
albicans and species of Candida were evaluated for their
susceptibility to this form of lymphocyte mediated growth
inhibition.

Figure 8 depicts the growth inhibitory effect of the
rIL-2 activated population on various clinical isolates of
Candida albicans, Candida tropicalis, Candida parapsilosis,
or Torulopsis glabrata. All isolates of C. albicans and
species of Candida evaluated were susceptible to growth
inhibition by rIL-2 activated populations, indicating the
anti-C. albicans activity observed by the rIL-2 activated
population is not limited to the strain of C. albicans
utilized in this study.

Characterization of rIL-2 Activated Populations

A. Flow cytometric analysis of rIL-2 activated populations
Figure 8.

Growth inhibition of rIL-2 activated populations for various Candida strains and species. rIL-2 activated populations were assessed for C. albicans growth inhibition against clinically isolated strains of C. albicans by the uridine method and presented as % inhibition and IU. rIL-2 activated populations were also assessed for growth inhibition against clinically isolated species of Candida. C. albicans and Candida species growth inhibition was assessed by the incorporation of [³H] uridine and data presented as % inhibition and inhibitory units per 10⁷ cells at 20% inhibition (IU). *, indicates ATCC 58716 strain of C. albicans utilized throughout study. Data are expressed as the mean ± S. D. for 3 independent experiments.
Figure 8.
In order to determine the phenotypic nature of rIL-2 activated populations and that of day 0 splenocytes, both populations were assessed for the expression of the phenotypic markers; ASGM-1, NK 1.1, Ig, Thy-1.2, L3T4, and Lyt-2.2.

Four experiments comparing phenotypic expression of day 0 splenocytes to rIL-2 activated populations are summarized in Table I. 52.7 ± 7.6% of day 0 splenocytes expressed Thy-1.2 and 40.9 ± 4.3% expressed ASGM-1. 19.7 ± 3.3% of the day 0 splenocyte population expressed the Lyt-2.2 T cell marker while 10.7 ± 2.7% expressed the NK 1.1 marker. The number of rIL-2 activated cells expressing Thy-1.2 increased to 92.0 ± 3.8% and while the number of rIL-2 activated cells expressing ASGM-1 increased to 85.7 ± 9.6% after 7 days in culture with rIL-2. 53.3 ± 8.3% of the rIL-2 activated population expressed the Lyt-2.2 T cell marker while 36.8 ± 6.1% expressed the NK 1.1 marker. The presence of Ig and L3T4 phenotypic markers were also examined for rIL-2 activated populations and day 0 splenocytes. Expression of both phenotypic markers decreased during time in culture. Ig expression decreased from 48.7 ± 6.3 to 4.3 ± 3.7% and L3T4 expression decreased from 30.1 ± 4.0 to 6.0% ± 3.2%.

These data indicate that following 7 days activation with rIL-2, a high percentage of lymphocytes express the markers Thy-1.2 and ASGM-1 while the percentage of cells expressing the markers NK 1.1 and Lyt-2.2 is intermediate.
Table I. Surface phenotype of day 0 splenocytes or day 7 rIL-2 activated population.

<table>
<thead>
<tr>
<th>Phenotypic Marker</th>
<th>Day 0 % Expressing$^a$</th>
<th>Day 7 % Expressing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thy-1.2</td>
<td>52.7 ± 7.6$^b$</td>
<td>92.0 ± 3.8</td>
</tr>
<tr>
<td>L3T4</td>
<td>30.1 ± 4.0</td>
<td>6.0 ± 3.2</td>
</tr>
<tr>
<td>LYT-2.2</td>
<td>19.7 ± 3.3</td>
<td>53.3 ± 8.3</td>
</tr>
<tr>
<td>ASGM-1</td>
<td>40.9 ± 4.3</td>
<td>85.7 ± 9.6</td>
</tr>
<tr>
<td>NK 1.1</td>
<td>10.7 ± 2.7</td>
<td>36.8 ± 6.1</td>
</tr>
<tr>
<td>Ig</td>
<td>48.7 ± 6.3</td>
<td>4.3 ± 3.7</td>
</tr>
<tr>
<td>FITC-RAMig$^c$</td>
<td>7.8 ± 2.2</td>
<td>8.5 ± 3.7</td>
</tr>
<tr>
<td>PE-avidin$^d$</td>
<td>8.9 ± 3.1</td>
<td>8.7 ± 4.2</td>
</tr>
<tr>
<td>FITC-GAMig$^e$</td>
<td>6.3 ± 4.4</td>
<td>4.6 ± 3.1</td>
</tr>
</tbody>
</table>

$^a$ As judged by flow cytometric immunofluorescence.
$^b$ Mean ± S. D. of 4 independent experiments.
$^c$ Secondary antibody for ASGM-1 (rabbit Ig).
$^d$ Secondary marker for Thy-1.2, Lyt-2.2.
$^e$ Secondary antibody for NK 1.1 (goat Ig).
within the population.

B. Phenotypic expression of NK 1.1 and Lyt-2.2

To determine whether the phenotypic markers Lyt-2.2 and NK 1.1 are expressed on the same population with the rIL-2 activated population, antibody and complement depletion experiments were performed, followed by flow cytometric analysis.

Flow cytometric analysis of rIL-2 activated populations treated with anti-Lyt-2.2 and complement show a decrease in cells staining for Lyt-2.2 from 48% to 13% (Figure 9) in a representative experiment. In contrast, the rIL-2 activated population treated with anti-Lyt-2.2 and complement displayed increased expression of NK 1.1 from 35% to 59%. The rIL-2 activated population treated with anti-NK 1.1 and complement showed a decrease in cells staining for expression of NK 1.1 (35% expression in untreated populations compared to 11% expression of NK 1.1 in treated populations) yet displayed an increase in expression of Lyt-2.2 from 48% to 59%. Evaluation of flow cytometric analysis utilizing this method is limited. Special considerations for the use of flow cytometric analysis must be made following the isolation of cell subpopulations using antibody and complement. Similar, if not identical antibody binding sites may be measured for immunofluorescence as well as for complement mediated depletions. Therefore, residual
Figure 9.

Flow cytometric histograms representing rIL-2 activated population following in vitro antibody and complement depletions. rIL-2 activated populations were treated with anti-Lyt-2.2 or anti-NK 1.1, and complement; or complement alone. Cells were then assessed for expression of Lyt-2.2 and NK 1.1 phenotypic markers. The left column shows rIL-2 activated populations stained with anti-Lyt-2.2 and the right column shows rIL-2 activated populations stained with anti-NK 1.1. The first row shows complement treated rIL-2 activated populations, the middle row rIL-2 activated populations treated with anti-Lyt-2.2 and complement, and the third row shows rIL-2 activated populations treated with anti-NK 1.1 and complement. Data are representative of 3 independent experiments.
Figure 9.
antibody to the cell population may block antigen expression as determined by flow cytometric analysis. Flow cytometric analysis was supported with analysis of percentage recoverable cells following antibody and complement depletions. The percentage of rIL-2 activated populations not expressing Lyt-2.2 as judged by flow cytometric analysis was 46.7 ± 8.3%. Following Lyt-2.2 antibody and complement treatment, the percentage of recoverable cells was 47.3 ± 5.9% in 3 separate experiments. The decrease in the percent recoverable cells following anti-Lyt-2.2 and complement treatment to a level near the original percentage of cells not expressing Lyt-2.2 indicates that antibody and complement removed nearly all cells expressing Lyt-2.2. Likewise, the percent of recoverable cells following NK 1.1 antibody and complement treatment was 74.7 ± 7.4%, approximately equal to the percentage of cells not expressing the marker (63.2 ± 6.1%) (Tables I and II).

These data are consistent with the expression of Lyt-2.2 and NK 1.1 on separate cellular subsets within the rIL-2 activated population.

C. Functional role of Lyt-2.2 and NK 1.1 depleted populations

The role of Lyt 2.2+ and NK 1.1+ cell populations in anti-fungal and anti-tumor cytotoxic activity was assessed following antibody and complement depletions of rIL-2
Table II. Percent recovery of rIL-2 activated population following treatment with antibody and complement.

<table>
<thead>
<tr>
<th>Effector Population</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>100.0 ± 3.6</td>
</tr>
<tr>
<td>Complement</td>
<td>89.0 ± 6.1</td>
</tr>
<tr>
<td>anti-Lyt-2.2 +</td>
<td>47.3 ± 5.9</td>
</tr>
<tr>
<td>Complement</td>
<td></td>
</tr>
<tr>
<td>anti-NK 1.1 +</td>
<td>74.7 ± 7.4</td>
</tr>
<tr>
<td>Complement</td>
<td></td>
</tr>
</tbody>
</table>

*Mean ± S. D. of 3 independent experiments.*
activated populations.

Populations depleted of Lyt-2.2 displayed significantly lower activity against both \textit{C. albicans} and P815 when compared to rIL-2 activated populations or complement treated controls (p<0.05) (Figure 10). In contrast, NK 1.1 depleted populations showed no difference in anti-fungal and anti-P815 activity when compared to either complement treated rIL-2 activated populations or untreated rIL-2 activated population. The activity of the NK 1.1 depleted population against the YAC-1 cell line was significantly reduced (p<0.05) yet still retained activity. No difference in activity against YAC-1 was observed between the anti-Lyt-2.2 treated population and either complement treated rIL-2 activated populations or untreated rIL-2 activated populations (p>0.05).

D. Functional activity of NK 1.1$^+$ and Lyt-2.2$^+$ selected populations

To confirm the experiments described above, Lyt-2.2$^+$ or NK 1.1$^+$ populations were selected by immunomagnetic beads and specific antibody 18 h prior to the assessment of anti-fungal and anti-tumor activity.

Against both \textit{C. albicans} and P815, NK 1.1 selected cells (NK 1.1$^+$ cells) and Lyt-2.2 unselected cells (Lyt-2.2$^-$ cells) displayed significantly reduced tumor cytotoxic and \textit{C. albicans} growth inhibitory activity when compared to rIL-
Figure 10.

Fungal growth inhibition and tumor cell cytotoxicity of rIL-2 activated population after in vitro antibody and complement depletions. rIL-2 activated populations were treated with complement plus anti-Lyt-2.2 or anti-NK 1.1 complement alone, or untreated. A. C. albicans growth inhibition was assessed by the incorporation of $[^3]H$ uridine. Data are presented as % inhibition and inhibitory units per $10^7$ cells at 20% inhibition (IU). B. YAC-1 cytotoxicity is expressed as % cytotoxicity as judged by $[^5]Cr$ release and by lytic units per $10^7$ cells at 20% cytotoxicity (LU). C. P815 cytotoxicity is expressed as % cytotoxicity as judged by $[^5]Cr$ release and by lytic units per $10^7$ cells at 20% cytotoxicity (LU). *, (p<0.05) Significant difference between activity of Lyt-2.2 antibody and complement treated rIL-2 activated populations compared to activity of untreated, complement treated, NK 1.1 antibody and complement treated rIL-2 activated populations; **, (p<0.05) Significant difference between activity of NK 1.1 antibody and complement treated rIL-2 activated populations to activity of untreated, complement treated, or Lyt-2.2 antibody and complement treated rIL-2 activated populations. Data are expressed as the mean ± S. D. of 3 independent experiments.
Figure 10.

A. C. albicans
- DAY 7: 395.9 ± 97.5 IU
- C: 427.4 ± 120.4 IU
- LYT+C: 21.1 ± 10.4 IU
- NK+C: 980.4 ± 150.4 IU

B. YAC-1
- DAY 7: 2066 ± 173 IU
- C: 2219 ± 308 IU
- LYT+C: 1619 ± 192 IU
- NK+C: 416 ± 62 IU

C. P815
- 289.4 ± 35.9 IU
- 269.9 ± 45.8 IU
- 59.4 ± 7.8 IU
- 372.4 ± 63.2 IU
Figure 11.

Fungal growth inhibition and tumor cell cytotoxicity of rIL-2 activated population after in vitro selection with immunomagnetic beads and specific antibody. rIL-2 activated populations were selected with anti-Lyt-2.2 or anti-NK 1.1 and immunomagnetic beads, or unselected populations which remained following selection of NK 1.1 or Lyt-2.2 and immunomagnetic beads, or immunomagnetic beads alone. A. C. albicans growth inhibition was assessed by the incorporation of [3H] uridine. Data are presented as % inhibition and inhibitory units per 10⁷ cells at 20% inhibition (IU). B. YAC-1 cytotoxicity is expressed as % cytotoxicity as judged by [51Cr] release and by lytic units per 10⁷ cells at 20% cytotoxicity (LU). C. P815 cytotoxicity is expressed as % cytotoxicity as judged by [51Cr] release and by lytic units per 10⁷ cells at 20% cytotoxicity (LU). *, (p<0.05) Significant difference of activity between either Lyt-2.2 selected or NK 1.1 unselected rIL-2 activated populations compared to untreated rIL-2 activated populations, Lyt-2.2 unselected, or NK 1.1 selected populations. **, (p<0.05) Significant difference of activity between either Lyt-2.2 unselected or NK 1.1 selected rIL-2 activated populations compared to untreated rIL-2 activated populations, Lyt-2.2 selected, or NK 1.1 unselected populations. Data are expressed as the mean ± S. D. of 3 independent experiments.
Figure 11.
activated populations (p<0.05, Figure 11). In contrast, significant enrichment of activity against both *C. albicans* and P815 was observed by both Lyt-2.2 selected cells (Lyt-2.2⁺) and NK 1.1 unselected or depleted cells (NK 1.1⁻) (p<0.05). The activity of these populations was significantly enhanced (p<0.05) compared to untreated rIL-2 activated populations, NK 1.1⁺ selected cells, and Lyt-2.2⁻ unselected (depleted) cells. The cytotoxic activity of the NK 1.1⁻ population against the YAC-1 cell line was significantly reduced (p<0.05) when compared to all other populations examined, yet retained activity. Selected and remaining unselected populations were examined for expression of Lyt-2.2 or NK 1.1 as judged by direct microscopic fluorescence. Lyt-2.2⁺ selected populations and NK 1.1⁻ unselected populations contained insignificant percentages of NK 1.1⁺ expressing cells. Lyt-2.2⁻ unselected populations and NK 1.1⁺ selected populations contained less than background levels of expression of Lyt-2.2 (Table III).

These data confirm the complement-depletional studies and demonstrate that the Lyt-2.2⁺ population mediates the anti-*C. albicans* and anti-P815 activity of the rIL-2 activated population while both Lyt-2.2⁺ and NK 1.1⁺ populations mediate anti-YAC-1 activity.
Table III. Percentage of rIL-2 activated population expressing Lyt-2.2 or NK 1.1 following selection with immunomagnetic antibody.

<table>
<thead>
<tr>
<th>Effector population</th>
<th>% Lyt-2.2⁺</th>
<th>% NK 1.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lyt-2.2⁺ (selected)</td>
<td>&gt; 95%</td>
<td>4.5 ± 3.4bc</td>
</tr>
<tr>
<td>Lyt-2.2⁻ (unselected)</td>
<td>3.1 ± 1.7d</td>
<td>N. D.⁵</td>
</tr>
<tr>
<td>NK 1.1⁺ (selected)</td>
<td>5.0 ± 3.6</td>
<td>&gt; 95%</td>
</tr>
<tr>
<td>NK 1.1⁻ (unselected)</td>
<td>N. D.</td>
<td>4.2 ± 2.2</td>
</tr>
</tbody>
</table>

* As judged by microscopic immunofluorescence.
₁ Mean ± S. D. of three experiments.
⁵ Secondary background 4.3 ± 3.1%.
⁶ Secondary background 5.7 ± 2.6%.
⁷ Not Done.
Cold Target Inhibition of Anti-fungal and Anti-tumor Activity

To assess whether the same cell population is capable of binding both fungal and tumor targets, cold target inhibition studies were performed.

In all experiments, homologous inhibitors provided maximum inhibition of their respective targets (Figure 12). In all studies, control populations for nonspecific inhibition were naive thymocytes. No cold target inhibition by thymocytes was observed in any experiment. Figure 12A depicts cold target inhibition of C. albicans. Both P815 and YAC-1 demonstrated the ability to inhibit the activity of rIL-2 activated populations against C. albicans. The competitive activity of both tumor cell lines was significant when compared to that of naive thymocytes (p<0.05, Figure 12A). Cytotoxic activity against the YAC-1 tumor cell line was inhibited by both the P815 tumor cell line and to a lesser extent by C. albicans. The inhibition by both P815 and C. albicans was significant at p<0.05 at the three highest ratios examined (Figure 12B). The cold target inhibition of the P815 cell line was nearly complete by the YAC-1 cell line with the inhibition significant at p<0.05 (Figure 12C). C. albicans significantly inhibited cytotoxicity for the P815 tumor cell line (p<0.05) (Figure 12C). To assure that the cold target populations were not mediating the inhibition through released factors, culture
Figure 12.

Unlabeled target competition studies with *C. albicans*, YAC-1, and P815. rIL-2 activated population were utilized at a 40 : 1 effector : target ratio for % competitive inhibition. 

A. Competitive inhibition of anti-fungal activity by *C. albicans*, YAC-1, and P815 as assessed by the uridine method. 

B. Competitive inhibition of Yac-1 cytotoxicity by *C. albicans*, YAC-1, and P815 as assessed by [51Cr] release. 

C. Competitive inhibition of P815 cytotoxicity by *C. albicans*, YAC-1, and P815 as assessed by [51Cr] release. Unlabeled homologous target inhibitors were shaded with squares representing *C. albicans*, circles representing YAC-1, and triangles representing P815. Thymocytes (diamonds) were utilized as irrelevant targets. Data are expressed as the mean ± S. D. of percent competitive inhibition for 3 independent experiments.
Figure 12.
Supernates from the target cell populations were collected and assessed for inhibitory activity. No effect on cytotoxic or inhibitory activity was observed for supernates (Data not shown).

These data suggest that the rIL-2 activated population which binds to C. albicans also binds to P815 and YAC-1. Reciprocally, the rIL-2 activated population subpopulation which binds and mediates anti-tumor activity also binds C. albicans.

Role of Cell Contact in C. albicans growth inhibition by rIL-2 activated population

A. C. albicans growth inhibitory activity of cellular supernates

To assess the anti-fungal activity of secreted substances and evaluate the necessity for cell contact in anti-fungal activity, rIL-2 activated population supernates and cellular sonicates were assessed for C. albicans growth inhibition.

Supernates concentrated 75 fold from populations treated for 3 h with C. albicans hyphae were assessed for C. albicans growth inhibition (Figure 13A). 200 - 12 µl of C. albicans stimulated supernates displayed an anti-C. albicans activity in a dose dependent manner (200 µl of concentrated supernate is equivalent to the supernate of 3.75 x 10^7 cells). Unstimulated culture supernates (concentrated 75
Figure 13.

Effect of rIL-2 activated population supernates and whole cell sonicates upon C. albicans. C. albicans growth inhibition was assessed by the uridine method and presented as % inhibition and IU. A. Concentrated supernates (200 ul to 12 ul) from C. albicans treated rIL-2 activated population, untreated rIL-2 activated population, and supernates from tissue culture media containing 1500 U/ml rIL-2 were assessed for C. albicans growth inhibition and compared to intact rIL-2 activated population activity. B. Activity of sonicates from C. albicans treated rIL-2 activated population, untreated rIL-2 activated population, day 0 spleen sonicates, and day 0 thymocytes were assessed for C. albicans growth inhibition at equivalent cellular ratios of 50:1 to 1:1, and compared to the intact rIL-2 activated population activity. *, (p<0.05) Significant difference between C. albicans growth inhibition of stimulated or unstimulated supernates or sonicates from rIL-2 activated populations compared to untreated rIL-2 activated populations (significant decrease in inhibitory activity) or control populations (significant increase in inhibitory activity). Data are expressed as the mean ± S. D. of 3 independent experiments.
Figure 13.
fold) also possessed inhibitory activity against *C. albicans* when compared to the inhibitory activity of supernates of cell culture media (with rIL-2) concentrated 75 fold (p<0.05).

These data demonstrate that *C. albicans* stimulated rIL-2 activated populations secrete substances which possessed activity against *C. albicans*. However, the anti-*C. albicans* was 225-fold less active than the intact rIL-2 activated population. A finding consistent with a requirement for cell contact for optimal *C. albicans* growth inhibitory activity.

**B. C. albicans growth inhibitory activity of cellular sonicates**

Cellular sonicates prepared from rIL-2 activated populations produced an enhanced *C. albicans* growth inhibition when compared to the concentrated supernates (Figure 13). Cell sonicates, equivalent to splenocyte numbers utilized for assessment of activity, from either *C. albicans* stimulated or unstimulated rIL-2 activated populations produced moderate inhibitory effects against *C. albicans*. The activity of either stimulated or unstimulated sonicates was significantly higher (p<0.05) than sonicates from either day 0 splenocytes or thymocytes. In contrast, the activity of either stimulated or unstimulated sonicates was significantly less (p<0.05) than the activity of rIL-2
activated populations.

These data demonstrate that cellular sonicates are one sixth as efficient in mediating *C. albicans* growth inhibition as the intact population, indicating an intact cell is required for maximum *C. albicans* growth inhibition.

C. Role of protease inhibitors in anti-fungal and anti-tumor activity

Contact and an intact cell are required for maximally efficient anti-fungal activity. Proteases are associated with contact-mediated cytotoxic activity for tumor cells. Therefore, three protease inhibitors were analyzed for their ability to inhibit the anti-tumor and anti-fungal activity of rIL-2 activated populations.

Figure 14 depicts the inhibition of anti-tumor and anti-fungal activity during treatment with the protease inhibitors, aprotinin 10%, PMSF 300 ug/ml, and TAME 10uM. Aprotinin produced a marked (p<0.05) decrease in activity for all targets examined when compared to rIL-2 activated populations. PMSF treated rIL-2 activated populations displayed numerically reduced activity for both *C. albicans* and P815 when compared to untreated rIL-2 activated populations. PMSF treated rIL-2 activated populations were unaffected in their activity for YAC-1. In contrast to PMSF and aprotinin, TAME treated rIL-2 activated populations showed no inhibition of anti-fungal or anti-tumor activity.
Figure 14.

Fungal growth inhibition and tumor cell cytotoxicity of rIL-2 activated population during treatment with protease inhibitors. rIL-2 activated populations and targets, as well as both C. albicans alone and rIL-2 activated populations alone were treated with aprotinin 10% (APRO), TAME 10uM (TAME), and PMSF 300 ug/ml (PMSF), or untreated (day 7). A. C. albicans growth inhibition was assessed by the incorporation of [3H] uridine. Data are presented as % inhibition and inhibitory units per 10^7 cells at 20% inhibition (IU). B. YAC-1 cytotoxicity is expressed as % cytotoxicity as judged by [51Cr] release and by lytic units per 10^7 cells at 20% cytotoxicity (LU). C. P815 cytotoxicity is expressed as % cytotoxicity as judged by [51Cr] release and by lytic units per 10^7 cells at 20% cytotoxicity (LU). Targets without effectors were treated with appropriate protease inhibitors and utilized to determine maximal incorporation of [3H] uridine for inhibition calculations. No differences in uptake of [3H] uridine were observed between treated targets without effectors and untreated targets without effectors (p>0.05), nor did treated effectors without targets display loss of viability as judged by trypan blue. *, (p<0.05) Significant difference between activity of aprotinin treated rIL-2 activated populations compared to untreated rIL-2 activated populations. Data are expressed as the mean ± S. D. for 3 independent experiments.
**Figure 14.**

A. *C. albicans*
- DAY 7: 104.8 ± 35.3 IU
- APROT: 0.0 ± 0.0 IU
- PMSF: 85.5 ± 23.6 IU
- TAME: 145.6 ± 81.2 IU

B. YAC-1
- DAY 7: 1979 ± 333 IU
- APRO: 78 ± 11 IU
- PMSF: 1645 ± 319 IU
- TAME: 1876 ± 234 IU

C. P815
- DAY 7: 162.5 ± 19.4 IU
- APRO: 14.5 ± 2.6 IU
- PMSF: 141.6 ± 16.7 IU
- TAME: 166.4 ± 14.3 IU
against either YAC-1, P815 or C. albicans as compared to the activity of untreated rIL-2 activated populations. The protease inhibitors were not directly cytotoxic.

pretreatment of rIL-2 activated populations with these protease inhibitors for 1 h failed to reduce the anti-fungal and anti-tumor activity (Data not shown).

These data demonstrate the anti-fungal and anti-tumor activity of rIL-2 activated populations to be affected by the serine protease inhibitor, aprotinin.

D. Effect of protease inhibitors on cellular sonicate and supernate activity

Sonicates and supernates treated with protease inhibitors from C. albicans stimulated populations were assessed for C. albicans growth inhibitory and anti-tumor cytotoxic activity either with or without the protease inhibitors PMSF (300 ug/ml) or aprotinin (10%) (Figure 11).

C. albicans growth inhibitory activity of rIL-2 activated population supernates (8.5 ± 2.5 IU) was not different when compared to supernates of rIL-2 activated populations treated with either PMSF or aprotinin. No difference in tumor cytotoxic activity was displayed by rIL-2 activated population cell supernates (14.3 ± 2.6 LU for YAC-1, 12.2 ± 2.3 LU for P815) compared to rIL-2 activated population supernates treated with either PMSF or aprotinin. Cellular sonicates of the rIL-2 activated population
Fungal growth inhibition and tumor cell cytotoxicity of cell supernates and sonicates during treatment with protease inhibitors. Cell sonicates (open symbols) at 50:1 to 6:1 equivalents and concentrated supernates (closed symbols) expressed at 200 ul to 50 ul, from C. albicans stimulated rIL-2 activated populations, were treated with aprotinin 10% ( , ) PMSF 300 ug/ml ( , ) or untreated ( , ). A. C. albicans growth inhibition was assessed by the incorporation of [3H] uridine. Data are presented as % inhibition and inhibitory units per 10^7 cells at 20% inhibition (IU). B. YAC-1 cytotoxicity is expressed as % cytotoxicity as judged by [51Cr] release and by lytic units per 10^7 cells at 20% cytotoxicity (LU). C. P815 cytotoxicity is expressed as % cytotoxicity as judged by [51Cr] release and by lytic units per 10^7 cells at 20% cytotoxicity (LU). Data are expressed as the mean ± S. D. for 3 independent experiments.
Figure 15.
demonstrated activity against all target populations (63.1 ± 9.3 IU for Ca. albicans, 105.5 ± 13.2 LU for YAC-1, and 115.2 ± 13.6 LU for P815). The protease inhibitors had no effect on the sonicates.

These data indicate that the protease inhibitor acts only against the intact cell and not any soluble factor released from the cell.
DISCUSSION

Fungal infections in general and \textit{C. albicans} infections in particular are important causes of morbidity and mortality in immunocompromised individuals. This study evaluated a novel mechanism by which to limit the development of fungal disease. It has been very difficult to understand the means by which the mammalian host deals with limiting the growth of fungal hyphae which reach lengths of 100 \( \mu \text{m} \) or larger, \textit{in vivo} (Soll, 1985). It is the hypothesis of this study that lymphocytes influence the growth of fungal hyphae. If true, then such cells may provide an important host defense mechanism by which fungi may be limited when normal defense mechanisms are disrupted. It was the purpose of this investigation to clearly identify the immunological nature, functional activity and laboratory conditions by which to generate anti-fungal effector lymphocytes. This identification will permit a direct assessment of the potential \textit{in vitro} biological activity of the lymphocyte cell population and will serve as the groundwork for a better understanding of the mammalian host response to \textit{C. albicans}.
A review of the literature shows that a correlation exists between in vivo resistance to *C. albicans*, the production of IL-2 and IFN gamma, and the genotype of specific inbred strains of mice (Ashman et al., 1990). In that study, resistance to *C. albicans* did not correlate with phagocytic cell activity of the murine strains (Marquis et al., 1986). Therefore, non-phagocytic cell populations from C57Bl/6 mice were evaluated for *C. albicans* growth inhibition. Due to the importance of the hyphal form of the fungus in disseminated infections, *C. albicans* growth inhibition was assessed against hyphae. Lymphoid cells with functional and phenotypic characteristics similar to "activated" T lymphocytes were found to inhibit the growth of the hyphal form of *C. albicans* (Figures 9-11). Lymphoid cells derived from mice and immediately assessed for hyphal growth inhibition showed little or no activity (Figures 1-2). This finding is consistent with previously published data (Djeu et al., 1988; Djeu and Blanchard, 1987). However, significant hyphal growth inhibition was observed when lymphoid cells were cultured with high concentrations of recombinant interleukin-2 (rIL-2) (Figures 3-7). Growth inhibitory activity was mediated by non-adherent lymphocytes which had the capacity to lyse the NK susceptible YAC-1 cell line and the NK resistant P815 cell line. Phenotypic marker selection showed the lymphocyte cell population to express Thy-1.2, Lyt-2.2, and ASGM-1. Other investigators have
failed to demonstrate lymphocyte mediated activity against *C. albicans* (Baccarini et al., 1983; Djeu and Blanchard, 1987). Those investigators have either used freshly isolated lymphocytes or lymphocytes activated with low concentrations of cytokines to inhibit *C. albicans* growth (Baccarini et al. 1983; Djeu and Blanchard, 1987). The results of this study are consistent with those previous observations but demonstrate that greater concentrations of rIL-2 are required to "activate" or enhance the capacity of lymphocytes to mediate growth inhibition of the fungus. With greater quantities of rIL-2 it is possible to "activate" lymphocytes, and/or expand a population of lymphokine activated cells which mediate fungal growth inhibition.

Multiple methodologies have been published with which to assess activity against *C. albicans* including; $^{51}$Cr release from the fungus, and direct microscopic examinations (Baccarini et al., 1985; Hashimoto, 1983). Although accepted methodologies, neither permits easy assessment of large sample numbers. Therefore, an alternative method was developed by which to measure growth inhibitory activity. This procedure is based upon previously published methodology (Yamamura et al., 1977), but includes an extremely important additional step which enzymatically removes the adherent *C. albicans* from plastic surfaces. The enzyme employed is lyticase. Lyticase cleaves beta 1,3
linkages of glucose, which are found in glucan, a major constituent of the cell wall of *C. albicans*. Alteration of the cell wall by lyticase reduces the binding capacity of *C. albicans* to plastic (Kennedy *et al.*, 1989; Hazen, 1989), and allows for easy harvest of the fungi with a PhD cell harvester. Therefore, large numbers of experimental samples can be processed in a manner similar to the harvesting procedure for lymphoproliferation assays. The assay employed measures the incorporation of $[^3\text{H}]$ uridine by *C. albicans* as an index of fungal cell growth. Very little is known about the metabolism of RNA during *C. albicans* growth. Wain *et al.* (1976) demonstrated that the RNA content, as measured by $[^3\text{H}]$ uridine uptake by *C. albicans*, increases continuously throughout the cell cycle. The predominant form of RNA found in *C. albicans* is ribosomal (Anderson and Soll, 1984). No measurement of messenger RNA synthesis have been reported. Nevertheless, following the conclusion of the three hour co-incubation period, $[^3\text{H}]$ uridine uptake is at a steady state of accumulation (Soll, 1985). The inhibition of $[^3\text{H}]$ uridine uptake directly reflects alteration of normal *C. albicans* growth (Soll, 1985). The assay system utilized takes advantage of this steady state incorporation of uridine and with the use of lyticase and the PhD cell harvester, is a simple, convenient and reproducible procedure. Furthermore, the obtained experimental results are similar to those of previously
published methodologies (Baccarini et al., 1985; Hashimoto, 1983; Beno and Mathews, 1990).

Anti-\textit{C. albicans} activity is presented as a percent inhibition of hyphal growth as described previously (Nabavi and Murphy, 1985). The hyphal form predominates in tissues infected with \textit{C. albicans} and is therefore considered to be the most relevant for examination. The assay utilized for assessment of growth inhibitory activity was developed to reflect inhibition within a three hour period. Whether the assay truly measures growth inhibition or is in fact a measure of direct cytotoxic activity is unknown. The decrease in countable disintergrations in the experimental samples might reflect cytotoxic activity. However this cannot be directly assessed by the results of this assay. The decrease in countable disintergrations can only be associated with a change in the steady state incorporation of \textsuperscript{3}H uridine. The decrease in radioisotopic incorporation has been associated with a decrease in RNA accumulation, protein synthesis, and cell proliferation (Anderson and Soll, 1984). Whether this is inhibition of the hyphal fragments resulting from damage of one or more of the individual segments of the organism or death of the entire organism is unknown. Regardless, the \textit{C. albicans} activity does not lead to complete destruction of the fungi in that countable disintergrations are incorporated in all experimental cases. Therefore, the lowered incorporation of
[\(^{3}H\)] uridine is defined as growth inhibition.

NK activity (as judged by anti-YAC-1 cytotoxicity) was present in murine splenocytes (Figure 1). In contrast, no *C. albicans* growth inhibition was detectable in murine splenocytes (Figure 1). These findings are consistent with previous studies which indicated no anti-*C. albicans* activity in murine splenocyte populations (Vecchiarelli et al., 1985; Zunino and Hudig, 1988). NK activity is associated with a morphological cell type, the large granular lymphocyte (LGL) (Ortaldo and Longo, 1988). LGL can be isolated in high purity from discontinuous Percoll gradients or enriched by passage over nylon wool columns (Hidore and Murphy, 1986). To enrich for NK activity, day zero splenocytes were subjected to discontinuous Percoll separation. Low density fractions which contained high concentration of LGLs showed a significant increase in NK activity when compared to the unseparated splenocyte population. No increase in *C. albicans* growth inhibition was observed with the LGL enriched cell population (Figure 2). Splenocytes were also depleted of adherent cells by passage over nylon wool columns as well as by adherence to plastic. NK enrichment and macrophage depletion by these procedures resulted in cells with no *C. albicans* growth inhibitory activity, despite an increase in NK activity when compared to untreated splenocyte populations. These data are consistent with previous studies indicating no
enrichment of C. albicans growth inhibition in cell populations enriched for NK activity (Djeu et al., 1988; Zunino and Hudig, 1988).

Previous studies using human LGL isolated from PBMCs failed to demonstrate C. albicans inhibitory activity following activation with low levels of rIL-2 (Djeu and Blanchard, 1987). rIL-2 activated human LGLs did not produce growth inhibition of C. albicans following activation with 100 U/ml rIL-2 for 18 h (Djeu and Blanchard, 1987). The current study found that murine splenocytes activated with 1500 U rIL-2 for 24 h showed no significant increase in C. albicans inhibitory activity despite a significant increase in NK activity (Figure 3). Splenocytes cultured for one day in culture medium without rIL-2 had no activity against C. albicans or P815 and the level of activity against YAC-1 was significantly lower than either day zero or day one. These data are consistent with previous findings demonstrating that splenocytes activated for a short time period with rIL-2 possess an enhanced NK activity but do not mediate C. albicans growth inhibition (Djeu and Blanchard, 1987). However, the results of this dissertation do demonstrate that with increased concentrations of rIL-2, the capacity to inhibit C. albicans is acquired by lymphocytes and is associated with the capacity to lyse NK sensitive and insensitive tumor cell lines (Figures 4, 5). These data are unique. No other
investigations have identified an enhanced *C. albicans* growth inhibition following rIL-2 activation.

This study sought to identify the length of time required for in vitro culture to acquire maximal *C. albicans* growth inhibitory activity. The appearance of the *C. albicans* growth inhibition coincided with the development of cytotoxic activity for the NK resistant tumor target, P815, and enhanced activity against the NK susceptible tumor target, YAC-1 (Figure 6). To identify the length of culture required for maximal cell recovery following activation with rIL-2, cell recovery and proliferation rate were evaluated. Splenocyte recoverability dramatically decreased following two days in culture with rIL-2 (Figure 7). Previous studies have demonstrated the loss of B lymphocytes and T helper lymphocytes over time in culture with rIL-2 (Peace et al., 1988). In the current study, as the recoverability decreased the rate of proliferation increased. Proliferation began at day two and peaked by day four. Proliferation failed to continue after day seven in this study. This finding is similar to the studies of Mule et al. (1987), who found no proliferation following eight days in culture with rIL-2. Splenocytes activated in rIL-2 for seven days were chosen for further study because both maximal cell numbers and anti-fungal activity were observed at this time period.

The extended culture period may permit the development
of a population with broad tumoricidal reactivity as evidenced by the concomitant appearance of cytotoxic activity for the NK insensitive cell line, P815. The development of this population could be the consequence of several factors. First of all, expansion of rIL-2 responsive populations which mediate C. albicans growth inhibition and anti-tumor activity may occur. Yang et al. (1986) found a concomitant decrease in the number of IL-2 unresponsive lymphocytes in such culture which paralleled an increase in the number of lymphocytes which were responsive to rIL-2. Alternatively, activation of an existing anti-fungal cell population may require an extended culture period for maximal activity. Studies by Merluzzi (1985) indicate that at least three days of rIL-2 activation are required for induction of anti-P815 activity with seven days required for maximal activity. Other studies of rIL-2 activation demonstrate that three to five days of culture are required for maximal tumoricidal activity (Grimm et al., 1982; Lafreniere et al., 1986). The results of this dissertation did not demonstrate an absolute requirement for the expansion of the rIL-2 activated population which mediates the C. albicans growth inhibition. However, the expansion of this populace cannot be dismissed due to the observed changes in cellular proliferation, cell recovery, and cellular phenotype during activation with rIL-2 (Figure 7, Table I). The results of this dissertation are
consistent with the activation of a rIL-2 activated population which mediates C. albicans growth inhibition.

Thus far this investigation has demonstrated that following extended activation with rIL-2, a population of lymphocytes arises which mediate C. albicans growth inhibition. The seven day rIL-2 activated cell population also mediates anti-tumor activity against both the NK-sensitive tumor cell line YAC-1, and the NK-insensitive tumor cell line P815. C. albicans growth inhibition does correlate with the acquisition of both anti-P815 and enhanced anti-YAC-1 activity suggesting that a MHC-unrestricted cytotoxic lymphocyte population is a portion of the cell population responsible for the observed C. albicans growth inhibition (Figures 3-5). Other studies of lymphocyte mediated anti-C. albicans activity have not observed anti-fungal activity because the requisite activation and expansion of the anti-fungal cell population was not permitted (Vecchiarelli et al., 1985; Zunino and Hudig, 1988). The current study demonstrates that seven days are required for maximal activation with 1500 U rIL-2.

The results of this study demonstrate that activation of splenocytes with rIL-2 augments not only the anti-tumor and anti-fungal activity of splenocytes, but also alters the phenotypic makeup of the cell population (Table I). Increased numbers of cells expressed the specific surface markers Thy-1.2, ASGM-1, Lyt-2.2, and NK 1.1 while cellular
expression of Ig and L3T4 decreased to background levels. This phenotypic marker expression has been demonstrated previously to be a consequence of rIL-2 activation (Yang et al., 1986).

Previously, rIL-2 activation of lymphocytes has been shown to induce MHC-unrestricted cytotoxic reactivity in vitro, which is mediated by lymphocytes expressing both Thy-1.2 and ASGM-1 phenotypic markers (Ballas et al., 1987; Merluzzi et al., 1986). Thy-1.2 is known to be expressed by lymphocytes with broad cytotoxic activity (Yang et al., 1986; Thiele and Lipsky, 1989) while ASGM-1 is present on a variety of cell types (Mule et al., 1989) and its expression has been shown to correlate directly with activation of MHC-unrestricted cytotoxic lymphocytes (Stout et al., 1987). Within the limits of experimental error, all seven day rIL-2 activated populations in this study expressed Thy-1.2 (Table I). In a similar manner, ASGM-1 was shown to be expressed by nearly all cells comprising the rIL-2 activated population (Table I). Therefore, ASGM-1 and Thy-1.2 are expressed simultaneously by the lymphocytes which comprise the rIL-2 activated population. This data is consistent with the scientific literature and the clear expression of both ASGM-1 and Thy-1.2 by the rIL-2 activated population.

The results of flow cytometric analysis (Table I) suggested the possible differential expression of Lyt-2.2
and NK 1.1 by cells within the rIL-2 activated population. Anti-NK 1.1 specifically identifies mature NK and other MHC-unrestricted cytotoxic lymphocytes in certain murine strains, including C57Bl/6 (Koo and Peppard, 1984), whereas antibody to Lyt-2.2 identifies cytotoxic T lymphocytes (Kalland et al., 1987). The current investigation sought to determine whether the anti-fungal activity segregated with a particular cellular phenotype within the rIL-2 activated population of cells. The differential expression of Lyt-2.2 and NK 1.1 was exploited to identify the phenotypic character of the rIL-2 activated population which mediates C. albicans growth inhibition. The results of those studies suggest that rIL-2 activated population mediating C. albicans growth inhibition express Lyt-2.2 and not NK 1.1 (Figures 6, 7, and 8). Differential expression of NK 1.1 and Lyt-2.2 within the rIL-2 activated population is similar to the studies of Peace et al. (1988) who showed LAK cells were divided into at least two mutually exclusive subsets, 1) a cytotoxic T lymphocyte (Lyt-2.2+, NK 1.1-) with MHC-unrestricted cytotoxic activity and 2) a NK (Lyt-2.2-, NK 1.1+) cell population with MHC-unrestricted cytotoxic activity. The results of this study show that these two separate and distinct subpopulations exist within the seven day rIL-2 activated population. The Lyt 2.2+ lymphocyte population mediates the anti-fungal activity of the seven day rIL-2 activated population.
This study has demonstrated anti-Lyt-2.2 and complement treatment to obliterate the growth inhibitory activity of the rIL-2 activated population for the hyphal form of \textit{C. albicans}. This treatment also reduced the lytic activity of the cell population for P815 but not YAC-1 (Figure 7). These data demonstrate the Lyt-2.2$^+$ population to be capable of activity against both \textit{C. albicans} and P815. NK 1.1 antibody and complement depleted cell populations mediated significantly (p<0.05) increased \textit{C. albicans} growth inhibition. Statistical analysis of the inhibitory units (IU) reveals significant differences between the cells treated with NK 1.1 and complement and complement alone, despite no apparent visual difference in the effector to target ratio data. In calculating the IU based on the lytic unit calculation, these data are significant because of the inclusion of the data at 6:1 effector to target ratio. The inclusion of this data alters the linear slope of the line which is used for IU calculation. Comparisons of NK 1.1 and complement treated cells with cells treated with complement alone reveal numerical differences in \textit{C. albicans} growth inhibition. These differences indicate that the loss of NK 1.1$^+$ cells results in an increase in \textit{C. albicans} growth inhibitory activity. These data calculated as IU also demonstrate that lymphocytes expressing the NK 1.1 determinant do not mediate the \textit{C. albicans} growth inhibition (Figure 7).
Although stringent conditions for antibody and complement mediated depletions were utilized, the completeness of these depletions may not be absolute (Mule et al., 1989; Kalland et al., 1987). Furthermore, antibodies used for such depletion may interfere with cellular functional activity (Peace et al., 1988). It is difficult, and perhaps impossible, to obtain a totally pure cell populations with any cell separation procedure, and it is even more difficult to accurately quantify the possible contribution of contaminating cells (Kalland et al., 1987). In this study, a small percentage of positively labelled cells were observed by direct fluorescent microscopy to remain in depleted cell populations (Table II). Thus, any remaining low levels of activity against *C. albicans* and P815 might be the result of residual cell populations not eliminated during complement-mediated depletions. However, the complement depletion studies do suggest that Lyt-2.2\(^+\) cells mediate *C. albicans* growth inhibition. Confirmation of these data was sought by positive selection of the same phenotypes.

Figure 8A demonstrates that the Lyt-2.2\(^+\) rIL-2 activated subpopulation, selected by magnetic antibody and anti-Lyt-2.2\(^+\), mediated significantly enhanced *C. albicans* growth inhibition when compared to unselected rIL-2 activated cell populations. Concomitant with the enhancement of *C. albicans* growth inhibition was an enrichment in
anti-P815 activity (Figure 8C). These data are consistent with the Lyt-2.2+ cell population mediating activity against both C. albicans and the P815 tumor cell line. No differences in anti-YAC-1 activity between the Lyt-2.2+ and Lyt-2.2- separated populations were observed (Figure 8B). Previous studies have demonstrated rIL-2 activated lymphocytes expressing Lyt-2.2+ to be capable of anti-YAC-1 cytotoxicity (Kalland et al., 1987; Gunji et al., 1989) as well as anti-P815 activity (Sensi et al., 1990).

The NK 1.1+ populations showed decreased anti-YAC-1 activity (Figure 8B). This is consistent with previous studies which demonstrate a majority of anti-YAC-1 activity resides within the NK 1.1+ population (Kalland et al., 1987). The absence of enriched anti-YAC-1 activity in the NK 1.1+ population is also consistent with previous studies which demonstrate both NK 1.1+ and Lyt-2.2+ populations to be capable of anti-YAC-1 cytotoxicity (Peace et al., 1988; Gunji et al., 1989). Figure 8A shows decreased C. albicans growth inhibition by the NK 1.1+ subpopulation selected by magnetic antibody compared to that of the Lyt-2.2+ selected subpopulations. NK 1.1+ selected populations showed significantly decreased C. albicans growth inhibition when compared to untreated rIL-2 activated populations. Concomitant with the decrease in C. albicans growth inhibition activity was a decrease in anti-P815 activity (Figure 8C). These data are consistent with the Lyt-2.2+ cell population
mediating activity against both *C. albicans* and the P815 cell line. No anti-fungal activity was observed for NK 1.1+ selected cells.

Lyt-2.2+ expressing cells have been demonstrated to mediate anti-P815 activity (Sensi et al., 1990). This finding is confirmed by the current study. However, the lack of NK 1.1+ mediated anti-P815 activity was surprising. Studies have demonstrated NK 1.1+ pan-cytotoxic activity (Ballas and Rasmussen, 1990). Those investigations employed the NK resistant targets, EL-4 and CL27a but not the P815. The lack of NK 1.1+ effector activity against P815 may reflect differences between these distinct target cell populations. However, the data unequivocally demonstrates the NK 1.1+ cell population to be unable to mediate anti-P815 activity (Figure 11).

The magnetic selection of rIL-2 activated subpopulations was completed on the sixth day of activation with rIL-2. This methodology was necessitated by the nature of the selection process. The major consideration was the possible inhibition of biological activity due to stearic hinderance by antibody-magnetic bead complexes on the cell surface. The differential functional activities observed with subpopulations separated magnetically attests to the appropriateness of the approach and its' superiority to that of complement-mediated, antibody depletions. Complement-mediated cell lysis, as compared to magnetic separation,
while based on the same spectrum of available antibodies is more time consuming, is restricted to cell depletion, and is less efficient with respect to the viability and purity of the resulting subpopulation (Pflueger et al., 1990). Therefore, populations selected by the magnetic separation technique were a more reliable index of biological activity.

The combination of data from Figures 6, 7, and 8 demonstrates that Lyt-2.2 and NK 1.1 are not expressed on the same cells within the seven day rIL-2 activated cell population. Both the total percentage of cells expressing each marker in the rIL-2 activated population along with the concurrent increase and decrease in the antibody treated populations argues for separate populations of Lyt-2.2+ and NK 1.1+ within the total Thy-1+ and ASGM-1+ population. Although a minor population expressing both Lyt-2.2 and NK 1.1 may exist. The *C. albicans* growth inhibition activity resides in the rIL-2 activated population which expresses ASGM-1, Thy-1.2 and Lyt-2.2, but not NK 1.1.

These results are consistent with one cell population mediating both an anti-*C. albicans* and an anti-P815 activity as well as an anti-YAC-1 activity. To assess whether the anti-fungal effector cells within the rIL-2 activated population also bind tumor targets, cold target inhibition studies were performed. Homologous cold targets mediated complete inhibition of growth inhibitory activity of *C. albicans* as well as cytotoxic activity against tumor targets.
(Figure 13). Heterologous cold targets inhibited biological activity for either tumor or fungal targets in a competitive and reciprocal manner. These data provide evidence that the rIL-2 activated population possesses the ability to recognize and bind all three targets. The absolute resolution of whether a population within the rIL-2 activated population mediates activity against both tumor targets and C. albicans will require the preparation of a cloned rIL-2 activated population. At this point, no known functional clone with similar activity to rIL-2 activated population is available and attempts to obtained cloned populations have not succeeded.

The findings of this study suggest recognition of targets, as judged by incomplete and varying levels of cold target inhibition, is not identical. However, similarities in recognition and binding do exist. The extent of inhibition of C. albicans activity was greater by P815 than YAC-1 suggesting the rIL-2 activated population which binds C. albicans is more reactive with P815 than with YAC-1. The greater inhibition by P815 might not necessarily reflect variation in target recognition, but rather might be a binding characteristic of the rIL-2 activated population. The rIL-2 activated population which mediates C. albicans growth inhibition might be more adhesive for certain tumor targets than cell populations which do not mediate C. albicans growth inhibition. The phenotyping data indicates
at least two populations possess activity against YAC-1, therefore the incomplete inhibition mediated by YAC-1 might occur because both subpopulations bind YAC-1 while only one subpopulation of the rIL-2 activated population binds \textit{C. albicans} and YAC-1. Isolation of the Lyt-2.2\textsuperscript{+} population and assessment of cold target inhibition is required to assess this hypothesis. The reciprocal inhibition of anti-YAC-1 activity by \textit{C. albicans} indicates partial recognition of \textit{C. albicans} by a portion of the rIL-2 activated population which mediates activity against YAC-1. The inhibition is not complete, further suggesting other rIL-2 activated populations, besides those reactive with \textit{C. albicans}, mediate activity against YAC-1. P815 partially inhibits YAC-1 activity, which is consistent with one of the two cytotoxic populations showing activity against YAC-1. The \textit{C. albicans} inhibition of YAC-1 activity is less than that of P815 competitive inhibition. The finding of stronger inhibition by P815 than \textit{C. albicans} suggests more similarity between the tumor targets than the fungal/tumor interaction. Alternatively, effector to target binding might occur through different cell surface receptors. Cold target inhibition studies of anti-P815 activity revealed that YAC-1 almost completely inhibits cytotoxic activity, suggesting recognition of both tumor populations by rIL-2 activated populations. The above data indicate that the rIL-2 activated population which mediates \textit{C. albicans} growth
inhibition possesses the ability to bind tumor targets. However, the binding of this population to tumor targets is not as easily inhibited with *C. albicans* as with tumor competitors.

The incomplete cold target inhibition observed by heterologous targets may be explained by several factors. The lower inhibitory activity of *C. albicans* tumor target inhibition may result from the heat inactivation of *C. albicans*. Affected heat labile receptors (Bouchara *et al.*, 1990) involved in recognition might alter binding of *C. albicans*. Alteration of receptors utilized in binding might lower the potential binding activity of the hyphae thus decreasing surface receptors, leading to less cold target inhibitory capability. Killing the *C. albicans* was necessitated for several reasons. First of all, live *C. albicans* possess the ability to incorporate released [51Cr] from the target cells. This would alter the amount of countable [51Cr] in the supernate thereby leading to a false diminution of cytotoxic activity. Secondly, *C. albicans* growth would alter the effector to target ratio during the assessment of cytotoxicity. Another consideration is the possible release of factors by *C. albicans* which may alter the binding and/or cytotoxic mechanism of tumor activity. Incomplete inhibition of *C. albicans* activity by cold tumor targets may also result from the characteristic growth of *C. albicans* in culture. The growth and elongation of *C.
*albicans* is accompanied by expansion of the hyphal length in culture. Growth increases the total surface area of the hyphae, allowing expression of more receptors for which rIL-2 activated populations may bind and mediate anti-fungal activity. As the assay continues the targets become larger, providing more binding sites and in effect, additional targets. Alternatively, the adherence of the hyphae on the plastic surfaces may provide a mechanism which allows preferential binding of rIL-2 activated populations to *C. albicans* rather than the tumor targets. Seeded and anchored *C. albicans* might preferentially enhance rIL-2 activated population binding. Another possible explanation is the construction of the *C. albicans* hyphae. It consists of separate organisms within the hyphae joined by an incomplete septum (Soll, 1985). Multiple hits on each hyphae may be required for growth inhibitory activity. This may force the rIL-2 activated population to remain longer on each hyphae before growth inhibition is observed. Furthermore, the mechanism of inhibition may require a longer time period before growth inhibition is observed and once bound, rIL-2 activated population may not dissociate from the cell wall and locate another target as quickly as in a tumor system. The incomplete inhibition of the fungus by rIL-2 activated populations affecting tumor targets could be the result of these physical and kinetic differences in *C. albicans*-rIL-2 activated population binding compared to tumor cell-rIL-2
activated population binding. In contrast to the simple plasma membrane where direct rIL-2 activated population-tumor cell interactions occur, the outer surface of fungi is substantially more complex. Studies of NK-cryptococcal interactions have shown NK-cryptococcal binding requires two hours prior to initiation of anti-fungal activity while NK-YAC-1 binding require only 20 minutes (Murphy, 1989). Furthermore, Cryptococcus neoformans conjugate formation is smaller, does not appear to interdigitate, and is generally much less intimate than is the broad area of interdigitating membrane contact observed in NK-tumor cell conjugates (Hidore et al., 1990). The interaction of the rIL-2 activated population with *C. albicans* might also require a longer time period for binding and initiation of anti-fungal activity thereby leading to the reduced competitive inhibition observed with *C. albicans*

The inhibition of biological activity by heterologous targets is statistically significant and demonstrates that both *C. albicans* and tumor cells competitively and reciprocally inhibit the activity of the rIL-2 activated population. These data demonstrate that an effector population within the rIL-2 activated population recognizes and mediates activity for two diverse target cell populations, *C. albicans* and tumor cells. Previous studies of fungal interactions of both Cryptococcus neoformans (Hidore et al., 1990) and *C. albicans* (Zunino and Hudig, 1988) have
demonstrated the ability of fungi to cold target inhibit NK activity. The current study does not imply similar mechanisms of binding, it does suggest that rIL-2 activated population possess the ability to bind all three targets in a competitive manner. Furthermore, the activity of the rIL-2 activated population is inhibited by each of these targets, suggesting that the rIL-2 activated population mediates activity against each of these target populations. The ability to bind all three targets, combined with proven activity against all three targets suggests a broad recognition and reactivity of the rIL-2 activated population.

The mechanism of \textit{C. albicans} growth inhibition is either mediated by direct cell contact or indirectly through secreted factors. The demonstration of recognition and binding of rIL-2 activated populations to \textit{C. albicans} suggests the anti-fungal activity to be contact mediated. Therefore, this became the hypothesis for a series of experiments. A method for limiting cell contact between effector and target cells, while allowing interaction of soluble factors was unavailable. Therefore, an assessment of absolute contact could not be accomplished.

A series of experiments was conducted to assess indirectly the role of cell contact in \textit{C. albicans} growth inhibition. The first of these experiments examined whether cellular supernates from rIL-2 activated populations
mediated growth inhibition against *C. albicans*. Unconcentrated culture supernates from rIL-2 activated populations possessed no growth inhibitory activity for *C. albicans*. Culture supernates were concentrated 75 fold and assessed for *C. albicans* growth inhibition (Figure 9). rIL-2 activated populations were stimulated with *C. albicans* in an attempt to induce the release of growth inhibitory molecules. Stimulated and concentrated supernates possessed a greater anti-fungal activity than unstimulated and concentrated supernates. However the activity was markedly less than that of the intact cell population (*p*<0.05). The stimulation of rIL-2 activated populations with *C. albicans* increased growth inhibition, but compared to intact cells, the inhibitory activity was negligible. These data are consistent with the hypothesis that contact is required for maximal *C. albicans* growth inhibition. Secreted molecules are not the most efficient mechanism of anti-fungal or anti-tumor activity (Theile and Lipsky, 1989; Murphy, 1989).

Minimal *C. albicans* growth inhibition by cell supernates suggests that growth inhibitory activity is most efficient when cell associated. Previous findings have demonstrated that LAK cells release membrane-damaging agents which remain cytotoxic for glial cells outside the contact area of effector target conjugates (Jaaskelainen *et al.*, 1990). However tumoricidal studies similar to this dissertation failed to demonstrate nonspecifically released
cytotoxins which generate comparable levels of activity to intact effector populations (Thiele and Lipsky, 1989).

The lack of supernate activity may be a consequence of a number of factors, e.g., the process of concentrating the freshly harvested supernates required three hours. Proteases may have destroyed functional activity of possible secreted cytotoxins in the fractions (Theile and Lipsky, 1988). Alternatively, the target cell, C. albicans, may be induced by the concentrated supernates to release a variety of proteolytic enzymes (Wilton and Lehner, 1981). The proteolytic enzymes may act in a protective manner to inactivate anti-C. albicans factors which are present within the concentrated supernate (Domer, 1989).

The concentration of rIL-2 within the media is not inhibitory to the measured activity since concentrated complete media containing rIL-2 failed to inhibit growth (Figure 13A). However, the activation of cells over seven days with rIL-2 could induce inhibitory molecules which reduce the growth inhibition of C. albicans. Such inhibitors might remain functional during the concentration process and inhibit C. albicans growth inhibition. These conditions may prevent supernates from demonstrating maximal C. albicans growth inhibition.

The results from these studies demonstrate that cell-free supernates concentrated 75 fold from rIL-2 activated populations stimulated with C. albicans were capable of
mediating anti-fungal activity, but at a much reduced rate when compared to intact rIL-2 activated populations.

The failure of cellular supernates to mediate comparable levels of *C. albicans* inhibitory activity to intact rIL-2 activated populations, lead to the next experimental series which assessed the requirement for an intact cell population to mediated *C. albicans* growth inhibition. rIL-2 activated populations were disrupted by sonication until cells were <99% viable as judged by trypan blue exclusion. Of primary interest was whether the disrupted cells possessed the ability to mediate the fungal growth inhibition. Therefore, rIL-2 activated populations used for the sonication experiments were placed in assay media at concentrations identical to those for assessment of *C. albicans* growth inhibition without sonication. Following sonication, equivalent starting numbers of sonicated rIL-2 activated populations to control rIL-2 activated populations were assessed for *C. albicans* growth inhibition. The sonicated cells of both *C. albicans* stimulated and unstimulated populations produced activity against fungal and tumor targets. However only one sixth of the *C. albicans* growth inhibition produced by the intact effector population was mediated against *C. albicans* by sonicated cells. The failure of sonicated cells to mediate identical levels of growth inhibitory activity against *C. albicans* suggests that the mediator(s) of *C. albicans* growth
inhibition are less effective at inhibiting *C. albicans* than an intact cell. Previous studies of tumoricidal activity have demonstrated granule extracts to be less efficient with respect to cytotoxicity than intact cells (Henkart *et al*., 1987; Hayes *et al*., 1989). The limited activity of granule extracts may be a consequence of dilution in that the activity in solution may be less than that of the intimate surroundings of the microenvironment formed by cell to cell contact. This effect is overcome by the polarized secretory mechanism that delivers a high local concentration of the active mediators to the appropriate target site (Hayes *et al*., 1989). Henkart *et al*., (1987) demonstrated that highly purified granules possess higher tumor cytotoxic activity than the activity from comparable numbers of unpurified cellular extracts. The activity of the cellular extracts was less than the activity of the intact cells. Furthermore, studies of purified NK granules revealed higher activity against the fungal target *Cryptococcus neoformans* than those of similarly treated unpurified extracts (Hidore *et al*., 1990).

Limited activity of sonicated rIL-2 activated populations in the current study could result from the methodology employed. For example, the process of sonication may inactivate any *C. albicans* growth inhibitory molecules. Furthermore, despite the presence of ice during sonication, the increase in temperature may inactivate the
inhibitory factors. The release of *C. albicans* inhibitory molecules from the stimulated rIL-2 activated populations which would otherwise possess enhanced activity might lower the potential inhibition. Furthermore, remaining *C. albicans* from the stimulation could alter the results in several ways. Remnants of *C. albicans* sonicated with the rIL-2 activated populations may act in a cold target manner to bind inhibitory molecules and lower the level of observable inhibition. Additionally, the sonicated *C. albicans* may release a variety of factors which may bind or inhibit the inhibitory molecules of rIL-2 activated populations. These conditions may prevent cell sonicates from demonstrating maximal *C. albicans* growth inhibition.

Cell sonicates from rIL-2 activated populations stimulated with *C. albicans* were capable of mediating both anti-fungal and anti-tumor activity, but at a much reduced rate when compared to intact rIL-2 activated populations. The finding that rIL-2 activated populations bind *C. albicans* (Figure 12), coupled with the absence of activity with sonicates (Figure 13A), and the failure of released mediators to affect fungal growth, suggests, but does not prove, that cell contact is required for maximal activity against *C. albicans*.

Based on the above findings, the implication of contact mediated activity was further assessed by the use of protease inhibitors. Cell surface associated proteolytic
enzymes (serine proteases and esterases), have been implicated in the lytic phase of the tumor cell cytotoxicity reaction (Lavie et al. 1985). Complex cellular mechanisms triggered by the contact and binding of the target cell via the receptor are involved in cellular cytotoxicity. Following recognition of a given target cell, the effector cell has the ability to activate any or all of several proteases which induce the cytotoxic mechanism. Protease inhibitors are known to diminish cell-mediated cytotoxicity against tumor cells (Jenne and Tschopp, 1987). Therefore, the effect of such inhibitors on rIL-2 activated population mediated C. albicans growth inhibition was examined. Proteases are generally regarded to require cell contact for functional activity (Jenne and Tschopp, 1988), therefore, the reduced C. albicans growth mediated by protease inhibitors in this dissertation implicates a contact requirement for maximal C. albicans inhibitory activity.

Representative protease inhibitors were selected for examination because of their differential ability to inhibit cytotoxic interactions of tumor targets by various effector populations (Lavie et al., 1985; Jenne and Tschopp, 1988; Scuderi, 1989). TAME, aprotinin, and PMSF were assessed. The concentrations utilized reflect maximum inhibition following titer evaluation and did not affect incorporation of [3H] uridine by C. albicans or affect the viability of the rIL-2 activated population utilized for calculation of
Aprotinin inhibited rIL-2 activated cell mediated *C. albicans* growth inhibition as well as tumoricidal activity against all targets examined (Figure 10). Aprotinin had a lesser capacity to diminish activity against YAC-1 than it did against other target cell populations. This could be the result of a differential effect on cell populations with activity against YAC-1. Aprotinin is a very stable molecule which inhibits a variety of enzymes, e.g., trypsin and chymotrypsin. Aprotinin binds to the active sites of the enzyme, forms a tight complex and renders the enzyme inactive (Hewlett, 1990). One model of aprotinin-mediated inhibition is through esterase modification in cytoplasmic granules (Hayes *et al.*, 1989). The modification of the esterase interferes with the regulation of the cytotoxic machinery and exocytosis of cellular constituents there by inhibiting cytotoxicity (Hayes *et al.*, 1989; Lavie and Zucker-Franklin, 1989). In the current study, the *C. albicans* inhibitory activity as well as the tumor cytotoxic activity is not completely eliminated by the effect of aprotinin. This may be as a result of incomplete saturation within the area of cell contact. However, concentration dependant evaluations failed to lower the remaining activity. Alternatively, the lack of inhibition may also be due to other cytotoxic mechanisms which are not inhibitable...
by aprotinin. Such mechanisms might be responsible for the residual \textit{C. albicans} growth inhibition of the rIL-2 activated populations.

PMSF inhibited the activity of rIL-2 activated populations against P815 and \textit{C. albicans}, but not YAC-1. PMSF has been reported to inhibit activity of both T cells and NK cells (Jenne and Tschopp, 1988). PMSF has been demonstrated to inhibit target cell DNA release by inhibiting a serine protease activity that resides in a low pH subcellular compartment, believed to be granzyme A (Hayes \textit{et al.}, 1989). Previous studies by that group have also demonstrated that PMSF substantially inhibits BLT esterase activity (Henkart \textit{et al.}, 1987). Whether PMSF is inhibiting either of these proteases in this study is unknown. The ability of PMSF to numerically inhibit growth of \textit{C. albicans} and cytotoxic activity against P815 but not YAC-1, does suggest a similar population or mechanism of activity is active in mediating anti-\textit{C. albicans} and anti-P815 activity, but not necessarily anti-YAC-1 activity. The inability of PMSF to reduce activity against YAC-1 might result from other cytotoxic populations which are not sensitive to PMSF. PMSF is slower to react with active sites of enzymes than aprotinin and is very susceptible to spontaneous hydrolysis and loss of activity (Gebhard \textit{et al.}, 1986). The activity of this relatively unstable molecule may have been altered during use and may contribute to the limited activity.
TAME failed to mediate any effect against either tumor targets or \textit{C. albicans}. While TAME may inhibit other proteases it has been specifically shown to inhibit the release of TNF from rIL-2 activated lymphocytes (Scuderi, 1989), and TNF mediated cytotoxicity (Suffys \textit{et al.}, 1988). Therefore, TNF most likely is not involved in the mechanism of growth inhibition of \textit{C. albicans}. This was not unexpected, for previous reports have shown TNF activity requires a longer time frame for tumor cytotoxic activity than the short term (four hour) assays utilized in these studies (Old, 1985; Havell \textit{et al.}, 1988). Furthermore, previous studies have failed to demonstrate anti-\textit{C. albicans} activity of TNF (Djeu \textit{et al.}, 1988). The observations with TAME are consistent with those findings.

Effector cells pretreated and washed of protease inhibitors failed to alter the normal cytotoxic and inhibitory activity of rIL-2 activated populations. These findings are supported by previous studies (Lavie \textit{et al.}, 1985). These findings are also consistent with the model proposed by Lavie in which surface-associated proteolytic enzymes that are concealed from the external environment become exposed during cytolysis and participate in target cell damage (Lavie \textit{et al.}, 1985). The model and results suggest that only within the contact microenviroment are the protease inhibitors active, further implying contact may be essential to the activity of rIL-2 activated populations.
This dissertation has demonstrated that \textit{C. albicans} growth is inhibited by a population of rIL-2 activated lymphocytes. According to Granger and Hobbs (1988), demonstration of the following criteria are required for indisputable proof that a cell population functions in defense against a fungal agent: 1) a recognition step occurs in which the effector cell forms a conjugate with the target cell; 2) contact-dependent interaction results in target cell killing or growth inhibition; 3) purified effector or cell molecules are cytotoxic or cytostatic for the microorganism; 4) experimental animals made specifically deficient in the effector cells show enhanced susceptibility to the microorganism; and 5) enhanced host susceptibility can be abrogated by adoptive transfer of effector cells.

By these criteria, several sets of experiments must be completed prior to absolute proof that rIL-2 activated populations mediate anti-fungal activity. Upon the completion of such studies, the role of rIL-2 activated populations in \textit{C. albicans} inhibitory activity will be answered. Until such time, the function of rIL-2 activated populations in mediating \textit{C. albicans} growth inhibition remains speculative.

The definition of the rIL-2 activated population as a biological activity and/or function against the hyphal phase of \textit{C. albicans} does not address its immunological nature. These studies strongly suggest that the rIL-2 activated
population is not an NK or an activated NK population due to the failure of these populations to mediate C. albicans growth inhibition (Figures 1, 2, and 3). Additionally, rIL-2 activated lymphocytes expressing NK 1.1 fail to mediate C. albicans growth inhibition (Figure 11). In contrast, rIL-2 activated populations do possess several characteristics of rIL-2 activated cytotoxic lymphocytes including; broad spectrum target cell activity, phenotype, and LGL morphology. The phenotypic expression of Thy-1.2+ and ASGM-1+ within the rIL-2 activated population, and Lyt-2.2+ within the rIL-2 activated population directly parallels LAK phenotypic expression (Mule et al., 1989). rIL-2 activated populations are clearly a lymphokine activated cell population. Whether they are a lymphokine activated killer cell (LAK) population or a subset of LAK is unclear. However, by essentially any definition, the anti-tumor activity of the lymphocyte population is LAK (Merluzzi et al., 1986; Mule et al., 1989). Various studies have revealed substantial heterogeneity in LAK cell activity following activation with rIL-2. The lytic activity of various types of LAK and their apparent specificity are highly dependent upon their state of activation. rIL-2 may alter the reactivity of LAK populations, and the high levels of rIL-2 utilized in this study may induce the C. albicans growth inhibition. No reports have previously demonstrated C. albicans growth inhibition or anti-fungal activity for
LAK populations.

The data presented in this study are the first to show that rIL-2 activation of splenocytes results in a population of lymphocytes capable of affecting the growth of \textit{C. albicans} \textit{in vitro}. This study is consistent with previous investigations (Vecchiarelli \textit{et al.}, 1985; Djeu and Blanchard, 1987) but demonstrates that higher concentrations of rIL-2 are required to obtain a splenocyte population capable of affecting the growth of the fungus \textit{in vitro}. Whether this phenomenon functions \textit{in vivo}, as a form of host defense, has yet to be established. It is possible that the elaboration of rIL-2 or other cytokines and the concomitant induction of activated lymphocytes may indeed serve as a line of defense against \textit{C. albicans}. Protection of the mammalian host against this form of fungal disease may at least in part account for the resistance of the C57Bl/6 mouse to challenge with the microorganism. Furthermore, these cells may serve as potential effectors during the development of fungal infections and may be considered as a possible therapeutic means by which to limit fungal infections in immunocompromised individuals.
CONCLUSIONS

1) Splenocytes activated with rIL-2 inhibit the growth of fungi, *in vitro*. They are CD8+ lymphocytes which must be activated with rIL-2 to exhibit fungal growth inhibition.

2) Optimal growth inhibition of fungi is mediated by intact lymphocytes, through a surface-active, serine protease.

3) Growth inhibition of fungi does not appear to be mediated by natural killer cells or activated natural killer cells.

4) *C. albicans* growth inhibition by rIL-2 activated cell populations is associated with enhanced tumoricidal activity against the NK resistant cell line YAC-1 and the NK resistant cell line P815, suggesting broad reactivity for heterogenous targets by rIL-2 activated cell populations.

5) The rIL-2 activated population which possess *C. albicans* growth inhibitory activity does not
necessarily possess anti-P815 activity despite correlative phenotypic, binding and cell associated protease susceptibility.
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