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Comparative Extracellular Killing of Candida and Tumor Cells by Macrophages at Varying Stages of Activation

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COMPARATIVE EXTRACELLULAR KILLING OF *CANDIDA* AND TUMOR CELLS BY MACROPHAGES AT VARYING STAGES OF ACTIVIATION

by

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CHAPTER I

INTRODUCTION

Macrophages play a vital role in the mammalian host immune response against a multiplicity of invasive agents including neoplasms (80), bacteria (87), protozoa (86), and fungi (79). The macrophage is capable of an extensive range of responses to these agents. The type of response is dependent upon the size and type of target. Targets such as tumor cells or large microorganisms are attacked in an extracellular manner while most bacteria, yeast, and small protozoans are phagocytosed and destroyed within the macrophage (2). Comparisons of extracellular tumoricidal activity and intracellular bactericidal activity of macrophages have been found to be highly dependent upon method of activation, target type, target state and duration of the experiment (41). Different populations of activated macrophages which have been defined by one functional criterion do not always possess other functional characteristics of activated macrophages (130). This has been shown in studies where different levels of macrophage cytocidal activity were observed for extracellular tumor cell killing versus intracellular fungal

(114) or parasite killing (88,95).

The purpose of this investigation has been to compare the extracellular fungicidal and tumoricidal activities of four macrophage groups. The groups were defined as described previously (82). These groups of macrophages were assessed for tumoricidal activity via release of ^{51}Cr from EL-4 tumor cells and for fungicidal activity by inhibition of yeast formation following induction of Candida albicans hyphae. Both of these assays measure the ability of macrophages to kill target cells in an extracellular manner (16,46). Such a comparison of extracellular cytocidal activity for both Candida and tumor cells, by identical groups of macrophages, has not been reported previously. In this study a correlation has been demonstrated between the acquisition and loss of extracellular tumoricidal and fungicidal activity by these four groups of macrophages.

CHAPTER II

LITERATURE REVIEW

C. albicans, is an opportunistic, dimorphic fungus commonly found as part of the normal flora of many mammals, including man. Diabetes, neoplasms, immunological defects, and immunosuppressive therapy are factors which favor the pathogenic invasion of Candida and the development of systemic infection. The cellular immune system is believed to be of vital importance in the host response to a Candida infection. As an example, it is well known that disseminated candidiasis occurs at a high frequency in patients with chronic granulomatous disease or myeloperoxidase deficiency. The antimicrobial capacities of phagocytic cells are impaired in these patients resulting in increased infections (69). Therefore, cells of the phagocytic lineage are regarded as being critical in the host defense against systemic candidiasis. The phagocytic response occurs despite the fact that invasive lesions caused by C. albicans characteristically contain hyphal forms which are typically too large to be ingested. Due to the large size of these hyphal fragments, phagocytic cells need to kill through an extracellular mechanism (26).

Cells of the phagocytic lineage are critical for host defense against fungi as well as neoplastic cells. Multiple fungicidal and tumoricidal mechanisms operate in normal monocytes (2,69). Damage to hyphae has been largely attributed to products of oxidative metabolism, although nonoxidative bactericidal activity of monocytes against Candida has been reported and may be important in some situations (25). Additionally, macrophages serve many roles in the immune response including; antigen processing, immune regulatory substance section, and the modulation of immune responsiveness (102). As a function of immune responsiveness, macrophages also have the ability to bind, react with, and kill neoplastic cells. This cytocidal event also occurs in an extracellular manner and is potentially mediated by various macrophage factors. Tumoricidal effects may be mediated by either oxidative or non-oxidative mechanisms. Whether the macrophage killing mechanism for Candida and neoplastic cells is identical remains unknown and will be discussed below.

Macrophages are known to possess varying capabilities to respond to stimuli. These variances in response to the stimuli are referred to generally as "activation states" or "levels of activation." An evaluation of the comparative extracellular killing ability of macrophages at various levels of activation

is the purpose of this study.

A. Macrophage activation

1. Levels of activation.

Macrophages play a major role in a wide variety of cellular defense mechanisms. Performance of these functions requires the simultaneous presence of effective activation signals and competent mononuclear phagocytes (83). These functional abilities are not a constitutive property of all mononuclear cells, rather such cells acquire these functions in response to various signals present in the immediate environment (44). Furthermore, macrophage-mediated resistance is localized, short-lived, and only regenerated by reinfection of the host (96).

The monocyte/macrophage maturation sequence can be divided into four different phases in accordance with the work of Meltzer: I. Precursor, II. Primed, III. Triggered, and IV. Postcytolytic (82). In vivo this maturation or differentiation sequence occurs in the following manner: Phase I: Macrophages which are recruited and differentiated from immature blood-derived mononuclear phagocytes into competent lymphokine-responsive cells by factors generated at the site of inflammation. Phase II: Inflammatory macrophages which have entered a priming stage in response to immuno-

modulating factors, during which they are not yet cytotoxic for infectious agents or tumor cells. Phase III: Macrophages which have developed full functional activity via a triggering signal and are cytotoxic for infectious agents or tumor cells. Phase IV: Macrophages which had been functionally active, but have now returned to a functionally quiescent level. These phases of macrophage activation have been also described as I. Resident, II. Elicited, III. Activated, and IV. Postcytolytic (82). Such phases have been characterized extensively *in vitro*, and will be discussed further using this terminology.

I. Resident

Resident macrophages are cells present at any given site in the absence of an exogenous or inflammatory stimulus (85). Resident macrophages do not constitute a stable cellular population, but rather are constantly renewed by an influx of blood-derived monocytes and the continuous division of immature monocytes (125). Complete turnover of the macrophage population varies between one and two weeks. Migration of circulating monocytes into a tissue site provides for the majority of new cells, since dividing cells account for only 35% of the total cellular population (22).

II. Elicited

The term, "elicited", indicates an accumulation

of exudate macrophages at a particular site. Elicited macrophages are attracted to a given site by certain stimuli. Investigations of *in vitro* macrophage activation have normally been carried out with macrophages obtained 3-4 days after induction of inflammation in the peritoneal cavity (65). These cells are a heterogenous population composed of resident macrophages and those macrophages derived from emigrating blood monocytes responding to the inflammatory stimulus (22). Such induction also results in the accumulation of other cell types, such as lymphocytes, which have the potential for modulating the functional activity of macrophages.

III. *Activated*

The term "activated" macrophage was originally coined by Mackaness to define the enhanced non-specific resistance to intracellular bacteria observed in animals recovering from infection with *Listeria monocytogenes* (125). Activated macrophages are cells with increased functional activity induced by a given stimulus which either increases one or more of the functional activities of the cell or induces a new functional cellular activity. The process of macrophage activation is not the result of a single interaction between the signal and cell, but appears to be the final step of an activation process which requires a defined progressive sequence of reactions for complete cellular activation

(82,83,105).

IV. Postcytolytic

Macrophages which have been activated for a prolonged period of time lose their cytocidal capabilities. These macrophages have been maximally stimulated previously and have returned to a normal or resident level of cytocidal activity. These cells are in a terminal maturation stage, yet still maintain responsiveness to proper stimuli, and can return to a cytocidal state (82).

The pathway of macrophage differentiation leading to the acquisition of nonspecific tumoricidal capability proceeds along a continuum from the unaltered resident, to the activated macrophage, returning to a noncytolytic cell (128). Accordingly, macrophages have adapted the ability to become directly responsive to microbes, and to molecules arising from microbial degradation (75). In addition, macrophages are responsive to lymphokines produced by other cellular defense mechanisms (35). The ability to respond in this manner is an evolutionary advantage which allows the macrophage to react in a varied context to different stimuli (68).

The induction of macrophage cytotoxic reactions by any of several different primary activation signals is regulated (either amplified or suppressed) in turn by a variety of unrelated secondary signals (81). There-

fore, not only can macrophages enhance their overall functional potential but also they can provide a selection of different functions. Each of these is capable of individual enhancement. This individual enhancement usually leads to concomitant suppression of other functions (2).

Differences in macrophage response to activation signals are not necessarily related to the number of activation signal receptors present on a given cell (81). A resident macrophage may be more, less or equally responsive than an elicited cell to an activation signal. The responsiveness of the cell depends directly on the stimulus and state of the elicited cell (81). The ability of macrophages cultured in vitro to respond to activation signals and kill neoplastic or microbial targets changes with time in in vitro cell culture (81). Macrophages cultured in medium prior to addition of lymphokines, gradually lose the capacity to be activated (83). It is believed that the immature monocyte is responsive to stimuli for only a short period of time. The lack of responsiveness may account for the progressive decrease in cytotoxic activity of macrophages cultured for days in vitro (83).

The cytocidal state of macrophages is mediated by specific signals such as lymphokines and lipopolysaccharide (LPS) but not by nonspecific inflammatory

stimuli such as thioglycollate (93). An increase in certain functions associated with the elicited macrophage, such as phenotypic markers or secretory proteins, are not accompanied always by an enhancement in bactericidal activity (117). Elicited macrophages have been shown to possess different bactericidal activities (109). Likewise, the types of macrophages which are elicited may also vary. The thioglycollate-elictied macrophage response is dependent upon the age of the mouse from which the cells were derived. Thioglycollate can recruit macrophages drawn from a pool of older preformed monocytes or macrophages (54). These thioglycollate-elicited macrophages phagocytose more bacteria and therefore provide a more efficient cell for potential intracellular microbial growth (85). These cells have been shown to have lowered listericidal activity (117), suggesting that thioglycollate may impair antimicrobial activity (54). Furthermore, the responsiveness of elicited macrophages may be modulated by endogenous microorganisms and/or microbial products (75). Therefore, the environment of the host may contribute to observed fluctuations in certain experimental systems following thioglycollate elicitation (68).

Some controversy exists as to the ability of thioglycollate macrophages to kill both tumor cells and

microbial targets (61). Furthermore, the ability of thioglycollate-elicited macrophages to be further activated to cytocidal states has been debated (96). Nathan (93) has found that peritoneal macrophages can be activated by LPS equally well whether they are resident or elicited. Other studies have found thioglycollate-elicited macrophages to kill very well and to be further activated to cytocidal activity by LPS (31). Still other investigators have found thioglycollate-elicited macrophages to lack cytocidal ability for a variety of targets (11,61). These discrepancies may be attributed to the agar contained within macrophages elicited with thioglycollate (32) and the interference by agar in macrophage metabolism (31). Despite comparable glycogen content, resident macrophages take up glucose while thioglycollate-elicited macrophages do not, suggesting metabolic alteration resulting from agar uptake (31). Finally, macrophage properties relegated to nonspecific inflammatory influences may be rapidly bypassed by lymphokines which induce a microbicidal macrophage fully capable of functional activity.

Macrophage precursors are induced by colony stimulating factors (CSF) to proliferate and differentiate into macrophages. Under defined <u>in vitro</u> conditions, a resident macrophage arises from the progenitor cell (39). Macrophages derived from bone

marrow and treated with lymphokines or LPS induce similar maturation changes to that of resident macrophages being activated (53). However, activated bone marrow macrophages mediated ten times the tumoricidal activity of resident macrophages, while maintaining the cytocidal effect for a longer period of time (7). CSF has been shown to induce distinct morphological and metabolic alterations in resident macrophages. These modifications are clearly distinct from those induced with gamma interferon and appear to affect all cells of the culture (7). CSF also affects mature macrophage functions and metabolism resulting in an increase in RNA, DNA, and cell size along with synthesis and secretion of a variety of substances such as PGE2, interleukin-1, and proteases (7).

2. Regulation of activation

Despite the nonspecific manner in which activated macrophages express their cytotoxic effects, the sequence of events leading to the activated state require precise interaction of macrophages and appropriate stimuli (66). Resident macrophages can be induced to the priming stage by lymphokines. But, another trigger, such as LPS, is required before macrophages become fully activated (105). Several groups have found full cytotoxic activity is best obtained when LPS and a lymphokine, such as gamma

interferon (100), are synergistically combined (99,128). Priming or triggering of macrophages by LPS occurs at concentrations as low as 0.1 ng/ml, and increases in a dose-dependent manner (103). The period of macrophage activation and/or responsiveness is short-lived - (83,105). In vitro macrophage activation to a tumoricidal state, in vitro by LPS has been shown to appear as early as 4 hours following stimulation. Maximal responsiveness is apparent for 6-16 hours and is lost by 36 hours (75,105). Similar observations have been made in vivo where macrophages have been shown to lose their cytotoxic capacity quickly following an antigenic challenge (83). This decrease in cytotoxic activity is irreversible, but is not a consequence of changes in cell viability. Rather, it is the result of terminal maturation and differentiation of the cell population (82).

It has been proposed by Pace and Russell that the role of the lymphokine is not to effect full activation but rather to increase macrophage sensitivity to the effects of LPS (99). Lymphokines acting alone, without LPS, fail to activate macrophages to cytocidal activity (99). Conversely, LPS has been shown by Weinberg to act as a signal for activation of macrophages in the absence of lymphocytes or their products (128). Furthermore, high doses (μg/ml) of LPS can activate an elicited

population of macrophages completely to both inhibit cell proliferation and provide a cytotoxic response without a requirement for lymphokines (75).

The mechanism by which LPS modifies macrophage responsiveness is unclear, but it is likely that it initially acts at the level of the macrophage plasma membrane (128). LPS has been shown to associate both with artificial (12) and biological (64) membranes. Evidence exists that LPS interacts with cell membranes by associating with the plasma membrane, intercalating in the phospholipid bilayer, after which it is endocytosed by the cell (60). These data suggest that modification of the macrophage membrane alter cytocidal capacities (124). Therefore, the insertion of LPS into the membrane and the perturbation of the membrane results in macrophage activation by transmitting the necessary signals from the membrane to the vacuolar system (60,124). The molecular basis of this phenomena is unknown. However, addition of LPS alters the cellular protein composition of the macrophage which then allows it to become functionally competent (44).

There are several other major modifications of the macrophage membrane during the process of activation. Lipid composition correlates specifically with development of tumoricidal activity. Total lipid content of tumoricidal macrophages is far greater than

that of noncytolytic macrophages (109). Loss of tumoricidal activity directly parallels the loss of esterified cholesterol, suggesting that the macrophage activation process may be modulated by cellular lipid content (109). A high-molecular-weight serum lipoprotein is responsible for modulation of macrophage cholesterol content (109). Macrophage activation to the tumoricidal state has also been shown to be calcium dependent (131). Calcium ionophore A23187 enhances secretion of a cytolytic factor from macrophages, while calcium channel blockers inhibit activation (131). This suggests calcium channel activity is linked with the biochemical reactions for devolvement and expression of macrophage functional activity. The release of the ionophore changes phosphoinositol lipid metabolism which results in the mobilization of calcium ions following activation (131).

According to Drysdale et al. there exists two pathways by which macrophages may be activated (28). The first, is unaffected by the prostaglandin (PG) synthesis inhibitor, indomethacin. The second, is inhibited by indomethacin and is a consequence of activation by LPS or lymphokines (28). Low levels of PGE1 or PGE2 will restore cytocidal activity in non-activated cells cultured in vitro (84). But, PGE2 inhibits macrophage activation as a negative feedback

signal, preventing nonspecific spreading of a cytocidal effect (110). Conditions which favor high PGE metabolism result in the activation of resident and elicited macrophages while inhibiting activated macrophages (111). PGE2 accumulation in macrophage culture media has been claimed to be responsible for the concomittent loss of macrophage-mediated tumoricidal activity. Resident and elicited macrophages however, become tumoricidal when pretreated with PGE (111). The mechanism does not prevent the development of macrophage mediated cytolytic activity, but rather shuts it off once it develops full cytotoxic potential. Cultures treated with indomethacin sustain high levels of killing for long periods of time (120). Therefore, prostaglandins have differential effects on macrophage antitumor activity depending upon the prior activation state of the macrophage (120).

Additionally, other suppressive accessory factors can be derived from endogenous and exogenous sources (81). Several studies have shown that tumor cell culture supernatants, tumor extracts, and sera from tumor-bearing animals possess suppressive properties for macrophages (60). Some neoplasms themselves contain increased amounts of PGs. As one example, factors released from the EL-4 cell line can suppress macrophage cytocidal activity against all microbial targets tested,

yet only selectively inhibit tumoricidal activity (81). Furthermore, suppression of the macrophage is limited to the site of the tumor (120), and products of activated macrophages that are normally associated with cytocidal activity have been implicated as possible mediators of the suppression (84).

3. Biochemical and functional capacities of macrophages

The process of macrophage activation results in extensive but selective alterations in macrophage properties (39). Resident, elicited, and activated mouse macrophages display marked phenotypic heterogeneity along with selective surface, secretory and endocytotic differences (39). This heterogeneity is observed consistently among these populations regardless of the site from which the macrophages were isolated (39).

Microscopically, the most salient feature of the activated macrophage is the larger size which is expressed as a result of increased protein content (22). Macrophages secrete in excess of 50 different proteins (54). The secretion of these proteins depends both on the physiological state and activation level of the macrophage (54). The age and origin of the macrophage is very important as well, since secretion profiles are modified upon maturation and differentiation (54). Many proteins are constitutively produced by macrophages at

all activation levels (54), and products unique to activated macrophages represent less than 8% of their total secretory activity.

Several groups of investigators have attempted to correlate various macrophage maturation markers (appearance of receptors, either biochemical or physiological) to distinct stages of activation. This work has proven difficult to interpret due to the variable nature of activation mechanisms utilized. However, several characteristics of macrophages at different levels of activation appear to be consistently observed. (See Table 1).

Macrophages which have been induced to the primed stage, share some, but not all of the properties common to activated cells (2). Activated and induced macrophages show an enhanced oxidative response to stimuli, relative to resident macrophages (63). Resident macrophages which functionally present some similarities to neutrophils, posses an efficient intracellular microbicidal activity but are less actively fungicidal than neutrophils (25). Additionally, resident macrophages generally lack the ability to kill extracellular pathogenic fungi (14).

B. Cytocidal capacities of macrophages

1. Candidacidal capacities

Table 1. Summary of Macrophage Characteristics

Macrophage characteristic	Level of activation I	II	III	(ref)
Intracellular enzymes				
Lysosomal hydrolases	+	++	+++	2
Alkaline phosphodiesterase	-	+	+++	30
B-galactosidase	+++	+++	+	75
Surface receptors				
Fc	+	++	+++	95
Complement	+++	+	+	34
Man-GlcNac	+++	+	+	112
Ia	+/-	+	+++	112
5'-Nucleotidase	+++	+/-	-	30
CSF-1	+++	+++	+	39
Secretory capacities				
Lysozyme	++	++	++	15
Neutral proteases	+	+	+++	4
ROI release				
O_2^-	-	+	+++	62
H_2O_2	-	+	+++	115
Arachidonic acid metabolites	+++	++	++	111
Complement	-	+	+++	34
Monokines				
IL-1	+	+	+++	52
TNF	-	+	+++	38
Cytotoxins	-	+	+++	61
Cellular characteristics				
Cell protein	+	++	+++	54
Spreading	-	++	+++	29
O_2 uptake	+	++	+++	112
Glucose uptake	+	++	+++	75
Phagocytosis	-	+	+++	29
Cytocidal capacities				
Bind tumor	-	++	+++	61
Kill tumor	-	+	+++	61
Kill intracellular organisms	+	++	+++	92
Kill extracellular organisms	-	+	+++	122

I, II, III, = Levels of activation. Incomplete data on level IV. -, +, ++, +++, = Relative levels of activity. +/- = Disputed, absent versus low levels.

Invasive Candida albicans infections normally result in hyphal forms which are too large to be phagocytized (25). Early work by Diamond demonstrated that PMNs could damage and kill C. albicans through non-phagocytic means by adhering directly to the hyphae (56). Similarly, studies of macrophage-Candida interactions have resulted in the same finding (26). Macrophages have a large and diverse arsenal with which to combat infectious agents. This is generally divided into two classifications 1) oxygen-dependent and 2) oxygen-independent. Reactive oxygen metabolites are responsible for much of the microbicidal (63) activity of macrophages, although killing by oxygen-independent mechanisms also occurs (72).

Oxygen-dependent systems are classified as such due to a decrease in microbicidal activity under anaerobic conditions (115). The generation and secretion of reactive oxygen intermediates (ROI) is the end result of a coordinated sequence of metabolic events termed the respiratory burst. This function is not constitutive, but must be acquired. Acquisition of competence to execute the respiratory burst involves modulation of the cellular and biochemical elements necessary for the production of ROI (62). Agents which activate macrophages alter the expression of cell surface receptors. The altered receptors then trigger

the respiratory burst (62). The respiratory burst can be modulated at a number of different mechanistic levels. These include; the amount and/or function of cell surface receptors, the amount and/or function of enzyme systems which generate the output, and the various elements which couple these two systems (2). All either directly or indirectly stimulate protein kinase C (PKC), suggesting PKC is a central element in stimulus-response coupling for the respiratory burst and secretion of ROI (62).

The value of ROI in defense against Candida infection has been elucidated in studies of cells from patients with chronic granulomas disease and hereditary myeloperoxidase deficiencies (69). These along with studies utilizing ROI scavengers have shown that ROI (superoxide, hydrogen peroxide, singlet oxygen, and hydroxyl-free radicals) produced by the respiratory burst, all act in concert to mediate hyphal destruction (25). For example, superoxide (O_2^-) itself does not directly damage hyphae, but serves primarily as an intermediate in H_2O_2 production important to the myeloperoxidase-halide system (26). However, iodination has been shown to not be essential for myeloperoxidase-mediated killing but is a non-casual sidelight of the candidacidal activity of macrophages (69). The candid-acidal effects of singlet oxygen can be diminished by

quenching agents, such as histidine, suggesting a possible role for the molecule in hyphal destruction (26). Additionally, activated macrophages have been shown to produce high amounts of O_2^- *in vivo* and therefore may protect the host against *Candida* infections (23). Hyphal damage appears to result from products of oxidative metabolism, especially the myeloperoxidase-H_2O_2-halide system, but myeloperoxidase-independent mechanisms are active as well (26). Furthermore, anaerobiosis significantly reduced, but did not eliminate, damage to hyphae by macrophages indicating at least some oxygen-independent mechanisms may be involved in target cell destruction (26).

Macrophages possess a variety of hydrolytic enzymes which function in an oxygen-independent manner (23). These fungicidal constituents, such as B-glucosaminidase, are found within macrophage cytoplasmic granules such as lysosomes (69). Lysosomal constituents have been shown to be released by macrophages following contact with target cells and to be microbicidal (2,24). Two microbicidal cationic peptides (MCP-1 and MCP-2) produced by rabbit alveolar macrophages have been found by Patterson-Delafield *et al*. These peptides are effective against diverse gram positive and negative bacteria and fungi, and act either extracellularly or within the phagolysozome (73). These proteins, rich in

arginine and cystine, are distinct from histones and have been found to suppress cellular oxygen consumption by Candida (72). In studies using partially purified fractions of macrophages enriched for lysosomal granules Candida hyphae where not damaged (26). These results suggest that cationic proteins and perhaps other granule constituents might act as ancillary antifungal mechanisms, enhancing effects of oxidative mechanisms (97). Despite findings that suggest a role for oxygen-dependent and oxygen-independent mechanisms for Candida killing, it remains unclear which intermediates or pathways are directly responsible for hyphal damage (25). Conflicting data regarding the ability of in vitro cultured macrophages to destroy C. albicans has been reported. The disparity in results is a consequence of the utilization of mononuclear cells derived from: various sites, such as the blood (69), the peritoneum (23) or the alveoli (71); from various animal species, including mouse (62), man (69), and rabbit (71); as well as elicitation of the cellular populations with various in vivo adjuvants (51). Analysis is further complicated by the use of monocytes (69) or resident (63), induced (51), lipopolysaccharide (LPS)-activated (71), or lymphokine-activated (14) macrophages. Furthermore, many studies have used the capacity for intracellular killing of ingested yeasts to determine candidacidal

activity. This may not reflect the ability of the macrophage to damage hyphae at the cell surface by nonphagocytic mechanisms (26). Such a non-standardized approach has made comparisons of macrophage activity difficult.

It is known that contact between the macrophage and hyphae cell is vital for destruction of fungi in macrophages (37). Electron micrographs of monocyte-Candida interactions have shown monocyte plasma membranes adhering directly to portions of hyphal cell walls. Sections of hyphae in contact with the macrophage were partially engulfed, damaged and disrupted (26). This hyphal damage was accompanied by damage to the monocyte itself, in that many hyphae had surface-adherent vesicles that were bounded by three-layer unit membranes (26). These studies did utilize peripheral blood monocytes and the lability of human monocytes compared with mature macrophages has been shown to be significant (19).

During infections, yeast forms of Candida are found in tissue, although the prevalent form is the hyphal stage (11). It has been suggested that hyphal forms of Candida are endowed with in vivo resistance to host phagocytic defenses via a variety of mechanisms (107). Adherence of macrophages to the hyphae has been shown to occur through a cell wall constituent, most

probably mannose, which then stimulates the respiratory burst (24). The release of this soluble material has been shown to then inhibit further macrophage activation resulting in suppression of macrophage function (90). Degradation by phagocytic cells, results in the reduction of target cell cytoplasm. Cell wall deformation and collapse are all a result of enzymatic breakdown of Candida cell wall mannan by macrophages (37). The most invasive adaptation of Candida is the ability to alternate between the yeast and hyphal forms during its multiplication. This has been related to its ability to evade host defenses (101). The transition between the two stages appears to be important in the expression of fungal pathogenicity, although no definitive evidence exists which compares relative pathogenicity with different morphologies. Possibly cell wall composition and architecture of the two forms under different conditions may force Candida toward morphological transition (70).

2. Tumoricidal capacities

Vital roles have been proposed for cellular immunity in the complex host reaction to neoplastic disease (47,102). Killing of tumor cells, at least in vitro, can be mediated by many different cell types. The tumoricidal activity of macrophages has been the object of a great deal of study. Mackannas defined

activation as: the enhancement, through immunologically specific reactions, of the immunologically non-specific capacity of macrophages to kill microbial pathogens (90). However, activation is often defined as the capacity of macrophages to mediate cytotoxicity towards tumor cells. Whether macrophages kill tumor cells *in vivo* as a mechanism of primary defense is still unknown. However, *in vitro* nearly all tumors are susceptible to macrophage cytotoxicity in a nonphagocytic, contact-dependent process (90). The interaction between activated macrophages and target cells often leads to selective destruction of the neoplastic cell. It has been shown *in vitro* that layers of tumor cells will grow adjacent to but not in areas of macrophage growth, suggesting that cytolysis is restricted to the immediate vicinity of the macrophage (32,45,49). The mechanisms of tumor cell destruction by macrophages have been studied extensively (36,49), and may be affected by many types of secreted products such as enzymes, reactive oxygen intermediates, and secretory products.

ROI are responsible for a great deal of candidacidal activity, however, the tumoricidal activity of ROI is not completely understood. Nathan has suggested that an oxidative mechanism may provide the means by which activated macrophages kill tumor cells (92). There is some evidence H_2O_2 is tumoricidal *in vitro*

(92). A possible role for myeloperoxidase in the killing of tumor cells by macrophages has also been suggested (18,104). However, it has been found that induction of tumoricidal function does not correlate with induction of the respiratory burst either as a matter of time or stage of macrophage activation (21). Additionally, a majority of macrophage tumoricidal effects have been assigned to oxygen-independent mechanisms despite the observation that the toxic effects of macrophages toward target cells were significantly reduced when cells were cultured under anaerobic conditions (115).

Extracellular secretion of proteins has been established as an important function of macrophages, suggesting the possibility that tumor cells may be destroyed by secreted products (2). Although primarily cytotoxic, these agents may serve to digest infectious agents which were killed by predominantly oxidative mechanisms (66). Possible mediators of macrophage oxygen-independent cytocidal activity include: acid hydrolases (2); alkaline phosphatase (30); arginase (4); and neutral proteases (1). Such secreted factors may play an important role in infectious agent destruction since killing of tumor cells normally precedes the degradative and destructive processes of phagocytosis (2).

Even in the absence of phagocytosis it is still possible that macrophages may transfer their lysosomal enzymes by a process of exocytosis (15). It has been shown that the content of secondary lysosomes are transferred to the tumor cell prior to cell lysis (48). Furthermore, it is known that lysosomal enzymes of activated macrophages mediate tumoricidal effects (48). The macrophage-tumor cell interaction is initiated by a recognition phase that results in polarization and eventual release of lysosomal constituents through macrophage exocytosis and membrane fusion (41). Lysosomes are transferred from activated macrophage to the cytoplasm of target cells (48,36). Biochemical changes which have been detected in the cell surface membrane of activated macrophages (e.g. 5'-nucleotidase) are similar to changes detected in the membranes of the phagosomes (66). Perhaps these or similar membrane changes are related to the capacity of the lysosomal membrane to fuse with the cell surface membrane prior to lysosomal exocytosis (35).

Macrophages also secrete multiple components of the complement system, including constituents of both the classic and alternative pathways (2). Macrophage-produced C3 can be cleaved into C3b, which activates macrophages to secrete hydrolytic enzymes, and C3a which is lytic for tumor cells (34).

Tumor necrosis factor (TNF) is a soluble molecule which may be involved in macrophage tumor cell killing (123). TNF is produced almost immediately following exposure to LPS and its production is dependent upon the continued presence of LPS (38). Exposure of macrophages to LPS results in the production of TNF (38). TNF itself is capable of lysing targets. Lymphotoxins or lymphocyte cytotoxic factors, are another set of macrophage cytotoxic products, which also directly lyse neoplastic cells (124). Lymphotoxins may be very similar or identical to TNF since studies have found that an anti-TNF antibody also removes the cytocidal activity of lymphotoxin (132). However, in short term assays only TNF is effective in tumor cell lysis, whereas both TNF and lymphotoxin are effective in long term assays (132). Many investigators appear to have isolated different cytotoxic factors. For example, different cytotoxic factors found in serum have different stabilities at room temperature (122) and cytotoxic factors have varying sensitivity to heat treatments and proteases (124). One cytotoxic factor secreted from a macrophage hybridoma appears to be distinct from all other cytotoxic factors since it is heat stable and acid labile while others are not (121). Additionally, macrophage hybridomas have been produced which secrete cytotoxic factors and Interleukin-1 (IL-1) (122).

During infection, macrophages with bactericidal and tumoricidal activity display a parallel increase in IL-1 and cytotoxic factor production (52).

IL-1 is a central mediator in the regulation of the immune response. Macrophages are a major source of IL-1 and release it during the activation process. Regulation of IL-1 is itself complex. Low levels of TNF induce increased IL-1 production, while the converse is also true (52). However, these two are not direct mediators of one another, as high TNF levels induce PGE which blocks IL-1 production (52).

The sensitivity of tumor cell lines to macrophage tumoricidal effects varies greatly and some tumor cells are even promoted to proliferation by macrophages (93). Completion of tumor cell lysis by macrophages requires direct binding to target cells (109). However, the glutathione oxidation-reduction cycle serves as a mechanism against oxidant injury, and accounts for varying susceptibility of tumor cells to oxidation (91).

The sequence of events for macrophage killing of tumor cells may then be summarized in the following way: a)contact occurs between macrophages and target cells; b) lysosomal enzymes of macrophages are polarized toward bound tumor cells; c) spreading of macrophages at the tumor cell surface occurs; d) oxidative mechanisms are

released which weaken the tumor cell membrane; e) lysosomal constituents are released and tumor cells are lysed (2).

C. Comparisons of extracellular killing

It has yet to be determined whether increased tumoricidal and increased microbicidal activity are always generated together as a consequence of the activation process (96). Some evidence exists which suggests that the two activities are separate (130). The possibility exists that macrophage activation occurs in a series of definite steps. This is indicated by the presence of one manifestation of activation occurring in the absence of others. The number of activation steps may be determined by the particular homeostatic functions which the macrophage perform (96). For instance synthesis and secretion of plasminogen may require a definitive number of steps. Metabolic changes required for this action may occur earlier than or without acquisition and expression of the more complex function of increased microbicidal or tumoricidal activity (96).

Whether populations of activated macrophages which display one functional activity also possess other functional capacities is not established (130). Experiments have been conducted to measure the ability of macrophages to inhibit tumor cell DNA synthesis and

intracellular multiplication of Toxoplasma gondii (130). Macrophages removed from Toxoplasma spiralis infected animals after 19 weeks were able to inhibit tumor cell DNA synthesis yet were unable to prevent intracellular multiplication of T. gondii (130). Macrophages taken from the animals infected with T. gondii at 16 weeks and used immediately, inhibited DNA synthesis of tumor cells but failed to inhibit T. gondii intracellular replication (130). However, following incubation for 24 h in vitro, the capacity of these same 16 week macrophages to inhibit tumor cell DNA synthesis was lost while T. gondii intracellular replication was inhibited (130). These results suggest that the functions of activated macrophages in a given population maybe identical at any given time. However, the time of infection, the elicitation agent or immunization agent, and the time in culture, may alter the observed functional capacities of any given macrophage population.

The functional differences in different macrophage populations which were observed by Wing (130) have also been observed by many other groups. Macrophages which express specific antitumor activity, or express antibacterial resistance, did not kill the intracellular organism Listeria (130). Therefore there is a clear dissociation of intracellular microbicidal activity from other macrophage functions (95,114). Conversely, it has

also been shown that host responses to intracellular infection results in an increased resistance to neoplasia (125). It has been proposed that at maximum stimulation, all functional properties of activated macrophages may be expressed (130). The properties might be lost at different rates and consequently, at various times after activation only certain properties of activated macrophages are manifest. The functions of a given population must be considered with the number of factors already discussed which may induce variance (130). Alternatively, there may exist separate subpopulations of macrophages which are activated differentially and posses distinct functional capabilities (130).

Mature macrophages from different sites may differ functionally depending on which function the macrophage is called upon to perform. One of these functions is the ability to kill. This functional heterogeneity in macrophage lineage is detectable at the precursor level (10). Bone marrow-derived macrophage precursors from CSF conditioned cultures possess the ability to kill, extracellularly, YAC-1, Candida albicans, and Leishmania (10). These nonadherent, nonphagocytic, macrophage precursors originate from 3 day bone marrow liquid cultures. These cells are normally located in the bone marrow prior to recruitment

to peripheral organs (9). These macrophage precursors have been proven to possess an overall candidacidal potential greater than that expressed by resident macrophages (8). The same cells are also active against YAC-1 lymphoma cells (8).

Bone marrow-derived macrophage precursors are able to mediate extracellular killing against tumor and fungi targets (8). Both of these activities are lost during the course of maturation to mature macrophages only to be regained later (8). For example, bone marrow-derived precursors can differentiate in the presence of CSF-1 which is deficient in interferon (IFN) to become resident macrophages. These mature bone marrow derived-macrophages are unable to kill Candida (8). The same cells when exposed to CSF-1 and IFN, can progress developmentally to a more differentiated state which has the capacity to kill (127). Upon activation, restoration of the cytocidal activity resumes in a process analogous to that observed for macrophages infiltrating an inflammatory site (127). The common precursors seem to lose or retain their activity depending on the environment of the anatomical site into which they migrate (8).

Despite common mechanisms for the induction and expression of macrophage effector responses within the transient cytotoxic reaction, separate signals may

activate different functional capacities (89). Studies by Nacy (89), have shown that lymphokines from stimulated spleen cells regulate cytotoxic reactions against various targets through different activation factors or lymphokines. For instance factors added from cultures of murine spleen cells induce microbicidal activities against the intracellular targets Rickettsia tsutsugamushi or Leishmania tropica. These same factors are completely ineffective for inducing cytocidal activity against extracellular targets such as tumor cells or Schistosoma mansoni (89). Furthermore, lymphokines which induce intracellular killing can be separated into three components or factors. The extracellular killing is induced by only one factor, separate from the factors which induce intracellular killing (89). If a population of the T-cell lymphoma $EL\text{-}4_{FARRAR}$ is stimulated with PMA, extracellular killing and intracellular killing were found to be exclusive (89). In this situation, lymphokines were found to contain two components which induced extracellular killing, whereas only one lymphokine component was found to induce intracellular killing. The component which induced intracellular killing appeared to be identical to one of the extracellular components (89). These data suggest that induction of an effector response is dependent upon specific activation factors. It is clear

that macrophage activation for cytotoxicity against tumor and schistosomula targets is coincident over a broad range of experimental conditions (59). Both resident and elicited macrophages can be activated to this parallel activity (59). Whether the mechanisms of macrophage cytocidal tumoricidal and schistosomulacidal activity induced by these lymphokines are similar remains to be clarified (58). Both cytocidal activities were induced by the same large molecular weight activating factors suggesting that identical effector function originates from the same activating signal (89). The identiy of these lymphokines is unknown. Whether the lymphokine activities are from unrelated molecules or aggregates of a single molecule also is unknown. What is most important is that macrophage cytocidal activity against two divergent extracellular targets, tumor cells and helminthic worms were induced by the same macrophage activating lymphokines (89). Equally important was the observation that cytocidal activity against two totally unrelated intracellular parasites that reside in different compartments (intracytoplasmic rickettsiae and intraphagolysosomal leishmania) was induced by a common mechanism (89). The extracellular cryptococidal and tumoricidal results of Granger closely parallel the cytostatic effects noted above (42). In fact, the kinetics of onset and the effector cell sensitivity to

LPS, again indicate a similar mechanism of activation (42).

It appears clear that regulation of macrophage cytotoxicity is controlled by qualitatively different factors (89). One which controls extracellular targets, another, which controls intracellular cytotoxicity (89).

The cytocidal activities of macrophages are influenced by many factors, including the level of activation of the macrophage. The capacity of macrophages to respond to stimuli and induce a functional response, such as extracellular killing, has been studied extensively (89). In comparisons of extracellular killing of schistosomes and tumor cells, cytocidal effects were found to take place upon activation with particular lymphokines (90). The activation of macrophages with LPS has shown similar results. Nonactivated states, (resident and elicited) displayed parallel tumoricidal and schistosomicidal activity, again emphasizing similar levels of activation for extracellular cytocidal activity.

In contrast, little work has been done comparing the candidacidal and tumoricidal effects of macrophages. Most studies of Candida have been performed with the yeast phase of the fungus which is phagocytosed and killed in an intracellular manner (108). Based on studies by Nacy et al., comparing differential activa-

tion of macrophages by certain activating factors for intracellular cytocidal activity as compared to activating factors which induce extracellular cytocidal activity (89), one would not expect to find similar levels of activity for the intracellular killing of phagocytozed Candida as compared to the extracellular killing of tumor cells. Instead it would be expected that the intracellular killing of phagocytosed Candida would be mediated by different activation mechanisms and different cytocidal mechanisms than that of the hyphal form of Candida which must be killed in an extracellular manner. The possibility exists that similar activation mechanisms for cytocidal activity would be demonstrated. However based on the wide differences encountered by the macrophages in comparing these two targets the possibility is unlikely. More likely is a separate and distinct activation mechanism for extracellular killing and intracellular killing.

Work on macrophage precursors has indicated that, in a nonphagocytic manner, these cells have the ability to kill Candida, Leishmania, and tumor cells. That study utilized the yeast phase of Candida, relying on the inability of the macrophage precursor to phagocytize for studies of extracellular killing (8) rather than studying the mature macrophage and its extracellular killing mechanisms.

No studies have been completed comparing the cytocidal effects of macrophages on Candida in the hyphal form to the cytocidal effects of macrophages on tumor cells. Furthermore, no study has been undertaken to evaluate the relative cytocidal capacities of macrophages at various levels of activation. This study compares the levels of candidacidal and tumoricidal activity of macrophages at set levels of activation.

CHAPTER III

MATERIALS AND METHODS

A. Tumor Cell Line.

The EL-4 lymphoma (40) was obtained from R. Herberman, National Cancer Institute, Bethesda, Maryland. EL-4 cells were maintained *in vitro* in Corning 25 cm^2 tissue culture flasks (Corning, NY) containing RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (low LPS) (Gibco, Grand Island, NY) penicillin 100 units/ml, streptomycin 100 µg/ml, and Fungizone (PSF) (Whittaker, Walkersville, MD) 0.25 µg/ml, and 2mM L-glutamine (Gibco, Grand Island, NY). Cells were passaged 3 times weekly with maximal concentration not exceeding 1 x 10^6/ml.

B. Radiolabelling of EL-4 Tumor Cells.

Tumor cells maintained *in vitro* were washed once in culture medium and resuspended to 2 to 5 x 10^6 cells/ml. The cells were subjected to centrifugation at 500 x g for 10 min, decanted, and the pellet resuspended in approximately 0.1 ml of culture medium. Then 100 µCi/ml of $Na_2{}^{51}CrO_4$ (New England Nuclear, Boston, MA) was added to approximately 1 x 10^7 cells in a final

volume of 0.2 ml. These cells were incubated at 37°C with 5% CO_2 for 1 h with frequent agitation (every 10 min). The cells were then washed four times in HBSS, resuspended to 1 x 10^6 cells/ml in culture medium and 0.01 ml aliquoted to each well of a 96 well assay plate (Falcon 3072, Oxnard, CA) (27,129).

C. Fungal Culture

Candida albicans ATCC 58716 obtained from T. Hashimoto, (Loyola University Medical Center, Maywood, IL) was used throughout this investigation. Cultures were stored at 25°C on Sabouraud 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 HBSS prior to inoculation into Dulbecco's modified Eagle medium (DMEM, 4.5 g/L glucose) (Whittaker, Walkersville, MD). Any culture with greater than 15% budding cells was discarded. Candida hyphal forms were obtained by incubation at 37°C in DMEM. The inoculum of 1 x 10^5 Candida yeast cells/ml yielded approximately 100% hyphal fragments ranging in length from 40-65 µm when incubated for 3 h at 37°C (46).

D. Preparation of Macrophage Elicitation Agents.

Proteose peptone broth No. 3 (Difco Laboratories, Inc., Detroit, MI) was prepared as a 10% sterile solution. Aliquots were placed in 5 ml batches and

frozen at -20°C until needed. Brewer Thioglycollate Medium (Difco Laboratories, Inc., Detroit, MI) used for intraperitoneal injections of mice was prepared as described previously (16,74). An autoclaved solution was allowed to become oxidized for six months in storage at room temperature. The media was distributed in 5 ml aliquots and frozen at -20°C for later use.

E. LPS Preparation.

LPS from Salmonella typhosa 0901 was purchased from Difco Laboratories, Inc., (Detroit, MI), in lyophilized form. After rehydration, the LPS was distributed into 1 ml aliquots at a concentration of 500 µg/ml and stored frozen at -20°C until needed (16).

F. Macrophages

Female C57Bl/6 mice (Jackson Labs, Bar Harbor, ME), 8-16 weeks of age, were injected intraperitoneally with 1 ml of freshly prepared proteose peptone broth or 1 ml of aged thioglycollate broth. After 4 days, the mice were sacrificed by cervical dislocation and the peritoneal exudate cells (PECs) removed by lavage with 10 ml of HBSS (74). The PECs were also removed from normal mice which were not injected with proteose peptone or thioglycollate. The PECs were washed twice in HBSS and resuspended in RPMI-1640, supplemented with 10% FBS, (Gibco, Grand Island, NY), penicillin 100 µg/ml, streptomycin (Whittaker, Walkersville, MD) 100

µg/ml, and 2mM L-glutamine (Gibco, Grand Island, NY). Fungizone was omitted from the medium. These PECs were then allowed to adhere as described below to plastic culture vessels, either 24 or 96 well plates (Falcon 3047 or 3072, Oxnard CA). The non-adherent cells were removed by withdrawing the medium and gently flushing once with fresh culture medium which was immediately removed. Viability was routinely determined by 0.04% trypan blue exclusion.

G. Macrophage Activation.

The groups of macrophages evaluated by this study were; I= Resident, II= Induced, III= Activated, and IV= Post-Activated (82). Group I macrophages were obtained from the peritoneal cavity of naive mice (27). Mice for groups II - IV were injected four days previously with 10% proteose peptone broth. Group II were proteose peptone-induced macrophages with no *in vitro* treatment. Group III were macrophages activated *in vitro* with LPS (10 µg/ml) for 18 h. Group IV were macrophages which were cultured *in vitro* with LPS (10 µg/ml) for 72 h. Mice injected with thioglycollate broth four days previously were termed group IIt-IVt and were treated as described above for proteose peptone-elicited macrophage states II-IV. Additionally, some group I macrophages were treated *in vitro* with LPS (10 µg/ml) for either 18 h or 72 h and were termed group IIIr or group IVr,

respectively.

H. Tumor Cytotoxicity Assay.

The methods employed for this assay are modifications of the procedure originally described by Doe and Hansen (16,27). PECs used for tumor cytotoxicity assays were placed in 96-well plates (Falcon 3072, Oxnard, CA) at 2 x 10^5 PECs/well. Two h later the non-adherent cells were removed by aspiration of the media and replacement with fresh culture medium (0.2 ml). The wells were approximately 70-80% confluent with macrophages for all groups. Following an additional 20-24 h of incubation at 37°C, nonadherent cells were removed and fresh medium added. These adherent PECs are referred to as macrophages. At this time, LPS was added in a final concentration of 10 µg/ml to each well for groups III and IV. After 18 h of incubation for groups I-III and 72 h of incubation for group IV, 1 x 10^4 labelled tumor cells were added to each well in a 0.01 ml volume. Following an additional 18-24 h, the plates were subjected to centrifugation for 5 min at 200 x g, 100 µl of the supernatant fluid was removed from the 0.2 ml volume contained in each well, and assessed for radioactivity by use of an automated gamma counter (Beckman, Gamma 4000, Irving, CA). Maximum release was obtained by adding 0.5% Nonident P-40 (Sigma Chemical Co., St. Louis, MO) to wells containing media and

macrophages without LPS.

Data for this assay was based on a 20:1 effector/target ratio and results are expressed as % cytotoxicity as judged by release of ^{51}Cr.

$$\% \text{ cytotoxicity} = \frac{(\text{mean experimental DPM}) - (\text{mean minimum DPM})}{(\text{mean maximum DPM}) - (\text{mean minimum DPM})} \times 100.$$

Results are expressed as the mean of triplicate cultures from four separate experiments.

I. Candida Cytotoxicity Assay.

Candidacidal activity of macrophages was quantified by the microcolony method of Hashimoto (46). This method takes advantage of the dimorphic nature of Candida. PECs were placed in 24-well plates (Falcon 3047, Oxnard, CA) at 1 x 10^6 cells/well, with a 12 mm circular cover slip (no. 1: Rochester Scientific Co. Inc., Rochester, NY) within each well. PECs were treated and activated as described above for the tumoricidal assay except, that culture medium was removed and the macrophages gently washed to remove fungizone prior to the addition of Candida in DMEM. The viability of individual C. albicans hyphae recovered from the culture wells was determined as follows: non-phagocytosable Candida hyphae were exposed to macrophages at an effector/target ratio of 10:1, and incubated on the surface of cover glasses placed in the

bottom of a well of a 24 well cluster plate for 1 h. The DMEM was removed. Sabauroud's dextrose broth containing 0.5% Nonident P-40 (Sigma Co. St. Louis, MO) was added to each of the wells. The cover slips were recovered from the wells, gently rinsed in Sabauroud's dextrose broth without Nonident P-40, and placed inverted on a slide containing a thin layer of SDA (10 mm thick). This procedure only partially lyses the macrophage membrane allowing essentially all of the Candida hyphae to adhere to the glass surface via remnants of the macrophage membrane. The microculture was incubated under conditions conducive to the yeast phase of growth at 37°C for 5 h in a moist chamber. The cultures were examined with a phase contrast microscope. Routinely, 200 hyphae were examined for each sample, and the percentage of nonviable hyphae in each sample calculated. Results are expressed as the mean of triplicate cultures and four separate experiments.

J. Statistics

The Student's t test was used to determine the statistical significance of results.

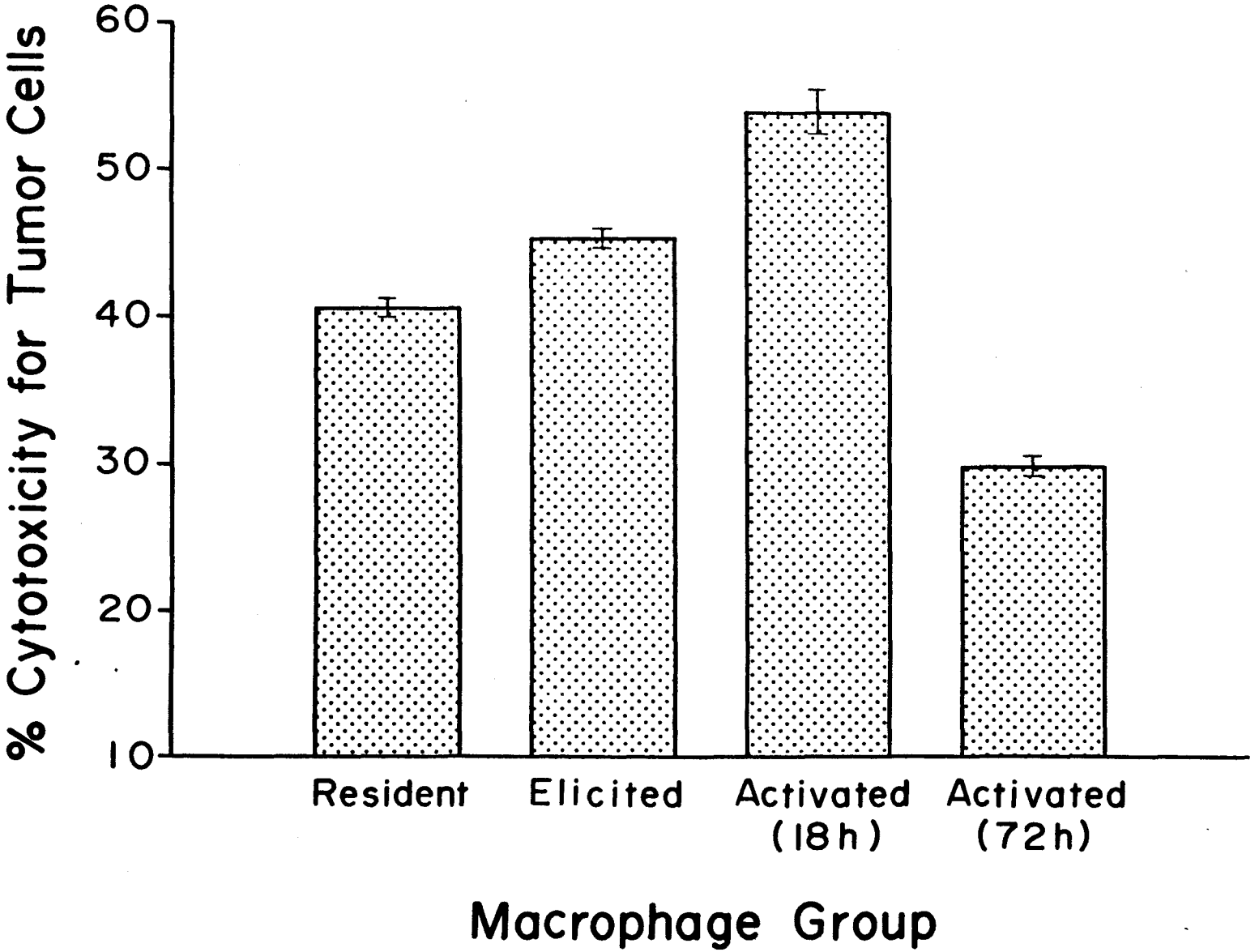
CHAPTER IV

RESULTS

Four different groups of macrophages were compared for their tumoricidal and candidacidal activities. Results which compare the tumoricidal activity of the four groups of macrophages are presented in Fig. 1. Compared to group I (resident macrophages), a significant increase in cytotoxic activity was observed for groups II (macrophages elicited from the peritoneal cavity with proteose peptone) and III (proteose peptone-elicited macrophages activated *in vitro* with LPS (10 µg/ml) for 18 h), while a significant decrease was observed for group IV (proteose peptone-elicited macrophagess incubated *in vitro* for 72 h with LPS (10 µg/ml). The cytotoxic activity of group I macrophages at 40.5% ± 0.7% release was exceeded by group II at 45.2% ± 0.6% release ($p<0.001$). Group III showed the greatest tumoricidal activity at 53.8% ± 1.5% release ($p<0.001$) when compared to group I. However, macrophages stimulated with LPS for 72 h (group IV) showed a significant decrease ($p<0.001$) in cytotoxic activity to 29.7% ± 0.7%, when compared to group I macrophages. A significant increase ($p<0.001$) in cytotoxic activity was observed between group III (53.8% ± 1.5% release) and group II (45.2% ± 0.6% release). There was no signifi-

Figure 1.

Comparison of macrophage groups as judged by 18 h ^{51}Cr release assay. Values are displayed as mean of 4 experiments performed in triplicate. Bars indicate ± standard deviation (SD). Macrophage populations are as follows; group I (resident macrophages), group II (proteose peptone-elicited macrophages), group III and group IV were proteose peptone-elicited and either treated with LPS for 18 h (group III), or treated with LPS for 72 h (group IV). Groups II and III are significantly ($p<0.001$) higher than group I, while group IV is significantly ($p<0.001$) lower than group I. Group II is significantly lower than group III ($p<0.001$).


% Cytotoxicity for Tumor Cells
60
50
40
30
20
10
Resident
Elicited
Activated (18h)
Activated (72h)
Macrophage Group

cant difference in cytotoxicity between group I resident macrophages cultured for 72 h without LPS stimulation and group I (data not shown).

As a comparison, macrophages from group I were exposed to LPS (10 µg/ml) in vitro for 18 h or 72 h. See Table 2. These macrophage groups were termed group III resident (IIIr) and group IV resident (IVr), respectivly. Cytotoxixicty by macrophages from group IIIr (54.8% ± 1.7% release) was not significantly different in tumoricidal activity from the experimental group III (53.8% ± 1.5 % release). Group IIIr was significantly more cytotoxic than either group II ($p<0.001$) or group I ($p<0.001$). Group IVr (31.3% ± 1.8%) displayed a significantly lower level of cytotoxic activity then group I while displaying no difference with regard to group IV.

In an identical series of experiments, macrophages were thioglycollate-elicited (group IIt), or thioglycollate-elicited and treated with either LPS (10 µg/ml) for 18 h (group IIIt), or LPS (10 µg/ml) for 72 h (group IVt). Tumoricidal activity of the group IIt macrophages (51.7% ± 1.7% release) showed a significant increase ($p < 0.001$) from both group I (40.5% ± 0.7% release) and group II (45.2% ± 0.6% release). There was no difference between group IIt and group III. Group IIIt showed a significant increase from group III ($p<0.05$)

Table 2. Comparison of treatment groups as judged by the extracellular tumoricidal assay.

	Cytotoxicity			
		Eliciting Agent		
Group Number	Resident	Proteose Peptone	None (r)[a]	Thioglycollate (t)[b]
I	40.5±0.7[c]	-	-	-
II	-[d]	45.2±0.6	-	51.7±1.7
III	-	53.8±1.5	54.8±1.7	57.6±1.9
IV	-	29.7±0.7	31.3±1.8	25.5±2.2

[a]Resident macrophages (r) activated with LPS.

[b]Thioglycollate (t)-elicited macrophages.

[c]Data expressed as the mean of 4 experiments performed in triplicate ± SD. Groups II-IV (including all resident activated; IIIr and IVr, and thioglycollate; IIt, IIIt, and IVt) displayed a significance of $p < 0.001$ when compared to group I. Additionally, groups IIt, III, IIIt, and IIIr displayed a significant increase ($p < 0.001$) to group II.

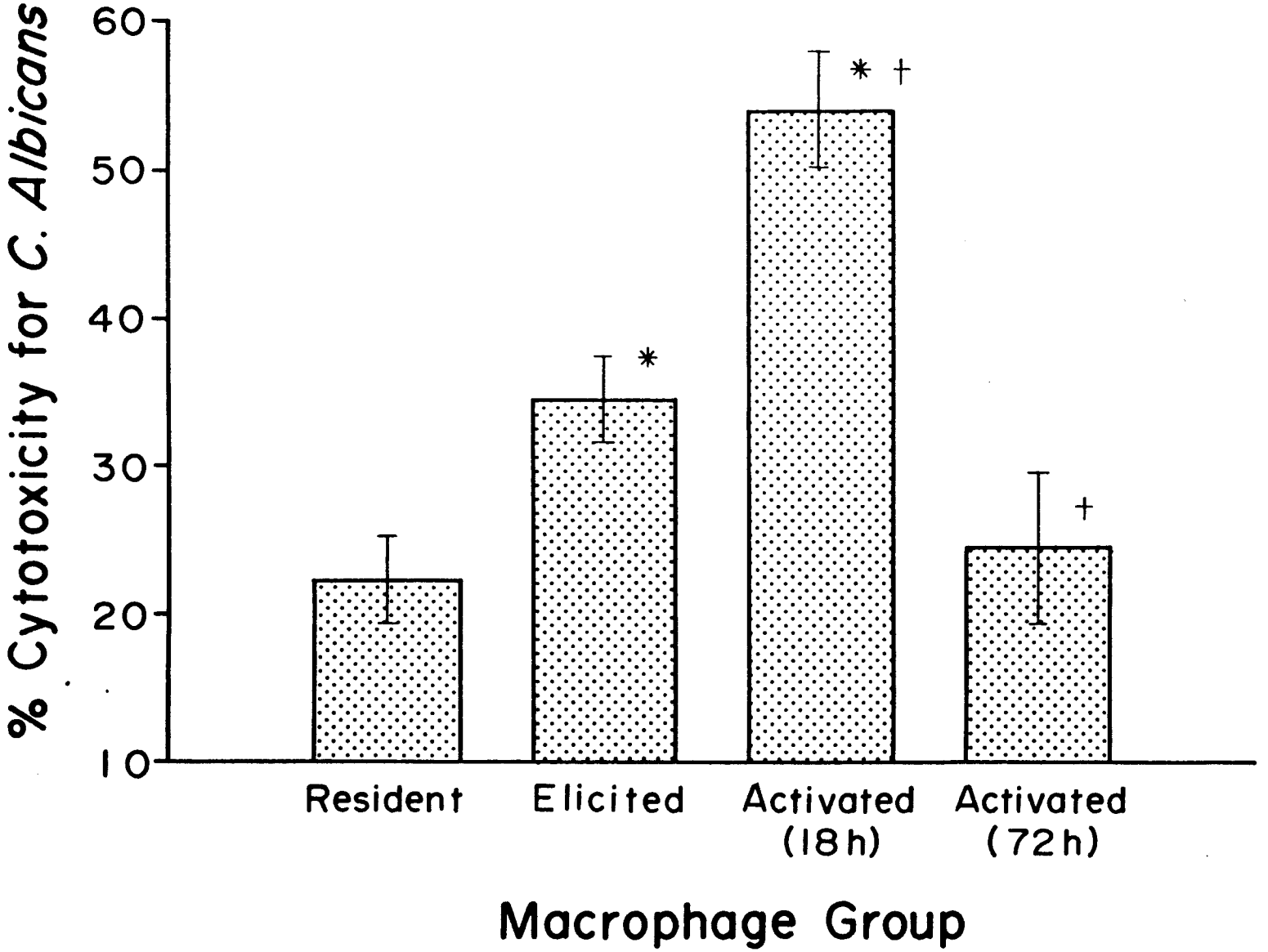
[d]Experimental values not determined due to protocol.

producing the highest level of cytotoxicity. Group IVt macrophages (25.5% ± 2.2% release) showed a significantly lower ($p<0.001$) level of tumoricidal activity as compared to those in group I. Additionally, group IVt (25.5% ± 2.2% release) was significantly ($p<0.05$) less cytotoxic than both normal group IV (29.7% ± 0.7% release) and resident group IVr (31.3% ± 1.8% release).

Simultaneously, candidacidal activity was measured with identical macrophage populations. Results are expressed as % cytotoxicity, defined as that part of the culture able to return to the yeast phase from the hyphal phase following a 1 h exposure to the macrophage populations. These studies showed that the level of candidacidal activity paralleled that of the tumoricidal assay. See Figure 2. The data show group I macrophage activity which resulted in Candida cytotoxicity of 22.2% ± 3.0%. This differed significantly from the 34.5% ± 2.9% cytotoxicity observed following exposure to group II macrophages ($p<0.001$). Treatment with LPS for 18 h (group III), significantly increased the candidacidal activity of these macrophages to 46.0% ± 3.9% cytotoxicity ($p<0.001$). Group II macrophages resulted in Candida cytotoxicity of 34.5% ± 2.9% which was significantly lower ($p<0.001$) than the cytotoxicity (46.0% ± 3.9%) observed with group III macrophages. However, unlike the tumoricidal results, there was no

Figure 2.

Comparison of macrophage groups as judged by the extracellular candidacidal assay. Values are displayed as mean of 4 experiments in triplicate. Bars indicate ± SD. Groups are described in the legend to Figure 1. Results are expressed as % cytotoxicity for Candida after 1 h exposure to the various macrophage populations (* = $p < 0.001$ to group I, + = $p < 0.001$ to group II).


% Cytotoxicity for C. Albicans
60
50
40
30
20
10
*
* †
†
Resident
Elicited
Activated (18h)
Activated (72h)
Macrophage Group

statistical difference between the cytotoxicity resulting after treatment with either group I or group IV macrophages.

No appreciable differences were found between macrophage candidacidal activity of either group IIIr and group IVr or group III and group IV, respectively (Table 3). In the candidacidal assay, group IIt macrophages produced a much higher cytotoxicity (43.2% ± 3.2%, $p<0.001$) when compared to group I macrophages (22.2% ± 3.0%). The cytotoxicity resulting after incubation with group IIt macrophages was significantly higher than that with group IIIt macrophages (47.1% ± 2.7%, $p < 0.001$). However, no difference in cytotoxicity following exposure to macrophages of group IIt (43.2% ± 3.2%) and group III (46.0% ± 3.9%) or group IIIr (40.2% ± 4.2%) was observed.

Table 3. Comparison of treatment groups as judged by the extracellular candidacidal assay.

Group Number	Cytotoxicity			
	Eliciting Agent			
	Resident	Proteose Peptone	None (r)[a]	Thioglycollate (t)
I	22.2±3.0[b]	-	-	-
II	-[j]	34.5±2.9[d]	-	43.2±3.2[d,i]
III	-	46.0±3.9[d,i]	40.2±4.2[d,g]	47.1±2.7[d,i]
IV	-	24.5±5.2[e,h]	17.7±2.7[e,i]	27.0±3.7[c,f]

[a]Resident macrophages activated with LPS. See footnotes of Table 2 for abbreviations.

[b]Data expressed as the mean of 4 experiments performed in triplicate ± SD. Following notations denote significant differences from stage I
c = $p < 0.05$ to stage I
d = $p < 0.001$ to stage I
e = $p > 0.05$ N.S.

Following notations denote significant differences from stage II
f = $p < 0.05$ to stage II
g = $p < 0.02$ to stage II
h = $p < 0.005$ to stage II
i = $p < 0.001$ to stage II

[j]Experimental values not determined due to protocol.

CHAPTER V

DISCUSSION

It has been speculated that the mechanisms of extracellular, non-specific, macrophage-mediated cytotoxicity are the same for any target (59). The results of this investigation indicate that the extracellular cytotoxic activity of macrophages for two distinctly different targets appears to be similar and to be coincidentally expressed. Parallel levels of extracellular macrophage cytotoxicity for both schistosomula and tumor cells have been demonstrated previously (67). The cytotoxicity was found to be coincident regardless of the method of macrophage treatment (59,67). In contrast, it has been noted that despite common mechanisms of macrophage induction and expression, the observed macrophage effector functions may be variable (88). Killing of the yeast form of C. albicans by peritoneal exudate cells, does not correlate with tumor cytotoxic activity (13). Other comparisons of activated macrophages have shown that extracellular tumoricidal activity did not correlate with intracellular candidacidal activity (114). Macrophage activating factors

which induced macrophage extracellular tumoricidal and schistosomulicidal activity have been shown to differ from factors which induce intracellular microbicidal activity (89).

The results of this investigation show that the capabilities of resident (group I), proteose peptone-elicited (group II), proteose peptone-elicited and LPS activated for 18 h (group III), and proteose peptone-elicited and LPS activated for 72 h (group IV) macrophages to be cytotoxic to Candida albicans paralleled their capacity to be cytotoxic to a cultured cell line, the EL-4 thymoma.

Resident macrophages placed in culture for 18 h were found to possess both extracellular fungicidal and tumoricidal activity. Previously published work has shown resident macrophage populations to be at a basal level of activation (82,128). In those experiments, resident macrophages were defined as cells derived from the peritoneal cavity of mice and immediately employed for cytotoxic activity against an extracellular target (128). In contrast, in this investigation macrophages were cultured for 18 h prior to assessment of macrophage predicted cytotoxicity. Under these conditions, resident macrophages do have the ability to kill both fungi and tumor cells in an extracellular manner.

Likewise, the tumoricidal assay showed levels of

resident macrophage activity which were higher than that which has been reported previously (105). This may be attributed to variations in target type, mouse strain, macrophage concentration, or the LPS employed. However, it is more probable that the relative activity of the macrophages cultured for 18 h prior to onset of the activation process have an enhanced cytotoxic activity. During the 18 h culture period, macrophages were adherent to plastic culture vessels and possibly exposed to small amounts of contaminating LPS contained within the serum of the media. Weinberg has shown LPS contamination of media culture products leads to a more activated macrophage state (128). Therefore, the definition of resident macrophages in this study does not necessarily equate with macrophage populations termed "resident" in other studies (27,105). The 18 h period of incubation was necessitated by the experimental design in that a simultaneous time course for all experiments was vital for comparative purposes.

In vitro, resident macrophages may be induced to the activated state by simulating conditions in vivo. For example, resident macrophages may be primed by an antigenic stimulus resulting in the release of lymphokines. Such a stimulus could serve as an eliciting agent, which has been reported previously to produce enhanced cytocidal effects (54). Macrophages induced by

sterile irritants have been shown to be potentially capable of cytolytic activity, if noncytolytic but "receptive" monocytes were present (103). Proteose peptone-elicited macrophages (group II) from both the tumoricidal and fungicidal assays displayed a parallel increase in the extracellular killing of both targets. A significant increase such as the one demonstrated here does not agree with previous work which has demonstrated proteose-peptone elicited macrophages do not reach a level of macrophage activation which results in microbicidal or tumoricidal activity (128). In those investigations, macrophages were removed from the peritoneum and utilized immediately (128). Macrophages used in experiments here were allowed to remain in culture for 18 h prior to the activation required for group III macrophages. Therefore, a total time of 36 h elapsed prior to evaluation of cytocidal activity. This treatment was identical to treatment received by group I or resident macrophages which also resulted in higher than expected cytocidal activity. Nevertheless, at a level of $p<0.001$ parallel differences have been found in each of the assays. These results indicate an increased cytocidal activity for the overall population of cultured macrophages.

When macrophages were treated with LPS (10µg/ml) for 18 h (group III), maximum tumoricidal and fungicidal

activity was found for both assays. The parallel increase in functional cytocidal activity was apparent. This is consistent with previous reports which show that LPS can induce cytotoxicity by macrophages which are already partly activated to maximum activity without lymphokines (16,128). To become cytocidal, less activated cells require large doses (10µg/ml) of LPS (16). Unactivated (resident) cells have been found to vary in this response (16). However this study has shown that resident macrophages do obtain cytocidal activity when treated with large doses of LPS. Group III macrophages were also observed to show an enhanced level of activity over group II macrophages in both systems. Again, these results suggest parallel levels of fungicidal and tumoricidal activity by the macrophage population. The parallel increases in cytotoxic activity suggest the cytocidal effect to be non-specific in nature. That is, the mechanisms employed appear to be capable of lysing diverse targets.

The one observed difference in which the relative tumoricidal and fungicidal activity did not parallel each other, was with group IV macrophages. It was expected that macrophages which had been maximally activated would return to a level of cytocidal activity at or near the unactivated or resident macrophage (2). Group IV macrophages did return to a level of fungicidal

activity which was not significantly different from resident or group I macrophages. However, the tumoricidal activity of group IV macrophages dropped to a level significantly below that of the resident macrophages. This may be attributed to the length of the tumoricidal ^{51}Cr release assay in that the macrophages were exposed to 10 µg/ml of LPS for 96 h. The responsive capability of the macrophages may have been overwhelmed by this continued stimulation. This is further supported by the observation that cultures of group I and group II macrophages which were not activated with LPS, but were simply cultured for 72 h, demonstrated a level of activity near group I for both the tumoricidal and fungicidal assays. The observation of a reduction in cytocidal activity provides a possibility that the macrophage may be a self-regulating population. The cells may produce a signal which operates in a manner similar to the process of activation. This regulatory product may be an arachidonic acid metabolite, such as prostaglandin E, which has been shown to down-regulate macrophage functional capacity by limiting expression of tumor cytolysis and certain other macrophage capacities. The suppressive effect may also be a result of tolerance to the original stimulatory signal. Clearly, a reduced response was observed. Whether the effect is a consequence of a metabolite

blocking a terminally differentiated or tolerant cell or simply a cell which has returned to its basal level of activity remains to be demonstrated. Nevertheless, both assay systems displayed significant decreases in cytotoxicity from groups II and III to group IV. In this respect parallel killing was observed for all four groups of macrophages.

When activated with LPS, thioglycollate-elicited macrophages displayed the highest levels of cytocidal activity in both assays. Thioglycollate-elicited macrophages displayed, in both the tumoricidal and fungicidal assays, an enhanced cytotoxic activity greater than that of macrophages elicited with proteose peptone. Thioglycollate broth is often used as an eliciting agent because it has the advantage of recruiting large numbers of cells to the site of inflammation (117). However, the microbicidal activity of these macrophages is variable, indicating the macrophages may not be totally activated (85). In contrast to previous studies which place thioglycollate-elicited macrophages into a functionally resident or primed classification (54,74), experimental evidence here indicates that thioglycollate-elicited macrophages may be in an activated state. There was no difference between these macrophages and proteose peptone-elicited macrophages activated with LPS. These results may be the conse-

quence of enhanced activation *in vivo* or as in the case of group I and group II macrophages, a higher level of activity induced during the activation period *in vitro*.

Miake has proposed that functional activities of macrophage populations vary dependent upon the elicitation agent employed (85). Miake further stated that macrophage populations may have different levels of cytocidal activity or activation. The results of this study have shown similar variability. Proteose peptone and thioglycollate broth both elicited macrophage populations which displayed significantly different tumoricidal and candidalcidal activity. Upon activation with LPS both of these populations displayed levels of activation which differed, indicating that levels of activation are dependent upon the elicitating agent and the activation signal.

Variable cytocidal activities have been reported for thioglycollate-elicited macrophages (16,61,90). Russell has shown that thioglycollate-elicited macrophages exposed to µg/ml concentrations of LPS will activate macrophages for tumoricidal activity, which is consistent with the study performed herein (105). Additionally, Russell has shown that thioglycollate-elicited macrophages do possess tumoricidal activity without stimulation of LPS. Other groups have reported

different findings (27,49). Any number of factors may contribute to this discrepancy. It has been proposed that enhanced macrophage cytocidal activity of thioglycollate-elicited macrophages may be the result of macrophage activation due to LPS-contamination (105). Additionally, the cytocidal response of thioglycollate-elicited macrophages has been shown to vary depending on the particular batch of thioglycollate utilized. Campbell (16) has demonstrated that Brewer's thioglycollate medium which was allowed to oxidize, produced macrophages which had enhanced cytocidal activity when cultured with LPS. The thioglycollate used in these experiments was allowed to oxidize for at least 6 months at room temperature prior to elicitation process.

Many other points of variability exist when considering elicitation with thioglycollate, including the observation that varying levels of LPS concentrations have also been shown to be required for activation of these cells to a cytocidal state (16). However, the high levels of LPS utilized in this study should allow for maximum activation. The age of the mouse may also vary the activation level of thioglycollate-elicited macrophages. Hopper has shown no difference in the number of macrophages elicited from the peritoneum of mice from various eliciting agents at 6-8 week (54). However, older mice display a different pattern of

macrophage inflammation and activity, thus displaying more variability. Therefore, variations in the ages of mice employed in previous studies may have resulted in variable cytocidal effects (54).

Bone marrow-derived macrophages have been described as a precursor cell which matures through the monocyte maturation pathway to become a fully functional macrophage (127). Previous investigators have shown bone marrow-derived macrophages, upon activation, become tumoricidal (127). If bone marrow-derived macrophages are driven to maturation prior to establishment of a resident state, they possess a variety of extracellular cytocidal activities against such targets as the yeast form of both Candida, Cryptococcus, Leishmania, and tumor cells (10).

It was the purpose of this investigation to evaluate the maturation sequence of bone marrow-derived macrophages after two weeks of in vitro cell culture. Macrophages which were allowed to remain in culture without the addition of LPS displayed levels of candidacidal activity which were not significantly different from resident macrophages. The candidacidal activity was slightly higher, and could result from the length of culture required to achieve maturation.

Identically treated bone marrow-derived macrophages were pulsed with 10 µg/ml of LPS 18 h prior to evalu-

ation of candidacidal activity. This group of macrophages obtained levels of activity similar to that of group II macrophages. There were significant differences between these macrophages and peritonital macrophages activated with LPS. It is possible that the bone marrow-derived macrophages activated with LPS are in a state of activation either between groups II and III or between groups III and IV. The 18 h activation period employed may have been inadequate and henceforth, would not allow sufficient time for proper activation to full cytotoxicity. Alternatively, LPS stimulation may have been either too strong or too delayed following stimulation. Either of these would result in the downregulation of function and place the macrophages into a level of cytocidal activity between groups III and IV.

The tumoricidal activity of the macrophage groups correlate with previous attempts to define discrete stages of macrophage activation. The terminology of resident, primed, and activated have been used to describe levels of activation with regard to tumoricidal activity (43). The methodology and treatment of the macrophages in these experiments was similar to the procedure employed in this investigation. Other stages of activation have been proposed, such as inflammatory (60) and responsive (61). However, these stages are difficult to evaluate because they are activated with

lymphokines and furthermore they are defined by appearance of certain receptors such as C3 and 5'-nucleotidase (61). These stages may indeed be apparent. However, the comparison of functional cytocidal activity versus the appearance of cellular maturation markers has not been made.

The regulation process of macrophage activation for functional activity is complex. Comparisons of functional activities must consider various aspects of the assays used to measure functions including: the origin of the responding cell, the activation signal, the susceptibility of the target to the macrophage, and nuances associated with the assessment of macrophage function (81). In this study these parameters have been addressed. An inbred strain of mouse was used to obtain the groups of macrophages. The macrophages were treated in an identical manner from elicitation through lavage to activation, both throughout the study and within each experiment. The macrophages were treated together until separated for the assays, at which point treatments received were identical. The same level of LPS was employed for all experiments (10µg/ml). This was based on work by Weinburger which showed LPS at 10 µg/ml to be effective in activating macrophages to tumoricidal activity. Additionally, <u>in vitro</u> experiments have shown LPS to stimulate macrophages to kill <u>Candida</u> at 10µg/ml

(98). Furthermore, both targets have been shown previously to be susceptible to the cytotoxic effects of macrophages.

Other considerations must be made regarding these assays such as the nature and concentrations of both the target and effector cells. Adherent peritoneal exudate cells have been defined in this study as macrophages. This definition implies only that non-adherent cells were removed from the culture and does not absolutely exclude the possibility that a small number of residual non-adherent cells may remain which either directly or indirectly influence the cytotoxic response of the macrophages. Campbell has shown that PECs were equally cytotoxic whether removed by simple washing techniques such as those employed herein, or by multiple vigorous washings. Additionally, if excess non-adherent spleen cells were added to the culture wells, no increase in cytotoxicity was noted (16). Furthermore, macrophages must be placed in culture, such that a less than confluent monolayer of cells results (16,99). This is important for two reasons. First, if the concentration is too high, macrophages tend to round up and spread poorly, resulting in inhibition of macrophage function. Second, if the macrophages are too scarce in the culture vessel, the cells fail to produce optimal cytocidal activity. This study utilized macrophage concentrations

similar to those employed by Cambell for the tumoricidal assay. Likewise, the concentration of macrophages used for the candidacidal assay was recommended by the author of the assay to provide a monolayer (46).

While some of the problems associated with each assay can be regulated, others cannot be as stringently controlled. For example, macrophage cytotoxicity assays must take into account LPS contamination (99). The responsiveness to LPS appears to correlate with the stage of macrophage functional differentiation, therefore, if macrophages with lower levels of functional activity were exposed to contaminating doses of LPS they may become further activated, as noted previously (127). Further variations such as the stage of differentiation and consequent functional responsiveness are parameters not easily controlled, and are likely to be responsible for variability among different systems (65).

A direct comparison of the data between the assays must take into account the differences in the two experimental systems. One employs direct examination of cytotoxicity while the other measures an index of cytotoxicity as judged by the release of radioisotope. The different methodologies involved with these assays may account for the different range of data obtained. Therefore, levels of activity between these assays cannot be compared directly. However, comparisons can

be made between groups within an experimental system and comparisons can be made between relative changes in both systems. The results of these experiments, have shown statistically that nearly parallel levels of extracellular cytocidal activity for fungi and tumor cells exist. The only exception was in post-cytolytic activity where the tumoricidal capacity of the macrophages dropped to a level significantly lower than group I. This was not the case for fungicidal activity which returned to group I levels. The observed difference may relate to the total time required to accomplish the two assays; measurement of fungicidal activity was 73 h in duration, while measurement of tumoricidal activity required a total of 96 h. Likewise, the difference in culture time and the difference in actual assay time may contribute to the varied results, but has not been explored. Macrophages possess numerous physiological properties that may be modulated during the activation process. An expression of one such function, extracellular cytocidal ability, may be related in some form to other macrophage functions. However, the parallel levels of cytocidal activity suggest that extracellular killing is the same for all macrophage groups employed in this investigation.

The mechanism by which macrophages recognize and lyse various targets is poorly understood (2). However,

it is possible to speculate on a mechanism of killing by which the macrophages employed within this study lyse two diverse targets. The concept of the nonspecific response of macrophages originally proposed by Mackannas (77), does not explain or demonstrate how a macrophage recognizes, binds to, and destroys targets recognized as foreign. The data obtained by many investigators offer a variety of theories regarding this matter (2,60,130). However, the data obtained in this study supports the concept of similar interactive and functional activities, regardless of the nature of the target.

Complex cytocidal mechanisms for macrophage-mediated destruction for a diverse set of target cells has been described (6). Each of a multitude of toxic substances has been proposed as the effector molecule for cytotoxic activity including; proteases, C3a, arginase, H_2O_2, TNF, and cytotoxins. The primary reaction of macrophage activation could result in increased synthesis of factors mediating the cytotoxic activity (88). Alternatively, precursor formation, or accessory system development could assist in cytocidal activities (88). Macrophages could release these effector or precursor molecules, resulting in lysis various targets.

The process of macrophage cytotoxicity is normally

considered to be cell-contact dependent. Therefore, upon recognition and binding of the target cell, a small surface area of intimate contact would be established (3). Such surface contact could trigger a series of metabolic events which allow the macrophage to secrete a vast array of cytotoxic compounds (5). These compounds would be localized between the macrophage and its target. The area of macrophage-target surface contact has been speculated to be protected from these compounds (3). Likewise, the effector molecules would be protected, both from dilution in the extracellular milieu, and from degradation by extracellular proteases or inhibitors (5). Under some conditions, the macrophage may not be protected from this concentrated assortment of toxic metabolites and several studies have shown deleterious effects of secretion products on macrophage membranes invoked during the course of target cell destruction (33).

Binding of targets to receptors on the macrophage is thought to serve several functions in cytolysis (119). Two types of tumor cell binding exist; low level and high level. Low level-binding is a nonselective attachment between many cell pairs while high level-binding allows only selective binding between tumor cells and activated macrophages (60). It is possible for macrophages to convert a weak nonselective binding

to stable binding if tight binding between macrophage and target cell occurs (113).

Macrophages selectively bind tumor cells with specific cell markers to their surface. The biochemical determinants of the macrophage tumor cell receptor are unknown except for the participation of a trypsin-sensitive cell-binding site which is very similar to a receptor (113). However, unlike normal receptor-ligand interaction, energy is required to bind the tumor cell. Certain tumor cells have adapted their markers in such a way as to be resistant to recognition by certain cell types in certain strains of animals (2).

Macrophages also possess receptors which recognize nonphosphorolated, terminal mannose, N-acetylglucosamine and fucose residues (24). Such a recognition mechanism provides a means for microbial binding prior to phagocytosis (11). The blockage of this calcium dependent receptor inhibits the binding of mannose by macrophages, indicating a specificity for mannose (119). This specificity is not enhanced or decreased by level of macrophage activation. Furthermore this mannose specific receptor diminishes in latter stages of activation (57).

Different effector molecules may exist for different targets. The secretion of the multitude of cytocidal substances allows both for the specific

killing of targets by one mechanism and also for molecular synergism, resulting in enhanced cytocidal activity. For instance, specific mechanisms such as ROI have proven very effective against Candida while nonoxidative mechanisms seem only to mediate partial cytocidal effects (26). Conversely, secreted products such as TNF and cytotoxic factors have proven effective against tumor cells, while oxidative mechanism for tumoricidal activity are equivocal (91). Oxidative and non-oxidative means of cytocidal activity have been shown to cooperate in tumoricidal systems. H_2O_2 and cytotoxins combine for greater tumoricidal effect than either produces separately (60).

The expression of nonspecific effector activities by macrophages differ with the nature of the target and its relationship to the macrophage. The activation for extracellular versus intracellular effector activity share many common characteristics but can ultimately be dissociated on the basis of the target, the responding cell, and inductive signal. The details of each effector function of activated macrophages are unique for each target and has been shown to be dependent upon the lymphokine which activates the macrophage (88). Therefore at one level, regulation may not be dictated by the target, but by the site, either intracellular or extracellular (88). For example, the alteration of

membranes associated with phagocytosis may regulate the cell and initiate intracellular killing mechanisms (96). It can be speculated that lymphokines which induce intracellular killing provide the same type of activating signal to the macrophage as that of phagocytosis.

The regulation of extracellular killing is much more complex. Maximum antimicrobial activities against intracellular organisms is induced within 4 h regardless of the sequence in which the target, macrophage, and signal are presented. However, maximal extracellular killing requires the presence of macrophage, target and activation signal at the same time (88). Furthermore, any ingested cell could be killed by a combination of cytotoxic products concentrated within the phagosome. However, due to the nature of the killing process, the macrophage must recognize which extracellular cytocidal mechanism to induce for effective killing of a nonphagosotized organism.

Evaluation of the extracellular killing mechanism employed in this study proves difficult for the following reason. The ability of the macrophage to kill two distinct targets, a single eukaryotic cell compared to a multiseptated hyphae implies utilization of different effector mechanisms. This is due to the variable susceptibility of fungal wall polymers of mannose as compared to the simple phospholipid bilayer

associated with tumor cells.

These mechanisms may be regulated at two different levels. At the first level, the cell activates either intracellular or extracellular mechanisms of cytocidal activity. Destruction of two different extracellular targets may be induced by the same cytolytic mechanisms, which are activated by signals which trigger extracellular killing mechanisms. Conversely, intracellular cytolytic activity would be activated by signals which would induce intracellular killing.

The secondary level is more complex. It must be noted that several possibilities exist. First of all macrophages may recognize target cells by binding to them as a result of certain receptors (i.e. mannose) present on macrophages. This binding may then activate a particular cascade of products which could allow the macrophage to respond in an appropriate and specific manner to a stimulus. Secondly, all products of the cytotoxic potential may be released to the extracellular environment. The regulation of this release would be time dependent. Therefore, only at various time points in the maturation process would certain effector molecules be released. This may limit specific killing of targets yet would allow for nonspecific killing, albeit at various time points.

Finally, functional subsets of macrophages which

were present upon maturation to macrophages would be activated against a specific target. These cells would be activated to mediate specific cytocidal effector mechanisms against specific predefined targets.

Data from this study which shows parallel levels of cytocidal activity in four different groups of macrophages, against these two targets does not support the last possibility, that functional subsets exist. However, many investigators favor this explanation (22,96,127).

Meltzer has proposed that all cytocidal mechanisms are enhanced first intracellular, then extracellular microbicidal activity, followed by tumoricidal activity (80). This study does not refute this concept, since the variance in time frames which may be required to observe this method of cytocidal activity utilized may not have been studied. However, in the context of this study, sequential effector function must be questioned since parallel increases and decreases in cytocidal activity were found with all groups of macrophages analyzed. Alternatively, extracellular killing for all targets may be parallel.

It is entirely possible that macrophages, once activated for extracellular cytocidal activity recognize specific markers on target cells such as mannose or tumor cell membrane markers. Once bound the macrophage

would then rechannel cytocidal activity to specific mechanisms based on which receptors are bound. It has been shown that macrophages which are strongly tumoricidal and then challenged with schistosoma are only weakly cytolytic (88). While it is possible that sequential levels of functional activity may be invoked, experiments to assess specific recognition have not been performed. Other studies have shown that macrophages activated for a particular tumoricidal response do show levels of activity for other organisms, thus implying that activity against these targets appears in a secondary or nonspecific manner.

Finally, this study has demonstrated that macrophages at various levels of activation possess varying levels of cytocidal activity. However, it is known that nonactivated macrophages possess higher levels of mannose specific receptors. Therefore, such macrophages would bind more readily to Candida. Once bound the Candida may activate the macrophage directly through binding, or indirectly through activation mechanisms similar to those described for LPS and lymphokines.

The results obtained in this study emphasize many of the problems encountered when studying macrophage activation. Grouping, elicitation, cell targets and functional differences found in the different stages of activation all contribute to variability among results

(88). Any given population of cells may also vary due to state of the host and dose of antigenic stimuli. Therefore, during active infection or simulated conditions of active infection, a maximum stimulus for macrophages occurs (130). Conditions of maximum stimulation, would be the most opportune time to comparitively study functional properties of macrophages. It has been suggested that these properties may occur and be lost at different rates within the population (130). This renews the theory that separate subpopulations of effector cells display activated properties and could modulate the population as a whole (126). Despite the merits of this theory (130) the data collected herein would contradict it, since parallel functional levels of activity have been found in both the candidacidal and tumoricidal systems at each level. The only exception to this was macrophages incubated with LPS for 72 hrs. In this case the macrophages from both assays did return to a level near that of the resident macrophages. The tumoricidal assay dropped to a level even lower, probably due to reasons already discussed. Alternatively, the mechanism by which macrophages produce their cytocidal effects may be similar, which could explain in part the parallel cytocidal activity (88).

A comparison of macrophage extracellular tumori-

cidal activity, as judged by ^{51}Cr release assay, and fungicidal activity, as judged by inhibition of Candida albicans yeast formation following hyphal induction, has resulted in a strong correlation of activity with four separate macrophage treatment groups. This correlation indicates parallel levels of extracellular cytocidal activity over a range of the macrophage maturation groups and suggests similar mechanisms of extracellular killing.

SUMMARY

This investigation compared the tumoricidal and candidacidal activity of four groups of murine macrophages. Resident macrophages (Group I) displayed the least cytocidal activity. Proteose peptone-elicited macrophages (Group II) showed a significant increase in cytocidal activity over group I ($p<0.001$) in both tumoricidal and fungicidal assays. Proteose peptone-elicited, 18 h LPS-treated macrophages (Group III) displayed a significantly greater level of activity than either group I ($p<0.001$) or group II ($p<0.001$) macrophages in both tumoricidal and fungicidal assays. Proteose peptone-elicited, 72 h LPS-treated macrophages (Group IV) showed a decrease in fungicidal activity to a level near group I, while the tumoricidal activity dropped to a level significantly below group I ($p<0.001$) macrophages. These data indicate a parallel relationship between the killing, via extracellular mechanisms, of both fungi and tumor cells by these four groups of macrophages.

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APPROVAL SHEET

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The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorportated and that the thesis is now given final approval by the Committee with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

4/14/87
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