Enhancement of the Candidacidal Activity of Cultured Peritoneal Exudate Cells by SM-1213, a Synthetic Immunomodulator

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ENHANCEMENT OF THE CANDIDACIDAL ACTIVITY OF CULTURED
PERITONEAL EXUDATE CELLS BY SM-1213,
A SYNTHETIC IMMUNOMODULATOR

by

Christine Joy Morrison

A Dissertation Submitted to the Faculty of the
Graduate School of Loyola University of Chicago in
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<tr>
<td>BCG</td>
<td>Bacillus Calmette-Guérin</td>
</tr>
<tr>
<td>BL</td>
<td>Boiled homogenate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cfu</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>CGD</td>
<td>Chronic granulomatous disease</td>
</tr>
<tr>
<td>CMC</td>
<td>Chronic mucocutaneous candidiasis</td>
</tr>
<tr>
<td>CMI</td>
<td>Cell-mediated immunity</td>
</tr>
<tr>
<td>Con A</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>EBSS</td>
<td>Earle's balanced salt solution (phenol red free)</td>
</tr>
<tr>
<td>EPO</td>
<td>Eosinophil peroxidase</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>FCC</td>
<td>Ferricytochrome c</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's balanced salt solution (phenol red free)</td>
</tr>
<tr>
<td>HMP</td>
<td>Hexose monophosphate</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HPO</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MDP</td>
<td>Muramyl dipeptide (N-acetyl-muramyl-L-alanyl-D-isoglutamine)</td>
</tr>
<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>NAD(H)</td>
<td>Nicotinamide adenine dinucleotide (reduced)</td>
</tr>
<tr>
<td>NADP(H)</td>
<td>Nicotinamide adenine dinucleotide phosphate (reduced)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>NAD(P)H</td>
<td>Nicotinamide adenine dinucleotide (phosphate), reduced</td>
</tr>
<tr>
<td>NAGA</td>
<td>β-N-Acetylglucosaminidase</td>
</tr>
<tr>
<td>NBT</td>
<td>Nitroblue tetrazolium salt</td>
</tr>
<tr>
<td>ND</td>
<td>Not determined</td>
</tr>
<tr>
<td>NI</td>
<td>Non-idet detergent</td>
</tr>
<tr>
<td>( ^1\text{O}_2 )</td>
<td>Singlet oxygen</td>
</tr>
<tr>
<td>( \text{O}_2^- )</td>
<td>Superoxide anion</td>
</tr>
<tr>
<td>•OH</td>
<td>Hydroxyl radical</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PEC</td>
<td>Peritoneal exudate cell(s)</td>
</tr>
<tr>
<td>PEC-O</td>
<td>Oil-elicited PEC</td>
</tr>
<tr>
<td>PEC-r</td>
<td>Resident PEC</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohemagglutinin</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol myristate acetate</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear leukocyte(s)</td>
</tr>
<tr>
<td>PRS</td>
<td>Phenol red solution</td>
</tr>
<tr>
<td>SBA</td>
<td>Soybean agglutinin</td>
</tr>
<tr>
<td>SDA</td>
<td>Sabouraud's dextrose agar</td>
</tr>
<tr>
<td>SDB</td>
<td>Sabouraud's dextrose broth</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
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<tr>
<td>SM-1213</td>
<td>1,2-O-Isopropylidene-3-O-3'-(N',N'-dimethylamino-n-propyl)-D-glucofuranose</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>SWA</td>
<td>Waymouth's medium supplemented with 2 mg/ml BSA and 5 μg/ml oleic acid</td>
</tr>
<tr>
<td>TX-100</td>
<td>Triton X-100 detergent</td>
</tr>
<tr>
<td>---------</td>
<td>------------------------</td>
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<tr>
<td>UND</td>
<td>Undetectable</td>
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CHAPTER I

INTRODUCTION AND REVIEW OF RELATED LITERATURE

Research interest has recently developed for a heterogeneous group of chemical compounds which have potential therapeutic usefulness as antimicrobial agents due to their capacity to augment responses of lymphocytes and macrophages. These compounds have been termed "pro-host" (40) because, rather than being directly microbicidal, they enhance the antimicrobial activities of host defense cells.

Included in such leukocyte-modulating substances are semi-synthetic or wholly synthetic agents such as muramyl dipeptide (MDP) (13,40), inosiplex (40,111) and levamisole (33,40). Another such agent is SM-1213 [1,2-O-isopropylidene-3-O-3'-(N',N'-dimethylamino-n-propyl)-D-glucofuranose, monohydrochloride], a synthetic, substituted monosaccharide which is reported to potentiate macrophage bactericidal activity (12,40). Recent studies describe antiviral, antitumor and immunomodulatory activities of SM-1213 (36,74), but few studies have examined for the effects of SM-1213 on an in vitro fungal infection of host phagocytes (83,84).
The present work examined for a potential enhancing effect of SM-1213 on the fungistatic or fungicidal capacities of oil-elicited guinea pig peritoneal exudate cells (PEC-O) and for the mechanism of such an effect against both the yeast and the hyphal forms of Candida albicans, a clinically important dimorphic fungus (44).

Candida albicans is an opportunistic fungus in that it is 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 (34,105). Host resistance to infection by C. albicans involves specific cell-mediated immune responses (54,55,68), as well as non-specific host defense mechanisms (47,82,120) in which phagocytic cells predominate (106).

Cell-mediated immunity (CMI) has been implicated as being of primary importance (54,55) in protection from the chronic mucocutaneous form of candidiasis (CMC). Positive therapeutic results were obtained after administration of a combination of antifungal antibiotics and injections of transfer factor or immunocompetent lymphocytes, suggesting a defect in thymus-dependent CMI in these patients (54,55). Candidal dissemination occurs rarely in CMC patients, however (58), and the mechanism whereby
candidal spread is prevented is not clear. For example, in congenitally athymic nude mice (108), resistance to experimental disseminated candidiasis was greater than that observed for their phenotypically normal littermates. Antibody-mediated immune responses were severely impaired and could not, therefore, account for the enhanced resistance of the athymic mice (108). It was suggested that high levels of activated macrophages contributed to the observed effects (108). This suggestion, along with the observation that disseminated candidiasis occurs in patients with chronic granulomatous disease (CGD) or myeloperoxidase (MPO) deficiency, where the antimicrobial capacities of phagocytic cells are impaired (60,64), implies that neutrophils or activated macrophages play the most important role in host defense against systemic candidiasis (108). [For reviews, see (30,107).] It has also been suggested, however, that phagocytes may actually promote the establishment of renal candidiasis in thymus-bearing mice (58). Therefore, in order to understand the critical role macrophages play in host resistance to candidiasis, several researchers have examined the capacity of mononuclear phagocytes to ingest and kill *C. albicans* in *vitro* (62,118,122).

Conflicting information regarding the ability of cultured macrophages to destroy *C. albicans* has resulted
from the utilization of mononuclear cells derived from various sites, such as the blood (62), the peritoneum (99,118) or alveoli (3,65), and from various animal species including mouse (118,122), man (62) and rabbit (3,65). Analysis is further complicated, since resident (118,122), elicited (99) or activated (65,118) macrophages have been employed under multiple culture conditions. Resident or normal macrophages and circulating blood monocytes are obtained from untreated animals, while elicited macrophages are derived from animals injected with an inflammatory stimulus, such as glycogen, thioglycollate, casein, proteose peptone, mineral oil, or a bacterial product such as lipopolysaccharide (LPS). Activated macrophages are obtained from animals vaccinated by injection of an agent such as bacillus Calmette-Guérin (BCG) or infected by intracellular parasites such as Mycobacterium or Listeria (48). During the later states of infection or after host recovery, activated macrophages possess an enhanced capacity to kill the same or unrelated organisms (72), and display such characteristics as an increase in size, enhanced spreading, greater numbers of lysosomal granules and an increase in content of hydrolytic enzymes (17,53). Elicited macrophages share some, but not all, of these properties. [For a review of the biochemical aspects of mouse peritoneal macrophages in various physiological
states as compared to resident macrophages, see Karnovsky and Lazdins (53).] The oxidative response of activated and elicited macrophages to a phagocytic stimulus is also enhanced in these cells relative to resident macrophages. [For a review of the evidence that the phagocytosis-associated oxidative burst is increased in elicited and activated macrophages, see Johnston (48).]

Production of reactive oxygen metabolites by macrophages mediates much of their microbicidal (50,118,127) and tumoricidal (92,97) activity, although some killing by oxygen-independent mechanisms also occurs (66,67,86). Oxygen-dependent systems are classified as such because of a decrease in microbicidal or tumoricidal activity under anaerobic conditions (63,81,97). However, some microbes (76), such as Bacteroides fragilis, Staphylococcus epidermidis and Clostridium perfringens, are normally killed by phagocytes in the absence of air. Therefore, oxygen-independent antimicrobial mechanisms must also exist. Possible mediators of macrophage oxygen-independent cytotoxic activity include acid hydrolases (19,117), alkaline phosphatase (29), lysozyme (11,52,66), a complement cleavage product, C3a (31), thymidine (121), arginase (24), neutral proteases (1, 2) and cationic proteins (67,101). Although cytotoxic, several of these agents may digest dead target cells (56) killed by predominantly oxidative mechanisms (15,76,118).
Particle ingestion by phagocytes is associated with a metabolic burst characterized by an increase in oxygen consumption, an enhanced oxidation of glucose via the hexose monophosphate shunt (HMP) pathway, and a substantial production of hydrogen peroxide \((\text{H}_2\text{O}_2)\) and superoxide \((\text{O}_2^-)\) (48). The superoxide anion, formed by the addition of an electron to molecular oxygen, can gain an additional electron to produce \(\text{H}_2\text{O}_2\) or lose an electron to produce molecular oxygen (4). Two superoxide radicals can be spontaneously dismutated to \(\text{H}_2\text{O}_2\) and oxygen or enzymatically converted to these products by the action of superoxide dismutase (SOD) (79):

\[
\text{O}_2^- + \text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2
\]

The resulting \(\text{H}_2\text{O}_2\) can also be removed enzymatically to produce \(\text{H}_2\text{O}\) and oxygen by the action of catalase.

The toxicity of \(\text{O}_2^-\) and \(\text{H}_2\text{O}_2\) for microorganisms was initially proposed by McCord et al (80), since aerobic organisms contain relatively high levels of SOD and catalase while strict anaerobes lacked these enzymes. The main function of these enzymes was hypothesized to protect microorganisms normally exposed to oxygen from reactive oxygen metabolites. This theory was supported by the finding that not only was superoxide anion capable of killing organisms, but exposure of \textit{E. coli} to high oxygen tensions induced an increase in microbial SOD levels (38).
However, it is not clear that microbial SOD or catalase can protect microorganisms from damage by external oxygen metabolites (61,118). While *C. albicans* contained ten times the concentration of SOD and catalase as *C. parapsilosis*, both species were killed equally by chemically-generated oxygen radicals (118). Also, Lehrer found that *C. albicans* was killed more easily by reagent H$_2$O$_2$ than was *C. parapsilosis* at all concentrations tested (61). On the other hand, SOD and catalase may be protective during the interaction of organisms with phagocytic cells. Neutrophils were reported to kill a low-catalase strain of *S. aureus* more easily than a high-catalase strain (77); catalase-deficient macrophages, whether the deficiency was genetic or chemically-induced, were better able to kill *Toxoplasma gondii* (88).

The production of H$_2$O$_2$ and O$_2^-$ by phagocytes may also lead to the formation of other reactive oxygen species (4) possessing antimicrobial activity (118). Such agents include hydroxyl radical (·OH) and singlet oxygen (¹O$_2$), which may be produced as a result of the interaction of O$_2^-$ and H$_2$O$_2$ in the following manner:

$$O_2^- + H_2O_2 \rightarrow ¹O_2 + OH^- + ·OH$$

The observed microbicidal activity of phagocyte-produced H$_2$O$_2$ and O$_2^-$ may therefore result from the formation of other highly reactive oxygen metabolites, as well (56).
Of the mononuclear phagocytes of various origin and physiological state assessed for candidal killing capacity, resident rabbit alveolar macrophages have been reported to be candidastatic (103) or candidacidal (65), while human blood monocytes (62,69) have been shown to be effectively candidacidal. Resident mouse (118) or rabbit (65) peritoneal macrophages have been demonstrated to be less candidacidal than resident rabbit alveolar macrophages. Stanley and Hurley reported that resident mouse peritoneal macrophages could ingest Candida yeast cells, but these macrophages were eventually destroyed by the intracellular elongation of hyphal elements (122). Others have demonstrated that casein-elicited mouse peritoneal macrophages suppress the metabolism and growth of phagocytosed yeast cells for 2-3 hours, but thereafter candidal growth proceeds, resulting in the destruction of the macrophage (99). In these instances, however, mouse peritoneal macrophages were maintained in culture for 24-48 hours before infection which may have resulted in a reduced fungicidal (59) capacity for these cells similar to that observed for other cultured mononuclear phagocytes.

The ability of resident macrophage populations to increase their fungicidal efficacy as a result of stimulation is also relevant. Sasada and Johnston (118) have reported that LPS-elicited or BCG-activated mouse peritoneal
macrophages killed 2-3 times more \textit{C. albicans} cells than did resident macrophages during a 2-3 hour period. In the host, resident macrophages may develop an enhanced capacity to kill \textit{Candida} cells via interaction with other cells or factors \cite{82,85}. For example, the required presence of lymphocytes for the development of activated macrophages and the subsequent development of normal cell-mediated immunity against such intracellular pathogens as \textit{Listeria} or \textit{Mycobacterium} has been demonstrated by Mackaness and others \cite{72,98}. The lymphokine responsible for the priming of macrophages for an enhanced oxidative and antimicrobial response to \textit{Toxoplasma gondii} has recently been identified as interferon-\textgamma\cite{94}. Maiti \textit{et al} \cite{73} demonstrated that mouse peritoneal macrophages activated \textit{in vivo} by BCG or \textit{in vitro} by exposure to phytohemagglutinin-induced lymphokines exhibited greater phagocytic and candidacidal activity than resident peritoneal macrophages. The highest phagocytic and candidacidal activity, however, was produced by exposure of macrophages to \textit{Candida}-induced lymphokines. Immune mouse serum also enhanced the phagocytic and candidacidal activity of resident, but not activated, macrophages. Therefore, humoral and cell-mediated mechanisms may be required for an effective macrophage-mediated control of candidal infection in the host.
Immunomodulatory agents such as LPS or MDP apparently act via a lymphokine-independent mechanism of macrophage stimulation (48). These agents can directly prime cultured mouse macrophages for an enhanced oxidative response (49) in the absence of lymphocytes or lymphokines (100). Evidence to support the lymphokine independence of this mechanism includes: 1) the rapid (16 hour) response of cultured macrophages to MDP and LPS; 2) the normal response of macrophages from nude mice lacking mature T lymphocytes; 3) the defective response of abnormal macrophages not correctable by the addition of normal lymphocytes; and 4) the responsiveness of the macrophage-like cell line J774.1 to LPS and MDP (100). MDP has been reported by others to enhance lymphocyte functions (14,123) as well, but the immediate target cell responsible for these effects was determined to be the macrophage (32). Others have also reported that lymphocytes are not required for LPS induction of tumor cell lysis (28) facilitated by an enhancement of macrophage oxidative responses (92).

In vivo priming for an enhanced oxidative response by cultured macrophages has also been achieved by subcutaneous injection of mice with MDP (23). Similar results were obtained with nude mice, indicating that in vivo priming can be accomplished in the absence of mature T cells. However, in the immunologically normal animal,
activation by this agent might result from a combination of lymphokine release and a direct effect on the macrophage.

The enhanced oxidative response induced by the elicitation or activation of macrophages by LPS, BCG, or MDP \textit{in vivo} directly correlates to the augmentation of candidal killing by macrophages \textit{in vitro} (23,118). In addition, administration of MDP prior to or immediately following infection with \textit{C. albicans} prolongs the survival of intravenously inoculated mice (23).

The reactive oxygen metabolites hydrogen peroxide ($H_2O_2$), superoxide ($O_2^\cdot$), hydroxyl radical ($\cdot$OH) and singlet oxygen ($^1O_2$) have all been implicated in macrophage candidacidal activity (118). \textit{Candida parapsilosis}, a less pathogenic species of fungi, elicited a greater oxidative response and was killed more efficiently by mouse peritoneal macrophages than was \textit{C. albicans} (118). Killing of both candidal species was inhibited by the exogenous addition of enzymes which remove $H_2O_2$ (catalase) or $O_2^\cdot$ (superoxide dismutase; SOD) or scavenge $\cdot$OH (benzoate) or singlet oxygen (azide) (118). Other data to support the requirement of oxygen metabolites in macrophage-mediated candidacidal activity include: 1) the macrophage-like cell line J774.1 produced less than 10% as much $O_2^\cdot$ as did resident mouse peritoneal macrophages and, although candidal ingestion was normal, candidal killing did not occur (118)
and 2) the greater candidacidal activity of LPS-elicited and BCG-activated macrophages relative to resident mouse peritoneal macrophages was associated with an enhanced capacity to release $O_2^-$ upon exposure to a membrane-perturbing agent, phorbol myristate acetate (PMA), or phagocytic particles such as opsonized zymosan (49) or Candida (118). In addition, reagent $H_2O_2$ has been demonstrated to be effectively candidacidal (118). The concentration of reagent $H_2O_2$ required for candidacidal activity was higher than that normally produced by resident or activated macrophages (92,118). However, the hydrogen peroxide-producing capacity of macrophages was measured in the extracellular environment, where culture medium diluted its absolute concentration (92,96). It has been hypothesized that, within the phagocytic vacuole or within the enclosed interstices which arise as a consequence of macrophage-to-target cell contact (93), hydrogen peroxide levels can reach effectively cytotoxic concentrations. Cytochemical techniques have documented that secretion of $O_2^-$ and $H_2O_2$ during phagocytosis is greatest at the region of cell surface contact (10).

An alternative explanation suggests that macrophage-produced $H_2O_2$ is cytotoxic because it is generated at a low concentration over a prolonged period of time and that this continued oxidative insult results in target cell
destruction (97). To support this hypothesis, Nathan et al (97) covalently coupled glucose oxidase to starch particles to facilitate contact between tumor cells in culture and the enzyme system. In the presence of glucose, this system generated \( \text{H}_2\text{O}_2 \) at a level equivalent to macrophages and was 100% tumoricidal. Evidence supporting the candidacidal effectiveness of a sustained production of oxygen metabolites arises from experiments in which a cell-free acetaldehyde-xanthine oxidase \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \)-generating system killed Candida and where candidal killing was inhibited by the exogenous addition of SOD or catalase (118).

Oxygen-dependent mechanisms are important to the candidacidal activity of the polymorphonuclear leukocyte (PMN) (60,63) and monocyte (62), as well as to the macrophage, since patients suffering from chronic granulomatous disease (CGD), a disease in which patients' phagocytes ingest organisms normally, but are deficient in \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) production (27,46), have an impaired ability to kill Candida. However, the oxygen-dependent mechanisms utilized by the PMN and monocyte may differ from that of the macrophage, since, unlike the macrophage, \( \text{H}_2\text{O}_2 \)-mediated killing by these cells is enhanced by a myeloperoxidase-\( \text{H}_2\text{O}_2 \)-halide system (56). Although peroxidase activity has been demonstrated histochemically (6) and biochemically (109) in macrophages, as circulating blood monocytes mature into
resident tissue macrophages there is a progressive loss in peroxidase activity (89). Although peroxidase activity in guinea pig peritoneal macrophages is located in the perinuclear cisternae, the Golgi vesicles and in some membrane-bound granules (22,25), the functional relevance of this enzymatic activity in relation to H$_2$O$_2$-mediated killing mechanisms remains to be established.

The enzyme primarily responsible for the respiratory burst in guinea pig macrophages has recently been identified as a membrane-bound oxidase that preferentially uses NADPH as substrate (8). Evidence supporting a membrane-bound NAD(P)H oxidase as the principle respiratory burst enzyme includes: 1) the 100,000 x g supernatants prepared from homogenates of both resting (resident) and PMA-stimulated macrophages oxidized very low levels of NADH and NADPH with the subsequent formation of O$_2^-$; 2) the 100,000 x g pellet prepared from homogenates of resting cells also oxidized NADH and NADPH with subsequent O$_2^-$ formation, but activity in homogenates from PMA-stimulated macrophages was substantially increased and higher with NADPH than with NADH as substrate; 3) the Km for NADPH was one-tenth that for NADH, and the maximum velocity of O$_2^-$ production was twice as great with NADPH as with NADH; 4) unlike myeloperoxidase (MPO), the oxidase was insensitive to azide and cyanide; 5) a direct correlation existed
between the degree of intact macrophage respiratory activity and the activity of the oxidase (8).

Of further interest, pre-incubation of oil-elicited macrophages with immunomodulatory agents such as LPS and MDP has been reported to result in an enhanced production of $O_2^-$ via the activation of the NAD(P)H oxidase of macrophages (51). NADPH-dependent $O_2^-$ production was enhanced in macrophages pre-treated with LPS or MDP for as little as 15 minutes prior to stimulation with cytochalasin E or wheat germ agglutinin as compared to macrophages which had not received LPS or MDP (51). Given this information and given that macrophage candidacidal activity has been directly correlated to the ability of stimulated macrophages to produce reactive oxygen metabolites such as $H_2O_2$ and $O_2^-$ (118), the potential enhancement of macrophage candidal killing by SM-1213, via the stimulation of macrophage NAD(P)H oxidase and the subsequent production of $H_2O_2$ and $O_2^-$, was examined and is described in the following report.
CHAPTER II

MATERIALS AND METHODS

A. Experimental Drugs

SM-1213 [1,2-O-isopropylidene-3-O-3'-(N',N'-dimethylamino-n-propyl)-D-glucofuranose, monohydrochloride] was obtained from Greenwich Pharmaceuticals, Inc., Greenwich, CT. Muramyl dipeptide (MDP, N-acetyl-muramyl-L-alanyl-D-isoglutamine·½ H₂O, grade A; Calbiochem-Behring Corp. La Jolla, CA) was used in some experiments in parallel with SM-1213, since both agents are substituted sugar moieties with immunomodulatory properties (13,35). The chemical structures of SM-1213 and MDP are presented in Figure 1. Drug solutions were endotoxin-free as determined by the Limulus amebocyte lysate assay (Sigma Chemical Co., St. Louis, MO).

B. Fungal Strain

Candida albicans, serotype A [a clinical isolate demonstrating typical carbohydrate assimilation, hyphal formation in serum and chlamydospore formation on cornmeal-tween agar (Uni-Yeast Tek system; Corning Medical Microbiology, Roslyn, NY)], was grown aerobically on Sabouraud's dextrose agar (SDA; Difco, Detroit, MI) for 24 h at 37°C.
Figure 1. A. The chemical structure of SM-1213 [1,2-0-isopropylidene-3-0-3'-(N',N'-dimethylaminon-propyl)-D-glucofuranose, monohydrochloride]

B. The chemical structure of muramyl dipeptide (MDP; N-acetyl-muramyl-L-alanyl-D-isoglutamine)
A. SM-1213

B. MDP
prior to use. Under these conditions, greater than 98% of the Candida cells were in the yeast phase.

C. Growth of Candida albicans

When Candida which had been grown on SDA were inoculated into Sabouraud's dextrose broth (SDB; Difco), greater than 98% of Candida grew as yeast. Therefore, to determine whether SM-1213 had a direct growth inhibitory effect on the yeast form of Candida, 500 ml side-arm Erlenmeyer flasks containing 100 ml of SDB were inoculated with $1 \times 10^5$ cfu. Flasks were incubated aerobically at $37^\circ$C on a rotary shaker. The increase in absorbance in flasks with the following concentrations of SM-1213 was monitored hourly over a period of 10 h: 0 (control), 1, 10, 100 and 1000 ng/ml. Absorbance readings were taken at 660 nm on a Spectronic 70 spectrophotometer (Bausch and Lomb, Northbrook, IL).

When Candida which had been grown on SDA were inoculated into serum-free Waymouth's medium (Flow Laboratories, McLean, VA) supplemented with 2 mg/ml bovine serum albumin (fraction IV, essentially fatty acid free; Sigma), 5 μg/ml oleic acid (Sigma) and 50 μg/ml gentamicin (Schering Pharmaceutical Corp., PR), greater than 98% of Candida developed hyphae after 6 h of incubation at $37^\circ$C. Candida cells displaying elongated germ tubes or pseudohyphae were defined as "hyphal" in form. To determine whether SM-1213
had a direct growth inhibitory effect upon the hyphal form of Candida, 250 ml flasks containing 50 ml of supplemented Waymouth’s medium (SWA medium) with the above concentrations of SM-1213 were inoculated with 1 X 10^5 cfu and incubated aerobically for 24 h on a rotary shaker at 37°C. Since mycelial mass formation prohibited growth analysis by absorbance measurements, hyphal growth was determined in each flask by dry weight analysis after Millipore filtration of the cultures. Three washes of the Candida were performed with distilled water during each filtration (0.45 μm pore size), followed by oven drying of the filters at 60°C for 48 h.

D. Leukocyte Harvest

One male guinea pig (Hartley strain, 750 g) was used in each of the experiments as a source of peritoneal exudate cells (PEC). To obtain oil-elicited peritoneal exudate cells (PEC-0), 20 ml of paraffin oil (light, white mineral oil; Fisher Scientific Co., Chicago, IL) was injected into the peritoneal cavity 72 h prior to animal sacrifice. PEC-0 were harvested from the peritoneum by five serial washes of 20 ml volumes of ice-cold Eagle’s basal medium (Flow) containing 50 μg gentamicin/ml (Scher ing) and 5 units sodium heparin/ml (Upjohn, Kalamazoo, MI). The washes were delivered into sterile polystyrene tubes and the cells were pelleted by centrifugation at 250 x g
for 5 min at 20°C in an International PR-6 centrifuge (International Equipment Co., Needham Heights, MA). The cells were washed by centrifugation in phosphate buffered saline (PBS, pH 7.3, % w/v in distilled water: NaCl 0.8, KCl 0.02, KH₂PO₄ 0.012 and Na₂HPO₄ 0.091), quantified in a hemacytometer, and diluted to the appropriate concentration in SWA medium. Differential counts of at least 200 PEC-0 were performed on Wright-Giemsa stained smears. The average cellular profile derived from multiple experiments was as follows: 77.2 ± 0.8% macrophages and monocytes, 2.6% PMN and 20.2% lymphocytes, mast cells and eosinophils. Total cellular harvest per animal averaged 1.17 (±0.14) X 10⁸ PEC-0.

To obtain resident peritoneal exudate cells (PEC-r), harvesting procedures were identical to those described above; however, no oil or other inducing agent was used. The average cellular profile for resident PEC was as follows: 93.0 ± 3.3% macrophages and monocytes, 0% PMN and 7.0% lymphocytes, mast cells and eosinophils. Total cellular harvest per animal averaged 3.07 (±0.61) X 10⁷ PEC-r. After 2 h in culture, greater than 95% of resident or oil-elicited adherent cells consisted of macrophages, as determined by Wright-Giemsa stain.

E. Cell Culture

PEC (>97% viable by trypan blue dye exclusion)
were seeded onto 60 mm diameter plastic petri plates (tissue culture grade; Becton Dickinson Co., Oxnard, CA) to a final concentration of 2 to 5 x 10^6 cells per plate in a total incubation volume of 3 ml. In some studies, 16 mm diameter wells (24-well, multiwell tissue culture plates; Becton Dickinson) were plated with 1 to 2.5 x 10^6 PEC per well in a total incubation volume of 2 ml. To allow attachment of PEC to the plates, cells were incubated in SWA medium with or without SM-1213 for 2 h at 37°C in 95% air-5% CO_2. After 2 h, non-adherent cells were removed by washing once with SWA medium and appropriate medium (with or without SM-1213) was replaced for those samples requiring further incubation. Final concentrations of SM-1213 in the incubation medium were: 0 (control), 0.1, 1, 10, and 100 ng/ml.

In experiments examining Candida-infected PEC, cell culture procedures were as described above except that yeast cell suspensions were inoculated into the culture medium directly following the addition of peritoneal cells in a Candida-to-PEC ratio of 1:5 to 1:10.

F. Candidal Hyphal Elongation

PEC were delivered into plastic petri plates (60 mm) containing two 2.2 cm square glass cover slips and were inoculated with Candida yeast cells as described above. At intervals of 2, 4 or 6 h, a cover slip with adherent,
infected PEC was removed from each of duplicate culture dishes, fixed in 95% methanol for 5 min, and inverted onto a microscope slide. The infected peritoneal cells were examined by phase contrast microscopy at 1000X for intracellular hyphal formation and elongation, since viable yeast cells converted to the hyphal form intracellularly. The hyphal lengths of Candida contained in 50 to 200 peritoneal cells were determined by a microscope eyepiece micrometer.

G. Candidal Viability

Hyphal viability was quantitatively determined by the microcolony method (45). This method took advantage of the dimorphic nature of C. albicans, which grew exclusively (>98%) in the yeast form when incubated aerobically on SDA. Briefly, a cover slip was removed from each of duplicate culture dishes and rinsed in successive baths of PBS and SDB. Washed cover slips were drained by touching the cover slip to filter paper. The cover slips were inverted onto SDA (3 mm thick) and incubated aerobically at 30°C. Similar results were obtained if PEC were first lysed with a solution of 0.5% Non-Idet detergent (Sigma) before placement of the cover slips on agar. The cultures were examined by phase contrast microscopy for Candida microcolony formation. Each Candida which produced at least one microcolony of four adjacent yeast cells was
defined as viable. The percentage of non-viable Candida was determined by counting 100 to 200 Candida-containing peritoneal cells:

\[
\% \text{ Candida killed} = \frac{\text{Number of Candida unable to form microcolonies}}{\text{Number of Candida able to form microcolonies} + \text{Number of Candida unable to form microcolonies}} \times 100
\]

Additional experiments were conducted to determine the effect of SM-1213 pre-treatment of PEC on their capacity to inhibit intracellular hyphal elongation and to reduce candidal viability subsequent to the removal of the drug. PEC were pre-incubated with drug for 2, 4, or 6 h and washed 3X in SWA medium to remove residual drug prior to inoculation with yeast cells. Hyphal elongation was determined microscopically by eyepiece micrometer and candidal viability was assessed by the microcolony technique previously described 2 h after infection of PEC.

In some experiments, both the microcolony technique and the methylene blue dye exclusion technique of Lehrer and Cline (63) were used for determining candidal viability.

H. Enzyme Assays and Protein Determination

Culture fluid and non-adherent cells were removed from culture dishes at 2 h and again at 4 or 6 h for samples incubated for 4 or 6 h, respectively. Values obtained at 2 h were combined with those obtained at 4 or 6 h to represent total non-adherent PEC or culture fluid values.
over the 4 or 6 h incubation period.

Non-adherent PEC and extracellular Candida, if present, were separated from the culture fluid by centrifugation at 500 x g, 4°C for 10 min. The pellet was treated with 3 ml 1% Triton X-100 detergent (TX-100; Sigma) for 10 min to disrupt non-adherent PEC or, in Candida-infected samples, with 0.5% Non-ident detergent (NI; Sigma). NI was used on infected samples since, at this concentration (0.5%), it disrupted PEC without affecting the viability of Candida cells. Following disruption of non-adherent PEC, cellular debris and Candida cells, if present, were removed by centrifugation at 1000 x g, 4°C for 10 min.

At 2, 4, or 6 h, adherent PEC were treated with 3 ml of 1% TX-100 or 0.5% NI for 10 min to disrupt the attached cells. A rubber policeman was used to facilitate the removal of cells from culture dishes. Cellular debris and Candida cells, if present, were pelleted by centrifugation at 1000 x g, 4°C for 10 min.

Culture fluid and supernatants from adherent and non-adherent cell lysates were either stored frozen at -40°C until assayed or, since freezing and thawing resulted in a decrease in catalase activity, samples to be assayed for this enzyme were assessed immediately after centrifugation. Enzyme activities for adherent and non-adherent PEC fractions were expressed in units per mg adherent cell or non-adherent cell protein, respectively. Enzyme activities
for culture fluid fractions were expressed in units per mg total cell protein. Enzyme assays and protein determinations were performed as follows:

1. Catalase (EC 1.11.1.6)

   Catalase activity was determined by the method of Beers and Sizer (7) in which the disappearance of reagent hydrogen peroxide (Mallinckrodt, Inc., Paris, KY) was followed spectrophotometrically at 240 nm. The decrease in absorbance was measured with a Gilford 2400 recording spectrophotometer for a period of 3 min at 25°C. Each cuvette contained 1.9 ml glass distilled H₂O and 1.0 ml 0.059 M H₂O₂ in 0.05 M KH₂PO₄ buffer (pH 7.0) to which 0.1 ml of the biological sample was added. Catalase activity in units was calculated using bovine liver catalase (thymol free, 17,000 U/mg protein; Sigma) as standard. One unit decomposed 1 μmole H₂O₂ per min at 25°C, pH 7.0, as determined from absorbance measurements at 240 nm using a molar extinction coefficient of 70 M⁻¹cm⁻¹ for H₂O₂ (7).

2. Peroxidase (EC 1.11.1.7)

   Peroxidase activity was measured as the decrease in sample fluorescence following the oxidation of scopoletin (7-hydroxy-6-methoxy coumarin; Sigma) by peroxidase and H₂O₂ (114). Fluorescence was monitored for a period of 5 min on an Aminco-Bowman spectrophotofluorometer (American Instrument Co., Silver Springs, MD) with an excitation
wavelength of 350 nm and an emission wavelength of 460 nm. Each cuvette contained 2.2 ml 0.05 M KH$_2$PO$_4$ buffer (pH 7.0), 0.1 ml 0.1 M H$_2$O$_2$ and 0.1 ml 0.2 mM scopoletin in phosphate buffer, to which 0.1 ml of the biological sample was added. Peroxidase activity was quantitated from a standard curve using horseradish peroxidase (HPO, type I, salt free powder, 105 U/mg solid; Sigma) as standard. One unit of HPO oxidized 1 µmole of o-dianisidine per min at 25°C, pH 5.0, as determined from absorbance measurements at 460 nm using a molar extinction coefficient of 1.13 X 10$^4$ M$^{-1}$cm$^{-1}$ (20).

3. $\beta$-N-acetylglucosaminidase (EC 3.2.1.30)

The method of Woolien et al (129) was used to measure $\beta$-N-acetylglucosaminidase (NAGA) activity in biological samples. The formation of p-nitrophenol from p-nitrophenyl-$\beta$-D-glucosaminide (Sigma) was determined spectrophotometrically at 400 nm. Borosilicate glass test tubes (16 X 125 mm), on ice, received 0.5 ml 0.1 M sodium citrate buffer (pH 5.0), 0.1 ml 3 mM p-nitrophenyl-$\beta$-D-glucosaminide in citrate buffer, and 0.5 ml of the biological sample and were incubated for 2 h at 37°C in a shaking water bath. Following incubation, the tubes were placed on ice, the reaction was stopped by the addition of 3.0 ml 0.2 M sodium borate buffer (pH 9.8) to each tube, and the absorbance at 400 nm was read. Enzyme activity was quantified in units using jack bean NAGA (Sigma) as stand-
ard. One unit hydrolyzed 1.0 μmole of p-nitrophenyl-N-acetyl-β-D-glucosaminide to p-nitrophenol and N-acetyl-glucosamine per min at 25°C, pH 8.0, as determined from absorbance measurements at 400 nm using a molar extinction coefficient of 1.67 X 10^4 M^{-1}cm^{-1} (41).

4. Lactate dehydrogenase (EC 1.1.1.27)

Lactate dehydrogenase (LDH) activity was determined by the method of Wacker et al (125) utilizing the principle that LDH catalyzes the conversion of L-lactate to pyruvate with the simultaneous reduction of a molar equivalent of nicotinamide adenine dinucleotide (NAD); the production of reduced NAD (NADH), then, as measured by the increase in absorbance at 340 nm, is proportional to the activity of LDH. Therefore, 3 ml of the following reaction mixture (Super Stat Pack; Calbiochem, La Jolla, CA) were delivered into each cuvette, followed by 0.1 ml of biological sample: 3.5 mM NAD, 0.05 M glycine buffer (pH 8.8), and 70 mM lactate. All components were pre-warmed in a water bath to the assay temperature of 30°C. LDH activity in units was calculated using rabbit muscle LDH (substantially free of pyruvate kinase, 900 U/mg protein; Sigma) as standard. One unit oxidized 1.0 μmole of lactate to pyruvate per min at 30°C, pH 8.8, as determined from absorbance measurements at 340 nm using a molar extinction coefficient of 6.22 X 10^3 M^{-1}cm^{-1} (131).
5. **Protein Determination**

Protein determinations on cell lysate supernatants were conducted according to the method of Lowry *et al.* (71) using bovine serum albumin (fraction IV, essentially fatty acid free; Sigma) as standard. Working reagents were prepared as follows: Reagent A consisted of 1.0 ml 2% w/v potassium-sodium tartrate in H₂O, 1.0 ml 1% w/v copper sulfate in H₂O, and 98.0 ml 2% w/v sodium carbonate in 0.1 N NaOH; Reagent B consisted of 2 N Folin-Ciocalteu Phenol Reagent (Sigma) diluted 1:1 v/v with H₂O. Biological sample (0.1 ml) was diluted with 0.4 ml H₂O and allowed to react for 10 min at 25°C with 2.5 ml of Reagent A. Reagent B (0.25 ml) was added and allowed to react for 30 min at 25°C. Test tubes containing the reaction mixture were centrifuged for 5 min at 1000 x g and the absorbance of the supernatant at 750 nm was determined.

I. **Determination of Hydrogen Peroxide and Superoxide Anion Generation**

Hydrogen peroxide generation was quantitated by the method of Pick and Keisari (104) based on the H₂O₂-mediated, HPO-dependent oxidation of phenol red. Oxidized phenol red was measured by its absorbance at 610 nm. PEC were plated onto 16 mm culture dishes and incubated for 2 or 6 h in SWA medium to which SM-1213 or MDP was added. Dishes were washed once with SWA medium at 2 h to remove non-adherent
cells. After incubation, adherent cells were washed 3X with phenol red-free Earle's balanced salt solution (EBSS; Flow) and covered with 2 ml per dish of pre-warmed phenol red solution (PRS). PRS was prepared immediately prior to use by dissolving 0.1 mg phenol red/ml (phenolsulfonphthalein, sodium salt; Sigma) and 8.5 U HPO/ml (type I, 105 units/mg solid; Sigma) in EBSS. Opsonized zymosan was added to a final concentration of 1 mg/ml and the dishes were incubated at 37°C. Plates which did not receive opsonized zymosan were assessed for phenol red oxidation and values were subtracted from those of test samples as background oxidation. After 30 min, the culture fluid was transferred to tubes which were centrifuged for 5 min at 1000 x g. Supernatants were made alkaline by the addition of 10 μl of 1 N NaOH per tube and the extracellular generation of H₂O₂ was determined by the absorbance at 610 nm. PEC adherent to the culture dishes were disrupted with 0.5 ml 1% TX-100, scraped with a pipette tip to dislodge cells, and incubated for 30 min at 37°C with 2 ml PRS. The H₂O₂ generation by the cell lysate was determined spectrophotometrically after centrifugation and alkalization of the supernatants as described above. Hydrogen peroxide concentration was determined using a standard curve which utilized PRS and a range of H₂O₂ concentrations up to 200 μM.
Superoxide anion generation was measured by one of two methods. Whole cell \( \text{O}_2^2 \) generation was determined by a modification of the quantitative nitroblue tetrazolium salt (NBT) reduction test described by Weiss et al (126). The major modification involved using oil-elicited guinea pig PEC adherent to culture dishes rather than human peripheral blood monocytes and neutrophils in suspension. After incubation for 2 or 6 h in SWA medium to which SM-1213 was added, adherent cells in 60 mm dishes were washed 3X with PBS and covered with the NBT reaction mixture. The reaction mixture contained 0.4 ml Hank's balanced salt solution without phenol red (HBSS; Flow), 0.4 ml 0.2% NBT in 0.85% saline, 0.1 ml 0.01 M KCN, and 0.1 ml opsonized zymosan or Candida. NBT reduction was allowed to proceed at 37°C, 95% air-5% CO\(_2\) atm for 15 min after which time the reaction was stopped by the addition of 10 ml of cold 0.5 N HCl. PEC were harvested from the plates with a rubber policeman, the suspension was centrifuged in test tubes at 1000 x g for 20 min, and the reduced dye was extracted from the pellet with 4 ml pyridine in a boiling water bath. The centrifugation was repeated and the supernatant absorbance was read at 515 nm. In some studies, cell-associated and extracellular NBT reduction were determined separately on the cell pellet and supernatant fractions, respectively.

The concentration of NBT reduced was calculated
from a standard curve constructed by reducing known quantities of NBT with a xanthine-xanthine oxidase system. This system reduced 11.4 nmoles NBT per min at 37°C, pH 7.0, as calculated from absorbance measurements at 515 nm using a molar extinction coefficient of $2.8 \times 10^3 \text{M}^{-1}\text{cm}^{-1}$ (126) for NBT and contained 0.4 ml of varying concentrations of NBT in 0.85% saline, 1.8 ml of $2.22 \times 10^{-4}\text{M}$ xanthine (Calbiochem), and 1.8 ml of $2.22 \times 10^{-8}\text{M}$ xanthine oxidase (grade III, from buttermilk, 1.3 U/mg protein; Sigma) in 0.05 KH$_2$PO$_4$ buffer (pH 7.0) containing $1 \times 10^{-4}\text{M}$ EDTA (Sigma). Xanthine oxidase (EC 1.2.3.2) concentration was quantitated from absorbance measurements at 450 nm using a molar extinction coefficient of $7 \times 10^4 \text{M}^{-1}\text{cm}^{-1}$ (78).

In some experiments, whole cell $O_2$ generation was measured spectrophotometrically by the reduction of ferricytochrome c (49). After incubation for 2 or 6 h in SWA medium to which SM-1213 was added, adherent cells in 60 mm culture dishes were washed 3X with PBS and covered with 3 ml of an 80 μM solution of ferricytochrome c (FCC; type III; Sigma) in phenol red-free HBSS. Opsonized zymosan was added to the culture dishes which were incubated for 90 min at 37°C in 95% air-5% CO$_2$. After incubation, culture fluid was transferred to tubes on ice, centrifuged for 5 min at 1000 x g, and the absorbance of the super-
The concentration of ferri-cytochrome c reduced was calculated using the molar extinction coefficient of $2.1 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ (49).

Ferricytochrome c or NBT reduction was used to quantitate NADPH-dependent (50,51) superoxide anion generation by cell-free PEC lysates. PEC were incubated in 16 mm dishes for 2, 4 or 6 h either in SWA medium containing SM-1213 or in SWA medium alone. After incubation, adherent cells were washed 3X with PBS and covered with the stimulant mixture: 1.6 ml phenol red-free HBSS, 0.2 ml 0.01 M KCN, and 0.2 ml opsonized zymosan. Samples were incubated at 37°C in 95% air-5% CO$_2$ to allow phagocytosis of zymosan particles. After 15 min, adherent cells were washed once with PBS, lysed by exposure to 0.5 ml 1% TX-100 for 10 min, and dislodged from the plates by scraping with a pipette tip. Cell lysates were incubated with the following reaction mixture for 15 min at 37°C in 95% air-5% CO$_2$: 1.5 ml phenol red-free HBSS, 0.2 ml 0.01 M KCN, 0.1 ml 0.2 M NADPH (Sigma), and 0.2 ml 0.8 mM ferricytochrome c or 0.2 ml 0.4% NBT in HBSS. In addition, cell lysates from samples initially incubated in SWA medium alone and not previously exposed to SM-1213 received drug in the above reaction mixture in order to determine the possible direct effect of SM-1213 on NADPH-dependent superoxide generation by cell lysates. Ferricytochrome c or NBT reduction in
supernatants was measured spectrophotometrically by absorbance at 550 or 515 nm, respectively, after transfer of samples to test tubes on ice and centrifugation for 5 min at 1000 x g.

In order to determine the percentage of NBT or ferricytochrome c reduction mediated by O$_2^-$, some samples were incubated in the presence of superoxide dismutase (SOD, from bovine blood, EC 1.15.1.1, 2800 U/mg protein; Sigma) at a final concentration of 3000 U/ml. Average SOD-inhibitable reduction, compiled from multiple experiments, constituted 72.7 ± 14.4% of total detectable reduction. SOD which had been heated for 10 min in a boiling water bath or autoclaved for 15 min, catalase (bovine liver catalase; Sigma) at a final concentration of 1700 U/ml, or bovine serum albumin (BSA; Sigma) was included in some samples measured for O$_2^-$ or H$_2$O$_2$ generation, as indicated in the Results section.

Opsonized zymosan or C. albicans was prepared by incubating 100 mg of zymosan (zymosan A from S. cerevisiae yeast; Sigma) or $1 \times 10^8$ Candida yeast cells with 6 ml of whole, normal guinea pig serum for 20 min at 37°C in a shaking water bath. The particles were washed by centrifugation in PBS for 5 min at 250 x g and resuspended to 50 mg zymosan/ml or $1 \times 10^7$ Candida/ml. Culture dishes received a final concentration of 0.5 to 1 mg opsonized
zymosan/ml or 0.5 to 1 X 10^6 opsonized yeast cells/plate. Opsonization of particles was indicated by direct agglutination testing using rabbit anti-guinea pig immunoglobulins (Accurate Chemicals, Westbury, NY).

J. Determination of the Candidacidal Activity of the NADPH-NAD(P)H Oxidase System

The effect of SM-1213 on the candidacidal activity of a cell homogenate-derived NAD(P)H oxidase O_2^-generating system was determined. After incubation in SWA medium for 2 or 6 h, adherent cells (2.5 X 10^6 PEC) in 16 mm culture dishes were washed once with PBS, covered with the following stimulant mixture, and incubated at 37°C in 95% air-5% CO_2: 1.6 ml phenol red-free EBSS, 0.2 ml 0.01 M KCN, and 0.2 ml opsonized zymosan. After 15 min, adherent cells were washed once with PBS, covered with 1.5 ml EBSS, and scraped with a pipette tip to disrupt and dislodge cells. Some samples received SOD at a final concentration of 3000 U/ml. Cell homogenates were transferred to glass test tubes (16 x 125 mm) containing: 0.1 ml SM-1213, 0.2 ml 0.01 M KCN, 0.1 ml 0.2 M NADPH, and 0.1 ml (8 X 10^5) Candida yeast cells in EBSS. Some cell homogenates were heated in a boiling water bath for 10 min and cooled to 37°C before addition to the above reaction mixture. After 90 min at 37°C in a shaking water bath, samples were serially diluted and plated in duplicate by the spread plate
method on SDA. Candidal viability was assessed by colony enumeration after plates were incubated aerobically at 37°C for 48 h. Alternatively, candidal viability was determined by methylene blue dye exclusion (63). Similar results were obtained if PEC were lysed with 0.5% NI rather than disrupted by physical means.

To determine the candidacidal activity of cell homogenates not dependent upon NAD(P)H oxidase, some samples did not receive substrate NADPH in the reaction mixture. In samples receiving SOD, the candidacidal activity of the NADPH-NAD(P)H oxidase system was inhibited an average of 62.5 ± 2.89% over three experiments.

K. Statistical Analysis

For candidal killing percentages, the Chi-square ($\chi^2$) analysis or the Student's t test was used; for all other averages, the Student's t test was utilized. Averages were represented plus or minus (±) the standard error of the mean. Values were considered significant when $P < 0.05$. 
CHAPTER III

RESULTS

A. Direct Effect of SM-1213 on the Growth of C. albicans

SM-1213 had no direct fungicidal or fungistatic activity against the yeast or hyphal form of Candida at any drug concentration tested over a 10 h (yeast) or 24 h (hyphae) incubation period (Table I).

B. Effect of SM-1213 on Hyphal Elongation of Phagocytosed C. albicans

Since the development of hyphal elements has been implicated in the pathogenesis of candidal disease (128), intracellular hyphal formation and elongation by phagocytosed yeast cells was measured. In SWA medium alone, 100% of Candida cells developed hyphae during the 6 h incubation period. All of the yeast cells phagocytosed by untreated PEC-0 initiated hyphal formation, while PEC-0 incubated in the presence of SM-1213 (1 ng/ml) reduced the percentage of hyphae formed to 92% (P < 0.05). In addition, of those yeast cells which initiated hyphal formation, the average hyphal length attained after 6 h of incubation was shorter in drug-treated than in untreated (control) PEC-0 (Figure 2). This effect was most pronounced at the 1 ng/ml
Table I. The effect of SM-1213 on in vitro growth of yeast and hyphal forms of *C. albicans*

<table>
<thead>
<tr>
<th>SM-1213 concentration (ng/ml)</th>
<th>Candidal growth (% of control)</th>
<th>Yeast form&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Hyphal form&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>101</td>
<td>105</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>101</td>
<td>105</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>100</td>
<td>103</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>101</td>
<td>98</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Yeast cell growth was monitored as the increase in absorbance of candidal cultures in SDB. Data shown are relative absorbance of control and drug-treated samples after 10 h of growth at 37°C and are representative of the absence of drug effect at earlier intervals.

<sup>b</sup> Hyphal growth was determined by dry weight analysis of candidal cultures as described in Methods section.
Figure 2. Elongation of C. albicans hyphae within control and drug-treated PEC-0. PEC-0 were infected with C. albicans yeast cells in vitro and incubated in the presence of various concentrations of SM-1213 for the designated time intervals. Hyphal elongation of phagocytosed yeast cells was significantly reduced by SM-1213 treatment of PEC-0 for 6 h, as indicated at right by an asterisk (*). Data represent means ± SEM derived from three experiments. Final drug concentration in incubation medium: (●), 0 ng/ml; (◇), 0.1 ng/ml; (■), 1.0 ng/ml; (▲), 10.0 ng/ml.
drug level, a drug concentration at which no direct antifungal effects were observed in the absence of PEC (Table I). No significant drug-induced differences in hyphal length were observed at any drug concentration tested after incubation for 2 or 4 h (Figure 2).

Although hyphal formation was initiated intracellularly, the majority of viable hyphae within control and drug-treated PEC-0 appeared to penetrate the phagocyte cell membrane by 6 h, growing into the extracellular medium. In these instances, then, inhibition of hyphal elongation was not solely "intracellular" although the mechanisms of growth inhibition may have been initiated while hyphae were wholly within PEC.

C. **Effect of SM-1213 on the Killing of Phagocytosed C. albicans**

Although essentially all of the *C. albicans* yeast cells phagocytosed by PEC-0 could initiate hyphal formation, some hyphae were eventually killed by the microbicidal action of the phagocytic cells. As shown in Table II, after incubation with *Candida* for 6 h, untreated PEC-0 (0 ng/ml) killed 22% of intracellular *Candida* while PEC-0 incubated with *Candida* and either 0.1 or 1.0 ng SM-1213/ml for 6 h killed 35.5 or 46% of the intracellular fungus, respectively. At the 10 ng/ml drug level, SM-1213 did not induce PEC to kill a significantly greater percentage of
Table II. The effect of SM-1213 on the viability of phagocytosed *Candida* albicans

<table>
<thead>
<tr>
<th>SM-1213 concentration (ng/ml)</th>
<th>% <em>Candida</em> killed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PEC-O incubated with drug&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Medium control&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.5 ± 0.5&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>0</td>
<td>22.0 ± 5.6</td>
</tr>
<tr>
<td>0.1</td>
<td>35.5 ± 2.5&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.0</td>
<td>46.0 ± 9.6*</td>
</tr>
<tr>
<td>10.0</td>
<td>25.0 ± 3.0</td>
</tr>
</tbody>
</table>

a) PEC-O were infected and incubated with drug for 6 h. The percent intracellular *Candida* killed was determined by the microcolony technique as described in Methods section.

b) Non-infected PEC-O were pre-incubated with drug for 6 h, washed, inoculated with *Candida* yeast cells and incubated for 2 h in the absence of drug. Percent intracellular *Candida* killed was determined as described in a) above.

c) Candidal viability was determined in PEC-free samples by the methylene blue dye exclusion technique (63).

d) Mean ± SEM

e) An asterisk (*) denotes P < 0.05 between untreated PEC (0 ng/ml) and drug-treated PEC samples.
intracellular Candida than controls. However, the initiation of fungal injury was suggested by a reduction in average hyphal length observed for this group (Figure 2), although growth inhibition was not statistically significant.

D. Effect of SM-1213 Pre-incubation of PEC-0 on Hyphal Elongation of Phagocytosed C. albicans

The results presented thus far suggest that SM-1213 could potentiate one or more intracellular candidacidal processes expressed by PEC-0. If such potentiation occurred via the modulation of the PEC-0 themselves and not via a direct effect on Candida cells, then pre-incubation of PEC-0 with SM-1213 and removal of drug prior to infection with Candida should result in enhanced candidal killing by drug-treated, relative to untreated, PEC-0. To test this hypothesis, PEC-0 were pre-incubated with SM-1213 for 6 h and adherent cells were washed to remove residual drug. Monolayers were infected with yeast phase Candida cells, incubated for 2 h in the absence of drug, and assessed for intracellular hyphal growth. Significant inhibition of intracellular hyphal elongation occurred during the 2 h incubation in PEC-0 pre-incubated with drug, at all concentrations tested, relative to PEC-0 pre-incubated in the absence of drug (Figure 3). No significant drug-
Figure 3. Hyphal length of phagocytosed *C. albicans* within pre-incubated control and drug-treated PEC-O. PEC-O were pre-incubated for 6 h in the presence or absence of drug. PEC-O monolayers were washed, infected with yeast cells, incubated for 2 h in the absence of drug and assessed for intracellular hyphal growth. Each bar represents the mean ± SEM for 200 intracellular hyphae. Hyphal length was significantly reduced in PEC-O pre-incubated with SM-1213, as indicated at right by an asterisk (*).
Average Hyphal Length (μm)

SM-1213 Concentration (ng/ml)
induced retardation of hyphal growth occurred when PEC-0 were pre-incubated with drug for less than 6 h prior to candidal infection (data not shown).

E. Effect of SM-1213 Pre-incubation of PEC-0 on the Killing of Phagocytosed C. albicans

PEC-0 were pre-incubated in culture for 6 h in the presence or absence of drug. Adherent PEC-0 were washed, infected with yeast cells, incubated for 2 h in the absence of drug, and assessed for candidal killing. PEC-0 pre-incubated in the absence of drug killed 16.5% of intracellular Candida during the subsequent 2 h infection period. In comparison, PEC-0 pre-incubated with 0.1 to 10 ng/ml SM-1213 for 6 h killed a significantly greater percentage of intracellular Candida (30-36.5%) than controls (0 ng/ml) during the 2 h infection period when drug was no longer present (P < 0.02, Table II). In all but the 10 ng/ml treatment group, pre-incubated PEC-0 killed an equal or somewhat lower percentage of intracellular Candida than did PEC-0 incubated with drug from the time of infection (Table II). However, killing of Candida within pre-incubated PEC-0 was substantial, since Candida were exposed to PEC-0 for only a 2 h infection period relative to a 6 h infection period for PEC-0 incubated simultaneously with Candida and drug.
F. Effect of SM-1213 on PEC Enzyme Activities and Protein Content

Since the work of Sasada and Johnston (118) and of Lehrer and Cline (64) emphasized the contribution made by active oxygen species such as $\text{H}_2\text{O}_2$ and $\text{O}_2^-$ to candidacidal systems of phagocytes, an examination of killing mechanisms involving these agents was undertaken. It was decided to focus first on possible drug-mediated effects on the efficacy, utilization and catabolism of $\text{H}_2\text{O}_2$. This might result, for example, from a drug-induced modification of the activity of peroxidase, an enzyme associated with an $\text{H}_2\text{O}_2$-mediated microbicidal system of phagocytes (62,64,102), or catalase, an enzyme associated with the abrogation of candidacidal activity of macrophages in culture (118).

PEC were incubated with SM-1213 for 6 h and adherent cell populations from resident (90-95% peroxidase positive), oil-elicited (45-50% peroxidase positive) and oil-elicited Candida-infected PEC were examined for enzyme activity (Table III). After 6 h of incubation with either 0.1 or 1.0 ng SM-1213/ml, significant drug-induced reductions in catalase activity occurred in adherent cell populations from both oil-elicited and oil-elicited infected PEC. Such reductions in specific enzyme activity were not attributable to an increase in adherent cell protein as a result of drug treatment. Catalase activity in resident
Table III. The effect of SM-1213 treatment of peritoneal exudate cells (PEC) on adherent cell enzyme activity

<table>
<thead>
<tr>
<th>PEC type(^b)</th>
<th>SM-1213 concentration (ng/ml)</th>
<th>Adherent cell enzyme activity (units/mg protein)(^a)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Catalase</td>
<td>Peroxidase</td>
</tr>
<tr>
<td>Resident</td>
<td>0</td>
<td>31.85 ± 0.69(^c)</td>
<td>10.23 ± 0.22</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>32.34 ± 0.46</td>
<td>11.00 ± 0.33</td>
</tr>
<tr>
<td>Oil-elicited</td>
<td>0</td>
<td>40.76 ± 2.64</td>
<td>0.55 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>25.97 ± 0.83(^d)</td>
<td>0.45 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>27.79 ± 1.95(^*)</td>
<td>0.64 ± 0.07</td>
</tr>
<tr>
<td>Oil-elicited infected</td>
<td>0</td>
<td>61.97 ± 1.35</td>
<td>N.D.(^e)</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>36.37 ± 1.95(^*)</td>
<td>N.D.(^e)</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>51.02 ± 1.25(^*)</td>
<td>N.D.(^e)</td>
</tr>
</tbody>
</table>

\(a\) Enzyme units and mg protein were determined on cell lysate supernatants from the isolated adherent cell fraction.

\(b\) Resident cells were obtained from guinea pigs on the day of the experiment by peritoneal lavage. Oil-elicited PEC were obtained by peritoneal lavage 72 h after elicitation of cells by i.p. injection of mineral oil. Oil-elicited PEC infected with yeast phase C. albicans at the time of PEC plating were designated as oil-elicited infected PEC. All samples were incubated with the designated concentrations of drug for 6 h. Culture fluid and non-adherent cells were removed prior to lysis and assay of adherent cell lysate supernatants.

\(c\) Data represent means ± SEM of triplicate dishes and are derived from one representative experiment for each PEC type.

\(d\) An asterisk (*) denotes \(P < 0.05\) between control (0 ng/ml) and drug-treated PEC samples.

\(e\) Not determined.
PEC was not affected by drug treatment (Table III). No significant drug-induced reductions in catalase activity were observed for any cell type incubated for less than 6 h or for PEC-O treated with >10 ng SM-1213/ml (data not shown). The observed drug-induced reduction in catalase specific activity in oil-elicited PEC types was selective, in that peroxidase levels were not affected, nor were levels of N-acetylglucosaminidase, a marker lysosomal enzyme (Table III). PEC lactate dehydrogenase activity and protein content (data not shown) were not significantly affected by drug treatment.

_Candida_ incubated for 6 h in medium alone followed by treatment with 0.5% NI for 10 min exhibited no leakage of catalase into the extracellular medium. No detectable protein was released from _Candida_ into the extracellular medium. Also, protein content was not greater in supernatants from infected adherent cell lysates than in supernatants from uninfected adherent cell lysates when PEC-0 were derived from the same experimental animal.

The drug-induced reduction of adherent cell catalase activity was not associated with a corresponding enhancement of non-adherent cell or extracellular activity (Figure 4), and thus could not be identified as representing a loss of cell adherence or of transfer of catalase from the intra- to the extracellular compartment. No
Figure 4. The effect of SM-1213 treatment of PEC-0 on adherent cell, non-adherent cell, and extracellular catalase activities in vitro. PEC-0 were incubated with the designated concentrations of drug for 6 h. Experimental conditions were as described in Methods section. Data represent the mean ± SEM of triplicate or quadruplicate dishes and were derived from one representative experiment. Significant drug-induced reductions in catalase activity were observed for the adherent cell population only, as indicated at right by an asterisk (*).
direct drug effect on standard bovine liver catalase activity was observed when drug was added during enzyme assay.

G. **Effect of SM-1213 Pre-incubation of PEC-0 on NBT Reduction by Phagocytes after Ingestion of Opsonized C. albicans**

An explanation for the reduction of catalase activity observed in drug-treated PEC-0 and the relationship of this reduction to the production of active oxygen species responsible for candidal killing by phagocytes was suggested by the work of Kono and Fridovich (57). They demonstrated that catalase activity was reduced by the action of $O_2^-$ on the heme portion of the enzyme. Similarly, the author has observed a reduction in catalase activity during exposure of the enzyme to a xanthine-xanthine oxidase superoxide generating system (unpublished observation, C.J. Morrison). It was therefore decided to examine the effect of SM-1213 treatment of phagocytes on their ability to generate $O_2^-$ and $H_2O_2$ in vitro. In all studies regarding the production of active oxygen species by phagocytes, oil-elicited PEC were pre-incubated in vitro in the presence or absence of drug for the indicated time intervals, washed, and then exposed to the assay conditions described.

Figure 5 depicts the significantly enhanced production of $O_2^-$ by PEC-0 pre-incubated with SM-1213, as assessed by NBT reduction during phagocytosis of opsonized
Figure 5. The effect of SM-1213 pre-incubation of PEC-0 on NBT reduction by phagocytes during ingestion of opsonized C. albicans. PEC-0 were pre-incubated for 2 or 6 h with the designated concentrations of drug, washed, and adherent PEC-0 were exposed to the NBT assay system with opsonized Candida as phagocytic stimulus. Data represent the mean ± SEM of triplicate dishes and were derived from one representative experiment. Significant drug-induced enhancement of SOD-inhibitable NBT reduction was observed, as indicated at right by an asterisk (*). UND = undetectable SOD-inhibitable NBT reduction. BSA or boiled SOD did not inhibit NBT reduction; Candida alone did not reduce NBT.
Candida. Although \( \text{O}_2^- \) production was reduced at 6 h relative to 2 h, SM-1213 treatment enhanced \( \text{O}_2^- \) production by PEC-0 at both time intervals (Figure 5). The reduction in \( \text{O}_2^- \) generation at 6 h was not due to a loss of PEC-0 viability as assessed by trypan blue dye exclusion or due to a reduced phagocytic capacity. SOD equally inhibited NBT reduction in control and drug-treated samples and drug-induced differences in SOD-inhibitable NBT reduction were, therefore, not due to a drug action on SOD itself.

In order to more fully understand the mechanisms whereby PEC-0 kill Candida, it was of interest to examine the location of \( \text{O}_2^- \) production by PEC-0, that is, to differentiate between NBT reduction occurring extracellularly and that associated with the cell. This was accomplished by transferring the NBT reaction fluid from the culture dishes to test tubes and by stopping the reaction separately in the tubes (extracellular) and dishes (cell-associated) after the 15 min incubation period. The majority of NBT reduction occurred extracellularly and accounted for an average of 74.4% of the total NBT reduced by all samples. In samples pre-incubated in the absence of drug for 6 h, 100% of the SOD-inhibitable reduction of NBT occurred extracellularly. In contrast, in samples treated with 1 ng SM-1213/ml, 66% of the SOD-inhibitable reduction of NBT occurred extracellularly. NBT reduction was not in-
hibited by boiled SOD or by BSA. *Candida* alone did not reduce NBT.

H. **Effect of SM-1213 Pre-incubation of PEC-0 on NBT and FCC Reduction by Phagocytes after Ingestion of Opsonized Zymosan**

PEC-0 were pre-incubated in the presence or absence of SM-1213 for 2 or 6 h, washed, and exposed to the NBT assay system with opsonized zymosan as phagocytic stimulus. NBT reduction by PEC-0 pre-incubated with SM-1213 was enhanced relative to PEC-0 pre-incubated in the absence of drug, as depicted in Figure 6. This drug-induced enhancement was most pronounced at the 1.0 ng/ml dose level. Opsonized zymosan (Figure 6) stimulated a greater reduction of NBT by PEC-0 than opsonized *Candida* (Figure 5) and was therefore used as the phagocytic stimulus in the remaining experiments.

In order to confirm the observed drug-induced enhancement of $O_2^-$ production by PEC-0, a second method typically used to assess $O_2^-$ production by phagocytes, ferricytochrome c (FCC) reduction, was utilized. Results were expressed as nmoles FCC reduced per mg protein per 90 min to conform to the standard reporting format. As Figure 7 depicts, pre-incubation of PEC-0 with SM-1213 resulted in a significantly enhanced reduction of FCC by phagocytes during ingestion of opsonized zymosan. As in
Figure 6. The effect of SM-1213 pre-incubation of PEC-0 on NBT reduction by phagocytes during ingestion of opsonized zymosan particles. PEC-0 were pre-incubated for 2 or 6 h with the designated concentrations of drug, washed, and adherent PEC-0 were exposed to the NBT assay system with opsonized zymosan as phagocytic stimulus. Data represent means ± SEM of duplicate or triplicate dishes and were derived from one representative experiment. Significant drug-induced enhancement of SOD-inhibitable NBT reduction was observed, as indicated at right by an asterisk (*).
Figure 7. The effect of SM-1213 pre-incubation of PEC-0 on ferricytochrome c reduction by phagocytes during ingestion of opsonized zymosan particles. PEC-0 were pre-incubated for 2 or 6 h in the presence or absence of SM-1213, washed, and adherent PEC-0 were exposed to the FCC assay system with opsonized zymosan as phagocytic stimulus. Data represent means ± SEM of triplicate dishes and were derived from one experiment. Significant drug-induced enhancement of SOD-inhibitable FCC reduction was observed, as denoted below by an asterisk (*).
Figures 5 and 6, although $O_2^-$ production was reduced at 6 h relative to 2 h, SM-1213 treatment enhanced $O_2^-$ production by PEC-O at both time intervals (Figure 7).

I. Effects of SM-1213 Treatment of PEC-O or PEC-O Lysates on NADPH-dependent Superoxide Generation by Cell Lysates

It is generally agreed that the enzyme primarily responsible for superoxide production by stimulated phagocytes is a cyanide-insensitive oxidase which utilizes a reduced pyridine nucleotide as substrate (8,16,91). NAD-(P)H oxidase has been demonstrated to be involved in $O_2^-$ production when phagocytes are stimulated by membrane-perturbing agents such as phorbol myristate acetate (8), or by phagocytic particles such as opsonized zymosan (91). In order to determine whether a drug-mediated effect on this enzyme was responsible for the observed enhancement of $O_2^-$ production by PEC-O pre-incubated with SM-1213, an examination of NADPH-dependent FCC and NBT reduction was initiated.

PEC-O were pre-incubated in the absence or presence of SM-1213 for various time intervals and washed prior to stimulation with opsonized zymosan. Following stimulation, PEC-O were lysed with detergent and cell lysates were assayed for NADPH-dependent NBT or FCC reduction in the presence of exogenously added NADPH.
Figure 8 depicts the significant enhancement of NADPH-dependent FCC reduction by lysates from PEC-O pre-incubated with SM-1213 for 6 h. Treatment at the 1.0 ng/ml drug concentration enhanced NADPH-dependent FCC reduction after 2 h to a level which approached significance ($0.1 > p > 0.05$).

A significant enhancement of NADPH-dependent NBT reduction by cell lysates was also observed (Figure 9) when PEC-O were pre-incubated for 6 h with 0.1 or 1.0 ng SM-1213/ml prior to cell lysis. No significant drug-induced increase in $O_2^-$ production was observed when PEC-O were pre-incubated for less than 6 h (Figure 9). PEC-O pre-incubated in the absence of drug for 6 h and receiving no substrate NADPH at the time of NBT assay demonstrated relatively minimal NBT reduction (Figure 9). The reduced activity observed at 0 h may have resulted from the presence of SOD (75) from minor red blood cell contamination present in PEC-O cell suspensions at this time interval only.

The reduction of $O_2^-$ production at 6 h relative to 2 h observed for whole cell NBT and FCC reduction (Figures 5, 6, 7) was not observed for $O_2^-$ production by cell lysates (Figure 9). Instead, an increase in $O_2^-$ production was observed at 6 h relative to earlier time intervals and only at 6 h was a significant drug-induced enhancement of NBT
Figure 8. Drug enhancement of NADPH-dependent FCC reduction by cell lysates from PEC-O pre-incubated with SM-1213. PEC-O were pre-incubated in the presence or absence of SM-1213 for 2 or 6 h and adherent PEC-O were washed prior to stimulation with opsonized zymosan and cell lysis. Cell lysates were assayed for FCC reduction as described in Methods section. Data represent means ± SEM of triplicate dishes and were derived from one experiment. Significant drug-induced enhancement of SOD-inhibitable NADPH-dependent FCC reduction was observed, as denoted at right by an asterisk (*).
NADPH-DEPENDENT NMOLES FCC REDUCED/10^6 CELLS/15 MIN

SM-1213 CONCENTRATION (NG/ML)
Figure 9. Time-dependent drug enhancement of NADPH-dependent NBT reduction by cell lysates from PEC-0 pre-incubated with SM-1213. PEC-0 were pre-incubated in the presence or absence of SM-1213 for 2, 4 or 6 h and adherent PEC-0 were washed prior to stimulation with opsonized zymosan and cell lysis. Cell lysates were assayed for NBT reduction as described in Methods section. At 0 h, PEC-0 in suspension (consisting of 77% macrophages, 3% PMN and 20% lymphocytes) were incubated with the opsonized zymosan stimulant mixture, washed by centrifugation in PBS and lysed. Lysates were assayed for NBT reduction as described in Methods section. Cell lysates from PEC-0 pre-incubated in the absence of drug for 6 h and receiving no substrate NADPH at the time of NBT assay are designated below as NO NADPH. An asterisk (*) denotes P < 0.05 between samples obtained from PEC-0 pre-incubated in the absence of drug and those from PEC-0 pre-incubated in the presence of drug. Data represent means ± SEM of the number of experiments denoted in parentheses ( ).
reduction observed (Figure 9).

In order to determine whether the time-dependent nature of the observed drug effect was a result of the time PEC-0 were exposed to SM-1213 or whether it was a result of the time PEC-0 were held in culture, PEC-0 were pre-incubated for 2 or 6 h in the absence of drug prior to stimulation with opsonized zymosan and cell lysis. Cell lysates were then assayed for NBT reduction in the presence or absence of SM-1213.

Table IV compares NADPH-dependent NBT reduction by cell lysates from PEC-0 pre-incubated with SM-1213 to cell lysates from PEC-0 pre-incubated without SM-1213 but receiving drug during NBT assay. When PEC-0 were pre-incubated for 6 h in the presence or absence of SM-1213, significant drug-induced enhancement of NBT reduction occurred in all drug-treated samples (Table IV). Pre-incubation of PEC-0 with drug resulted in an average percent increase in NBT reduction by cell lysates of 50.2% relative to cell lysates from PEC-0 pre-incubated in the absence of drug. Pre-incubation of PEC-0 without drug followed by incubation of cell lysates with drug during NBT assay resulted in an average percent increase in NBT reduction by cell lysates of 28.7% relative to cell lysates from PEC-0 pre-incubated in the absence of drug. When comparing NBT reduction by cell lysates from PEC-0 pre-incubated with SM-1213 to cell
Table IV. NADPH-dependent reduction of NBT by cell lysates from oil-elicited PEC (PEC-0)

<table>
<thead>
<tr>
<th>SM-1213 concentration (ng/ml)</th>
<th>nmoles NBT reduced/10⁶ cells/15 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PEC-O pre-incubated for:</td>
</tr>
<tr>
<td></td>
<td>6 h with drug²</td>
</tr>
<tr>
<td></td>
<td>(% drug effect)⁴</td>
</tr>
<tr>
<td>0</td>
<td>58.59 ± 1.12¢</td>
</tr>
<tr>
<td>0.1</td>
<td>87.05 ± 3.91*£</td>
</tr>
<tr>
<td>1.0</td>
<td>88.72 ± 3.91*</td>
</tr>
<tr>
<td>10.0</td>
<td>88.16 ± 5.02*</td>
</tr>
<tr>
<td>Average</td>
<td>(50.2)</td>
</tr>
</tbody>
</table>

a) PEC-O were pre-incubated with the designated concentrations of SM-1213 for 6 h and washed to remove residual drug prior to stimulation with opsonized zymosan and cell lysis. Cell lysates were assayed for NBT reduction as described in Methods section.
b) % drug effect = nmoles NBT reduced by: drug-treated - control (0 ng/ml) samples / nmoles NBT reduced by control (0 ng/ml) samples × 100.
c) PEC-O from the same cell source as in a) were pre-incubated in the absence of drug for 6 h prior to stimulation with opsonized zymosan and cell lysis. Cell lysates were assayed for NBT reduction in the presence of the designated concentrations of SM-1213 as described in Methods section.
d) % increase = nmoles NBT reduced by: a) - c) / nmoles NBT reduced by c) × 100.
e) Mean ± SEM of triplicate dishes from one representative experiment.
f) An asterisk (*) denotes P < 0.05 between untreated PEC (0 ng/ml) and drug-treated PEC samples.
lysates treated with drug during NBT assay only, NBT reduction was increased an average of 22.7% in cell lysates from PEC-0 pre-incubated with drug (Table IV). No significant drug enhancement of NBT reduction was observed for either type of cell lysate after pre-incubation for less than 6 h (i.e., 2 h with drug: 0 ng/ml, 62.50 ± 1.00; 0.1 ng/ml, 68.08 ± 3.08; 1.0 ng/ml, 61.38 ± 1.42. 2 h w/o drug: 0 ng/ml, 62.50 ± 1.12; 0.1 ng/ml, 65.29 ± 3.35; 1.0 ng/ml, 64.17 ± 2.23).

When resident PEC were tested in the same manner, no significant drug-enhanced O_2^- production was observed in either cell lysate type after either 2 or 6 h of pre-incubation (Table V). NBT reduction was greater in oil-elicited PEC lysates (Table IV) relative to cell lysates from resident PEC (Table V), an effect which was most pronounced in lysates from PEC pre-incubated for 6 h (cell lysates from PEC-0 pre-incubated in the absence of drug were increased an average of 19.7%, while cell lysates treated with drug during NBT assay or cell lysates from PEC-0 pre-incubated in the presence of drug were increased an average of 82.9%).

J. Effect of SM-1213 Pre-incubation of PEC-0 on Hydrogen Peroxide Generation

The results presented thus far suggest that SM-1213 treatment of PEC-0 or of PEC-0 lysates induces an increase
**Table V. NADPH-dependent reduction of NBT by cell lysates from resident PEC (PEC-r)**

<table>
<thead>
<tr>
<th>SM-1213 concentration (ng/ml)</th>
<th>nmoles NBT reduced/10⁶ cells/15 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PEC-r pre-incubated for:</td>
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<tr>
<td></td>
<td>2 h with drug&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Experiment #1</td>
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<tr>
<td>0</td>
<td>59.86 ± 1.55&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.1</td>
<td>54.70 ± 0.52</td>
</tr>
<tr>
<td>1.0</td>
<td>53.15 ± 2.06</td>
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a) PEC-r were pre-incubated with the designated concentrations of SM-1213 for 2 or 6 h and washed to remove residual drug prior to cell lysis. Cell lysates were assayed for NBT reduction as described in Methods section.

b) PEC-r were pre-incubated in the absence of drug for 6 h prior to cell lysis. Cell lysates were assayed for NBT reduction in the presence of SM-1213 as described in Methods section.

c) Mean ± SEM of duplicate or triplicate dishes.
in \( O_2^- \) production by enhancing the activity of phagocyte NAD(P)H oxidase. It follows that \( H_2O_2 \) generation would also be increased by SM-1213 treatment of PEC-0, since dismutation of \( O_2^- \) produced by phagocytes results in the production of \( H_2O_2 \) \( (104) \). It was decided to examine \( H_2O_2 \) generation by SM-1213-treated PEC-0, since the production of this active oxygen species by phagocytes has been shown to be involved in the tumoricidal \( (92,97) \), as well as the microbicidal \( (87,95,118) \), activity of phagocytes. In addition, the possible modulation of phagocyte \( H_2O_2 \) production by another related immunomodulatory drug, muramyl dipeptide (MDP), was assessed, since this agent increases the NAD(P)H oxidase activity of phagocytes \( (51) \).

PEC-0 were pre-incubated for 2 or 6 h in the presence of SM-1213 or MDP, washed to remove residual drug, stimulated with opsonized zymosan, and assayed for extracellular \( H_2O_2 \) production by the oxidation of phenol red. Extracellular \( H_2O_2 \) production by phagocytes was enhanced by pre-incubation for 2 h with 1 ng/ml of either drug and by pre-incubation for 6 h with 0.1 ng MDP/ml or 1 ng SM-1213/ml \( (Figure \ 10) \).

\( H_2O_2 \) production by cell lysates, assessed by the oxidation of phenol red after disruption of PEC-0 with detergent, was significantly increased in samples treated with either drug at all concentrations tested \( (Figure \ 11) \).
Figure 10. The effect of SM-1213 pre-incubation of PEC-0 on extracellular H$_2$O$_2$ production during phagocytosis of opsonized zymosan. PEC-0 were pre-incubated for 2 or 6 h with the designated concentrations of SM-1213, washed to remove residual drug, and adherent PEC-0 were exposed to the phenol red assay system with opsonized zymosan as phagocytic stimulus. After incubation for 30 min, medium was assessed for extracellular H$_2$O$_2$ production as described in Methods section. Data represent means ± SEM of triplicate dishes and were derived from one representative experiment. An asterisk (*) denoted P < 0.05 between samples from PEC-0 pre-incubated in the absence of drug and those from PEC-0 pre-incubated in the presence of drug.
EXTRACELLULAR

2 HR 6 HR

[150]

[110x208]N

[107x162]_. ·:·:·:·:·:· 0

[412x139]~

[195x122]··········

[618x115]0

[200x97]0 0.1 1.0 0 0.1 1.0

DRUG CONCENTRATION (NG/ML)

NMOLES H₂O₂/MG PROTEIN/30 MIN

0 50 100 150

0 50 100 150

* * * * * *

SM-1213

MDP
Figure 11. The effect of SM-1213 pre-incubation of PEC-O on cell lysate H₂O₂ production following phagocytosis of opsonized zymosan. Experimental conditions were as in Figure 10, except PEC-O were lysed with detergent following ingestion of opsonized zymosan and exposed to the phenol red assay system. Cell lysates were assessed for H₂O₂ production as described in Methods section. Data represent means ± SEM of triplicate dishes and were derived from one experiment. An asterisk (*) denotes P < 0.05 between samples from PEC-O pre-incubated in the absence of drug and those from PEC-O pre-incubated in the presence of drug.
CELL LYSATE

2 HR

6 HR

SM-1213

MDP

NMOLES \( \text{H}_2\text{O}_2 / \text{mg protein} \) / 30 min

DRUG CONCENTRATION (NG/ML)

0 0.1 1.0 0 0.1 1.0

0 100 150 0 100 150

*
In samples from PEC-O pre-incubated for 6 h in the absence of drug, 85.7% of the total \( \text{H}_2\text{O}_2 \) produced by phagocytes was extracellular, while 14.3% was produced by cell lysates. In contrast, in all samples from PEC-O pre-incubated for 6 h in the presence of drug (MDP or SM-1213), an average of 56.4% of the total \( \text{H}_2\text{O}_2 \) produced was extracellular (range 47.1-66.1%), while 43.6% was produced by cell lysates (range 33.9-52.9%). While extracellular \( \text{H}_2\text{O}_2 \) production either increased or decreased between 2 and 6 h, \( \text{H}_2\text{O}_2 \) production by cell lysates increased in all drug-treated samples an average of 30% during this interval (Figures 10 and 11). \( \text{H}_2\text{O}_2 \) production by samples from PEC-O pre-incubated in the absence of drug remained unchanged over time (Figure 11).

The addition of catalase to the \( \text{H}_2\text{O}_2 \) assay mixture resulted in a 94.6% reduction in phenol red oxidation, while boiled catalase reduced oxidation by 31.6% and SOD increased oxidation by 26%.

K. Effect of SM-1213 on the Candidacidal Activity of the NADPH-NAD(P)H Oxidase System

Since SM-1213 treatment enhanced the NADPH-dependent production of \( \text{O}_2^- \) by PEC-O lysates (Figure 9), it was decided to examine the effect of SM-1213 on the NADPH-dependent candidacidal activity of PEC-O homogenates.

PEC-O were pre-incubated \textit{in vitro} for 2 or 6 h in
the absence of drug, stimulated with opsonized zymosan and homogenized. Cell homogenates were tested for SOD-inhibit-
able, NADPH-dependent candidal killing in the presence or absence of SM-1213. The results are depicted in Figure 12. Significant drug-induced enhancement of SOD-inhibitable candidal killing was observed by cell homogenates from PEC-0 pre-incubated for 2 or 6 h in the absence of drug and subsequently treated with 0.1 or 1 ng SM-1213/ml during the killing assay (Figure 12). Cell homogenates from PEC-0 pre-incubated for 6 h in the absence of drug and subse-
quently treated with 10 ng SM-1213/ml also demonstrated enhanced candidal killing. When cell homogenates were boiled before addition to Candida or when no NADPH was added to the incubation mixture, some killing was observed (Figure 12). SM-1213 did not enhance killing by boiled cell homogenates or by samples receiving no NADPH.

The enhanced killing of Candida by drug-treated cell homogenates was not an artifact resulting from the increased clumping or sticking together of yeast cells in the presence of drug as determined by phase contrast micros-
copy.

Candidal killing by cell homogenates in the pres-
ence or absence of SM-1213 was also assessed by the meth-
ylene blue dye exclusion technique (63). Although overall candidacidal activity in all samples was lower (4-17.5%
Figure 12. The effect of SM-1213 on NADPH-dependent candidal killing by cell homogenates. PEC-0 were pre-incubated in vitro for 2 or 6 h in the absence of drug prior to stimulation with opsonized zymosan and cell homogenization. Candida yeast cells were incubated with cell homogenates and substrate NADPH for 90 min in the absence or presence of the designated concentrations of SM-1213. After incubation, serial dilutions of samples were plated on SDA for enumeration of cfu 48 h later. Cell homogenates from PEC-0 pre-incubated in the absence of drug and receiving no NADPH during incubation are designated below as NO NADPH. Cell homogenates which were boiled for 10 min before incubation with Candida in the assay system are designated below as BL. Data represent the mean percent SOD-inhibitable Candida killed relative to Candida incubated in buffer alone from duplicate dishes ± SEM and were derived from one experiment. An asterisk (*) denotes P < 0.05 between drug-treated and untreated (0 ng/ml) samples.
SM-1213 CONCENTRATION (NG/ML)

PERCENT CANDIDA KILLED

2 HR

6 HR
dead), the enhancement of candidal killing by drug-treated cell homogenates or cell lysates (lysed with 0.5% NI) was evident using this assay system \( (P < 0.05) \). The methylene blue assay system may, however, have given artificially high values for the number of apparently viable *Candida*. For example, others have demonstrated that as many as 27% of *Candida* killed by activated mouse macrophages (as assayed by microcolony formation) did not lose their ability to exclude dye during the assay period (45).
CHAPTER IV

DISCUSSION

In the absence of PEC, SM-1213 had no direct fungistatic or fungicidal effect against the yeast or hyphal form of Candida albicans (Table I). However, SM-1213 was found to be effective in inducing PEC-0 to inhibit the intracellular formation and elongation of C. albicans hyphae (Figure 2) and to reduce the percentage of viable Candida remaining after phagocytosis (Table II). Significant drug-induced candidastatic and candidacidal effects were also observed when PEC-0 were pre-incubated with SM-1213 and washed prior to infection with Candida (Figure 3, Table II). These results indicate that SM-1213 was able to potentiate one or more candidacidal processes expressed by PEC-0.

When PEC-0 were infected and incubated with SM-1213, the most significant drug-induced reductions in hyphal growth and candidal viability occurred at the 1 ng/ml drug level (Figure 2, Table II). Higher or lower concentrations of drug were less effective, reflecting the bell-shaped dose response curve frequently observed for immunomodulatory drugs (35,40). In addition, others (12)
have reported that SM-1213 enhanced the effectiveness of mouse resident peritoneal macrophages against *Mycobacterium tuberculosis* when macrophages were treated with SM-1213 concentrations as low as 0.01 ng/ml and as high as 100 ng/ml. However, the most effective antimycobacterial drug concentration examined by a dose response curve was 1 ng/ml (12). Also, Hadden *et al* (40) reported that SM-1213 was capable of significantly increasing the killing of intracellular *Listeria monocytogenes* within oil-elicited guinea pig macrophages treated with the lowest SM-1213 concentration tested, 0.12 µg/ml.

While PEC-O treated with 10 ng SM-1213/ml did not kill a greater percentage of *Candida* than untreated (0 ng/ml) PEC-O (Table II), intracellular inhibition of hyphal growth, although not significant, was suggested (Figure 2) and may reflect the initiation of hyphal injury. Hyphal inhibition was greatest relative to controls at 4 h, as opposed to at 6 h, in PEC-O receiving lower drug concentrations (Figure 2). It is not known at present whether SM-1213 is taken into and accumulated within PEC-O over time. Although requested from Greenwich Pharmaceuticals, radiolabelled SM-1213 was not available at the time the present studies were conducted. If, however, SM-1213 was taken into and accumulated within PEC-O, then PEC-O treated with 10 ng SM-1213/ml could attain effectively candida-
static drug levels at an earlier time interval (4 h) than PEC-O treated with lower drug concentrations. The mild reduction in candidastatic action at 6 h relative to at 4 h was not due to a toxic effect of SM-1213 upon phagocytes at the 10 ng/ml concentration for several reasons: 1) uninfected PEC-O incubated in the presence of SM-1213 were >97% viable by trypan blue dye exclusion after 6 h in culture; 2) others have demonstrated that the antibacterial activities of macrophages were significantly enhanced at this (12) or higher (40) drug concentrations; and 3) others have reported that drug concentrations 10-1000 times higher did not affect the viability of WI-38 cells (37) or macrophage monolayers (40).

The occurrence of maximal drug-induced effects at 6 h for PEC-O treated with 0.1-1 ng SM-1213/ml suggests that modulation of PEC-O anticandidal mechanisms are either initiated at an earlier time interval but enacted slowly (over 6 h) or not initiated until 6 h for reasons which may include the unresponsiveness of PEC-O to drug action at earlier intervals or the accumulation or processing of drug by PEC-O, resulting in drug effectiveness at 6 h. Others have suggested that a small but sustained oxidative attack by phagocytes results in effective target cell injury and death over time (97,118). Upon phagocytosis of opsonized Candida, PEC-O pre-incubated in the absence or
presence of drug were capable of producing as much or more $O_2^-$ after 2 h in culture as after 6 h in culture (Figure 5). PEC-0 pre-incubated in the presence of SM-1213 demonstrated enhanced $O_2^-$ production at both time intervals relative to controls (Figure 5). These data suggest that SM-1213 can enhance the oxidative anticandidal mechanisms of PEC-0 after only 2 h of drug treatment and that sustained oxidative injury may be responsible for the observed candidastatic and candidacidal effects at 6 h. However, the effect of culturing PEC-0 over time may also influence candidacidal mechanisms and the modulation of these mechanisms by SM-1213.

When PEC-0 were pre-incubated with SM-1213 for 6 h and washed prior to infection, all drug levels tested induced significant and relatively similar anticandidal effects (Figure 3, Table II). It is not surprising that PEC-0 pre-incubated in the presence of drug prior to the introduction of an infectious agent were equally receptive to modification by all levels of drug rather than one or two levels as observed for PEC-0 exposed to drug at the time of infection (Figure 2, Table II). Other immunomodulatory agents, such as MDP and LPS, are totally ineffective stimulators of an enhanced oxidative response, unless phagocytes are pre-incubated with these agents for 15 min to 4 h prior to the addition of a phagocytic or
chemical stimulus (51,100). Therefore, SM-1213 has the added capacity to enhance the candidacidal action of PEC-0 when administered at the time of phagocyte infection (Figure 2, Table II). These actions can be related to in vivo effects as well, since MDP is most effective in enhancing the survival of mice infected intravenously with \textit{C. albicans} when administered prophylactically 18 h prior to inoculation (23) while SM-1213 is most effective when drug is given 3 to 5 days after infection (83).

The mechanism whereby SM-1213 enters into PEC-0 pre-incubated in the presence of drug, as opposed to PEC-0 exposed to drug during infection, may provide an explanation for the observed differences in PEC-0 candidacidal effectiveness at the 10 ng/ml drug level. For example, PEC-0 pre-incubated in the presence of SM-1213 may use pinocytotic or sugar transfer mechanisms to transport SM-1213 into PEC-0. PEC-0 exposed to SM-1213 at the time of infection would also take drug into the phagocytic vacuole along with \textit{Candida} cells. Within phagocytic vacuoles, the drug may be retained in high concentration and remain localized in this cellular compartment.

Alternatively, during pre-incubation SM-1213 may not enter the cell, but may bind to the exterior of or induce a modification of the cell membrane. The lymphokine, interferon-\(\gamma\), has been reported to be an effective
stimulator of macrophage reactive metabolite production when macrophages are exposed for as little as 10 min to this agent, washed, and stimulated with PMA three days later (94). Therefore, the exposure of PEC-0 to SM-1213, followed by washing of the cells, may provide stimulation without subsequent inhibition of candidacidal mechanisms as a result of excess drug concentration.

At the time of infection, pre-incubated PEC-0 were composed of an entirely adherent cell population, while non-pre-incubated PEC-0 were composed of freshly plated adherent and non-adherent cells. It was therefore conceivable that differences in PEC-0 cell types present or the state of cell adherence at the time of initial candidal infection caused differences in the observed candidacidal activity of pre-incubated versus non-pre-incubated PEC-0. However, this was probably not the case, since PEC-0 allowed to adhere to culture dishes for 2 h and washed prior to infection gave virtually identical results to those obtained for unseparated PEC-0 (unpublished observation, C. J. Morrison).

After 6 h of pre-incubation in the presence or absence of drug, only a 2 h incubation period was required for PEC-0 to kill percentages of Candida almost as great or greater than PEC-0 incubated with Candida for 6 h (Table II). This suggests that, once PEC-0 are primed by pre-incubation in culture for 6 h, they become more effectively
candidacidal upon presentation of the infecting microorganism. However, there is also evidence to indicate that the candidacidal (59) and microbicidal (88) activities of phagocytes may actually be reduced by time in culture and, as depicted in Figure 5, upon stimulation with opsonized Candida the production of $O_2^-$ by PEC-O cultured for 6 h was reduced relative to those cultured for 2 h. Since $O_2^-$ production by phagocytes has been correlated to their candidacidal activity (118), a reduced production of $O_2^-$ at 6 h should result in a reduced anticandidal capacity for PEC-O at this time interval relative to at 2 h. Indeed, for PEC-O pre-incubated in the absence of drug, no $O_2^-$ production was observed at 6 h upon addition of opsonized Candida (Figure 5), although >97% of PEC-O were viable. Whether this reduction at 6 h was due to a non-responsive-ness of PEC-O at this time interval or whether Candida actively "turned off" or failed to initiate $O_2^-$ production by these cells is not known. However, PEC-O pre-incubated in the absence of drug were able to respond oxidatively at 6 h when opsonized zymosan was used as the phagocytic stimulus (Figures 6 and 7), although the response was somewhat reduced relative to at 2 h. This indicates that PEC-O were less responsive to C. albicans than to zymosan at 6 h due to an inherent property of the microbe.

Others have demonstrated that, although two candidal
species were phagocytosed equally, *C. albicans* stimulated only half the quantity of $O_2^-$ production by macrophages as the less virulent species, *C. parapsilosis* (118). Also, the survival of the intracellular pathogen, *Toxoplasma gondii*, within human macrophages was reported to be the result of ingestion of the organism without a stimulation of the production of reactive oxygen intermediates (127). Similarly, in studies presented here, PEC-O pre-incubated in the absence of drug phagocytosed opsonized *Candida* normally at 6 h (unpublished observation, C. J. Morrison), although no stimulation of phagocyte $O_2^-$ production was detected.

The ability of opsonized *Candida* to stimulate $O_2^-$ production by control PEC-O, pre-incubated in the absence of drug for 2 h, may relate to a state of PEC-O "pseudo-activation" as a result of agitation during cell harvesting and plating. Activated macrophages have been shown to generate increased amounts of reactive oxygen metabolites upon phagocytosis of *Candida* (118), and thus the 2 h pseudo-activation of phagocytes might result in a priming of control PEC-O for an enhanced oxidative response upon stimulation with *Candida*. It is hypothesized that, after 6 h in culture, the initial pseudo-activation of PEC-O would diminish so that opsonized *Candida* would no longer stimulate an oxidative response. Cultured human macro-
phages have been reported by others (86,94) to gradually lose $\text{H}_2\text{O}_2$-producing capacity with time in culture, while resident mouse macrophages have been reported to gradually increase this capacity over a 72-h period (87). Others have reported an increase followed by a decrease in $\text{H}_2\text{O}_2$-producing capacity for human macrophages (89).

Results of studies with monocytes from a patient with X-linked CGD and his heterozygous mother were reported to indicate that oxygen-dependent mechanisms were important to the early and efficient killing of \textit{T. gondii}, but that non-oxidative reserve mechanisms which were less efficient were capable of killing this microbe (127). Similar results were reported for the killing of \textit{Leishmania donovani} by CGD monocytes and, upon exposure to lymphokines, CGD macrophages (86). The observed killing of \textit{Candida} at 6 h by PEC-O pre-incubated in the absence of drug (Table II) without detectable $\text{O}_2^-$ production (Figure 5) may, therefore, be due to the non-oxidative killing mechanisms of phagocytes. PEC-O pre-incubated in the presence of drug demonstrated a reduced but substantial production of $\text{O}_2^-$ at 6 h relative to at 2 h (Figure 5). Drug-treated PEC-O may therefore utilize both oxidative and non-oxidative killing mechanisms, resulting in the observed overall enhancement of candidal killing for drug-treated PEC-O relative to control PEC-O (Table II).
Candida cells may also possess protective mechanisms whereby they "turn off" the anticandidal systems of macrophages. Ozato and Uesaka (99) observed that intracellular candidal growth was temporarily inhibited by casein-elicited macrophages but, after 2-3 h, candidal growth resumed, resulting in the outgrowth and survival of Candida cells. The production of endogenous scavengers of reactive oxygen metabolites such as catalase or superoxide dismutase (SOD) may be induced in microorganisms exposed to high oxygen tensions (38). Therefore, Candida may interfere with the anticandidal capacities of macrophages via the production of these enzymes. As detailed in the introduction to this paper, candidal catalase or SOD in and of itself may not be sufficient to protect fungi from damage by external oxygen metabolites (118). However, the exogenous addition of catalase or SOD to macrophages ingesting Candida can inhibit phagocyte cidal activity (118) and, in conjunction with PMN (77) or macrophages (88), microbial or phagocyte catalase may antagonize phagocyte-mediated killing. For example, Murray et al (88) reported an increase in intracellular macrophage catalase activity with time in culture that was associated with a decreased ability of phagocytes to kill T. gondii. In studies concerning the effect of SM-1213 on PEC-0 catalase activity, no increase in phagocyte catalase activity was
observed for resident or oil-elicited PEC between 2 and 6 h of culture (unpublished observation, C. J. Morrison). Although no increase in catalase activity was observed over time, control (0 ng/ml) PEC-0 demonstrated a 1.5-fold greater catalase activity when infected relative to uninfected PEC-0 (Table III). This suggests that, after phagocytes produce reactive oxygen metabolites in response to an infectious agent, PEC-0 initiate an enhanced endogenous production of catalase to protect against autooxidative damage (112). As a result, phagocytes may reduce their own antimicrobial effectiveness or the antimicrobial effectiveness of neighboring phagocytes upon autolysis and release of endogenous catalase. In vivo, however, infections may be resolved as a result of the sequential reinforcement or renewal of phagocytes induced by cellular immunity (82) or the release of inflammatory mediators (124).

Another possible explanation for the moderately higher catalase activities in the infected versus uninfected PEC-0 may be due to a contribution of catalase activity from \textit{C. albicans} cells. Sasada and Johnston (118) suggested that intracandidal SOD or catalase did not affect the level of detectable \textit{O}_2^- produced by macrophages, since intact, viable \textit{Candida} showed no detectable release of these enzymes. Upon sonication, however, substantial
candidal catalase (320 U per mg protein) and SOD (21.8 U per mg protein) activity was detected (118). It is therefore plausible that killing of candidal cells by PEC-0 could result in some leakage of intrafungal catalase. However, it is unlikely that this is the case, since: 1) SM-1213-treated PEC-0, which killed more Candida, did not demonstrate greater catalase activity after cell lysis than cell lysates from control PEC-0 (Table III); 2) the increased catalase activity of infected PEC-0 was selective in that no other enzyme activities tested were increased (unpublished observation, C. J. Morrison, and Table III), including enzymes commonly found in fungi (21,119); and 3) infected PEC-0 lysates did not contain more protein than cell lysates from uninfected PEC-0.

Since an increase in phagocyte catalase activity has been correlated to a reduced microbicidal capacity (88), the observed reduced capacity of control PEC-0 to kill C. albicans cells (Table II) relative to drug-treated PEC-0 may be the result of not only a relatively reduced production of reactive oxygen metabolites (Figures 5,6,7, 10,11), but a relatively greater production of catalase (Figure 4, Table III) by control PEC-0. It has been reported by others (57) that $O_2^-$ can interfere with the heme portion of the catalase molecule and, as a result, inhibit its activity. It may be, then, that the reduction in
catalase activity observed for drug-treated PEC-0 (Figure 4) is the result of the increased production of $O_2^-$ by these cells (Figures 5,6,7).

Resident PEC catalase activities were unaffected by drug treatment, suggesting that the physiological state or the state of differentiation of PEC affects PEC responsiveness to drug modulation. For example, elicited cells contain a larger proportion of more newly recruited cells (monocytes) than resident cells, which consist predominantly of established cells (macrophages)(42). Upon presentation with a phagocytic or chemical stimulus, macrophages from uninflamed tissues secrete little $O_2^-$ or $H_2O_2$ (49,96), while cells primed by exposure to inflammatory agents or lymphocyte mediators secrete high levels of these agents (49,87). Therefore, a similar mechanism of PEC priming may be necessary before maximum SM-1213-induced effects are invoked. As reported for MDP (23), SM-1213 administered in vivo can also modulate PEC activities in vitro. PEC-0, obtained on day three from guinea pigs treated intramuscularly with 10 mg SM-1213 per kg per day for three days, demonstrated a significant reduction in catalase activity in vitro relative to PEC-0 from animals which did not receive drug (unpublished observation, C. J. Morrison). In addition, significant drug-induced reductions in catalase activity were observed in lysates of the
phagosomal fraction of homogenized guinea pig PEC-0 treated with 1 ng SM-1213/ml (P. Gordon, personal communication). Thus, enhanced intraphagosomal killing of Candida may result from a reduced catalase activity (or enhanced $O_2^-$ production?) at this site due to drug treatment of PEC-0. Although reduced, cytosolic catalase activity may remain sufficiently active to protect PEC from auto-destruction due to the leakage of reactive oxygen products from the phagosomal compartment (112).

The ratio of cell-associated to extracellular $O_2^-$ and $H_2O_2$ produced was significantly increased in SM-1213-treated PEC-0 relative to control PEC-0. In control PEC-0, only 0 to 14% of total oxidative metabolites were cell-associated, while, in drug-treated PEC-0, 34 to 44% were cell-associated. Therefore, if oxidative candidacidal mechanisms are initiated intraphagocytosally or intracellularly, drug-treated PEC-0 would be expected to kill a greater percentage of phagocytosed Candida than untreated PEC-0. Also, since 100% of $O_2^-$ and 86% of $H_2O_2$ produced by control PEC-0 were extracellular, not only would intracellular candidal killing be reduced in these cells, but oxidative damage to surrounding tissues (116) and phagocytes (112) would be increased. The anticandidal and anti-inflammatory effects of SM-1213 have been demonstrated in vivo where intramuscularly infected mice treated with
SM-1213 produced smaller candidal abscesses relative to control mice without a concomitant reduction in candidacidal activity (83).

Since SOD is a particularly efficient inhibitor of candidacidal activity, others have suggested that $\text{O}_2^{-}$ may be an especially important reactive oxygen metabolite in phagocyte anticandidal activity (118). However, several researchers have suggested that $\text{H}_2\text{O}_2$ is the oxidative agent primarily responsible for phagocyte tumoricidal (92,97) and microbicidal (86,95) activity. Both SM-1213 and the structurally related (Figure 1) immunomodulatory drug, MDP, effectively enhanced total $\text{H}_2\text{O}_2$ production by PEC-O (Figures 10 and 11). This enhancement was evident at 2 and at 6 h. $\text{H}_2\text{O}_2$ production increased between 2 and 6 h by an average of 30% for PEC-O pre-incubated in the presence of drug, while $\text{H}_2\text{O}_2$ production remained unchanged over time for PEC-O pre-incubated in the absence of drug (Figures 10 and 11). The increase in $\text{H}_2\text{O}_2$ production between 2 and 6 h correlates with the decrease in detectable $\text{O}_2^{-}$ produced during this time interval (Figures 5,6,7). This suggests that a greater quantity of $\text{O}_2^{-}$ is being converted into $\text{H}_2\text{O}_2$ (reducing detectable $\text{O}_2^{-}$ levels) at 6 h than at 2 h in drug-treated PEC-O. These observations would provide an explanation for the reduced production of $\text{O}_2^{-}$ over time in culture while PEC-O viability was intact. However, this explana-
tion is relevant to drug-treated PEC-0 only, since no increase in H₂O₂ production over time was observed for control PEC-0 (Figures 10 and 11). Not all O²⁻ produced was spontaneously dismutated to H₂O₂, however, since the addition of SOD to the assay system increased detectable H₂O₂ production by 26%.

In addition to significantly increasing the production of active oxygen species by phagocytes, SM-1213 and MDP can significantly enhance the candidacidal and candidastatic activity of a cell-free xanthine-xanthine oxidase (115) O₂⁻-generating system (unpublished observations, C. J. Morrison). Complete protection of Candida from growth inhibition occurred when SOD was included in the system and substantial (83%) protection occurred when catalase was included. Therefore, O₂⁻ and H₂O₂ were important mediators of the anticandidal activity of this system. Scavengers of ·OH and singlet oxygen did not protect Candida. Preliminary experiments indicate that SM-1213 can enhance the binding of xanthine oxidase to Candida, resulting in an enhanced killing capacity which may be due to the closer proximity of the O₂⁻-producing machinery to the target cell. Others (130) have reported that binding of myeloperoxidase (MPO) to Candida cells is required for MPO-mediated killing of fungi and bound eosinophil peroxidase (EPO) has been demonstrated to effectively enhance the
microbicidal activity of EPO-mediated killing of *T. gondii* (70).

It has been reported that soluble cell wall mannans bind to myeloperoxidase (MPO), and thereby competitively inhibit MPO-mediated killing of *Candida* in a dose-dependent manner (130). In addition, soluble candidal mannans demonstrated a similar dose-dependent inhibitory effect on neutrophil-mediated candidacidal activity (130). In macrophages, which may or may not contain peroxidase activity (9,22,25), it could be speculated that soluble cell wall mannans may act as competitive inhibitors for the interaction or binding of the $O_2^-$-producing NAD(P)H oxidase. This would be especially important if close proximity or binding of the NAD(P)H oxidase to the target cell was required for NAD(P)H oxidase-mediated candidal killing, as binding is required for MPO-mediated candidacidal activity (130). Such binding would allow for the generation of short-lived $O_2^-$ in close proximity to the target cells, so as to increase the probability that the microorganism would become substrate for this reactive product and others (130).

Alternatively, the action of NAD(P)H oxidase may be "turned off" by the binding of soluble cell wall mannans. This hypothesis would explain the observed absence of $O_2^-$ production at 6 h by untreated PEC stimulated with opsonized *Candida* (Figure 5), but would not explain the lack of
an inhibitory effect at 2 h. In addition, zymosan, derived from cell wall components of the yeast *Saccharomyces cerevisiae* and composed largely of mannan (5), is a potent stimulator of phagocyte oxidative responses when presented in the opsonized form (49,104) and to a lesser extent in the unopsonized form (26). Also, mannans have been reported to enhance $H_2O_2$ production by PMN, while mannose inhibited its production (26). It is likely, then, that candidal mannans "turn on" the NAD(P)H oxidase, rather than turning it off.

Since the oxidative responses of phagocytes can be stimulated by a number of soluble agents (92,104,113), the initiation of the metabolic burst has been reported to occur at the plasma membrane (43,90). Molecules which can stimulate the oxidative burst, such as concanavalin A (Con A) (110), are reported to bind to mannose residues of phagocyte plasma membrane glycoproteins, resulting in a perturbation that invokes the oxidative burst (104). It has been proposed by others (26) that the stimulation of the oxidative burst of PMN upon presentation with *C. albicans* blastospores involves a receptor-ligand interaction at the plasma membrane. This hypothesis was based on a mannose-specific mechanism, since oxidative stimulation by *Candida* (but not by PMA or opsonized Sephadex) could be competitively inhibited upon the addition of mannose (26).
Since the NAD(P)H oxidase of phagocytes resides in the plasma membrane (8,91), either it or glycoproteins associated with it may be cross-linked by the binding of *C. albicans* or fungal cell wall components. Therefore, binding of *Candida* to the NAD(P)H oxidase may be required for the initiation of the oxidative burst and the subsequent killing of *Candida* by phagocytes via locally produced oxygen metabolites.

However, binding of *Candida* to the membrane may not be sufficient for maximal activation of the NAD(P)H oxidase. For example, the stimulation of the oxidative burst by Con A will not occur unless this agent is in the tetrameric form (104). Also, phytohemagglutinin (PHA), soybean agglutinin (SBA) and *Lotus* lectin all bind to macrophages (39), but do not stimulate $O_2^-$ production (104). Therefore, the configuration of the stimulating agent, as well as its binding to phagocytes, influences oxidative responsiveness. This was evident in studies conducted here in that opsonized *Candida* did not stimulate $O_2^-$ production by PEC-O after 6 h in culture unless PEC-O had been pre-incubated in the presence of SM-1213 (Figure 5). Opsonized zymosan, however, could stimulate $O_2^-$ production at 6 h by control PEC-O (Figures 6 and 7) and SM-1213 pre-treatment of PEC-O enhanced this production. Thus, even though zymosan and *Candida* contain mannan and/or mannose,
the manner in which they interact at the plasma membrane level differs somewhat. Similarly, the greater resistance of *C. albicans* to macrophage killing relative to other species has been suggested to result from the less favorable stimulation of the respiratory burst enzyme in the plasma membrane by *C. albicans* (118).

Since pre-incubation of PEC-0 with SM-1213 enhances the subsequent stimulation of PEC-0 oxidative metabolism upon the addition of either zymosan or *Candida* (Figures 5, 6,7), its actions may involve: 1) interacting with or alteration of the plasma membrane or the membrane oxidase; 2) changing the configuration of the stimulating agent; 3) changing the configuration of opsonins or the response of PEC-0 to opsonins; or 4) enhancing the binding of stimulating agents to the membrane. SM-1213 may enhance the binding of *Candida* to the membrane oxidase in a manner similar to that proposed by Wright *et al* (130) for the binding of MPO to *Candida*. It was suggested (130) that the binding of MPO to *Candida* was facilitated by electrostatic forces, since MPO is a cationic enzyme and the surface of *Candida* is anionic. Similarly, the alkylaminoalkyl substitution of SM-1213 is cationic at physiological pH (P. Gordon, personal communication) and may serve to bind SM-1213 to candidal surfaces. Drug treatment may thereby change the surface of *Candida* so that the manner in which
fungi are presented to PEC-O or are bound by PEC-O is altered. Experiments conducted on the NADPH-dependent production of \( \text{O}_2^- \) by cell lysates revealed that, in the absence of Candida, SM-1213 could enhance NADPH-dependent \( \text{O}_2^- \) production (Figures 8 and 9, Table IV). Therefore, although candidal binding, candidal configuration, or opsonic configuration may be influenced by drug, the activity of the NAD(P)H oxidase enzyme or the membrane in which it is located is altered by SM-1213 treatment as well. However, opsonins would probably not be affected, since the enhanced killing of Candida by drug-treated PEC-O occurred in their absence (Table II).

The NADPH-NAD(P)H oxidase system of PEC-O was directly modified by SM-1213 treatment (Table IV). Superoxide anion production by cell lysates was enhanced an average of 28.7% in the presence of drug, and this enhancement was increased to 50.2% if PEC-O were pre-incubated with drug prior to cell lysis (Table IV).

Drug-enhanced NADPH-dependent \( \text{O}_2^- \) production by cell lysates was time dependent (Figures 8 and 9) and was not apparent until the 6 h time interval. This was the case whether PEC-O were pre-incubated with drug prior to lysis (Figures 8 and 9) or pre-incubated without drug and exposed to SM-1213 during the time of NBT assay only (Table IV). Therefore, PEC-O become more receptive to drug modification
with time in culture. Indeed, unlike $O_2^-$ production by whole PEC-0, $O_2^-$ production by cell lysates was enhanced at 6 h relative to at earlier time intervals (Figure 9). The reduced activity at 0 h may have resulted from the presence of contaminating red blood cells which contain SOD (75). Pre-incubation of PEC-0 with drug prior to cell lysis increased $O_2^-$ production by cell lysates an average of 22.7% relative to cell lysates treated with drug during NBT assay only. Thus, pre-treatment of PEC-0 with drug, as well as incubation of PEC-0 in culture, primes phagocytes to produce greater quantities of $O_2^-$. The significantly higher quantities of $O_2^-$ produced by cell lysates (Figure 9) relative to whole PEC-0 (Figure 6) may relate to the availability of the $O_2^-$-producing enzyme to activation. Although located in the plasma membrane (8), NAD(P)H oxidase, once liberated by cell lysis, may have more access to activation by substrate. Also, the exogenous addition of substrate in excess may allow for a more complete expression of enzyme activity. Others have reported (50) an increased production of $O_2^-$ by cell lysates relative to intact phagocytes, particularly in phagocytes from patients with CGD. Intact CGD phagocytes were markedly deficient in $O_2^-$ production, while cell lysates from CGD phagocytes were only mildly deficient (50). It was suggested that the enzyme or enzymes controlling $O_2^-$ production
were present within CGD phagocytes, but that they were not strategically placed or not fully activated upon stimulation with a phagocytic particle (50).

NADPH-dependent O$_2^-$ production by cell lysates from resident PEC was not affected by SM-1213 treatment (Table V). Also, the quantities of O$_2^-$ produced by PEC-r lysates were generally lower than for PEC-O lysates (Figure 9, Table IV and V). The bases for these differences between resident and oil-elicited cell lysates may relate to the character of each of these cell types. The resident cells of the peritoneal cavity, which are primarily mature macrophages, have been shown by others (6) to differ from newly recruited inflammatory cell types, which are primarily monocytes. The maturation of mononuclear phagocytes into macrophages is associated with alterations of plasma membrane proteins, ecto-enzyme activity, and surface receptor expression (17,18). Although not readily apparent in our studies, differences in cell size (18) may also account for differences in the relative O$_2^-$-producing capacities of PEC-r and PEC-O. Therefore, as mentioned earlier in reference to the modulation of PEC catalase activity, the physiological state and the state of differentiation of PEC can influence phagocyte functions and, in this instance, the O$_2^-$-producing capacity of PEC and the modulation of this capacity by SM-1213.
The significant relationship between the drug-enhanced production of PEC-O reactive oxygen metabolites and the increased killing of Candida by drug-treated phagocytes was demonstrated upon incubation of Candida with an NADPH-cell homogenate derived NAD(P)H oxidase system (Figure 12). SM-1213 enhanced the candidacidal capacity of this enzyme system. The mechanism for this enhancement is not known, but data from experiments previously discussed using cell lysates (Figure 9, Table IV) suggest that SM-1213 can directly enhance the $O_2^-$-producing capacity of phagocyte NAD(P)H oxidase. Experiments are underway to determine if SM-1213 can also enhance the binding of Candida to NAD(P)H oxidase, and thereby enhance candidal killing upon addition of NADPH. Since 50-88% of candidal killing by this system was inhibitable by SOD, the reactive oxygen intermediate responsible for candidacidal activity was most probably $O_2^-$. However, not all killing was due to oxidative mechanisms, since some killing occurred in the absence of substrate NADPH and when cell homogenates were boiled prior to addition to the system (Figure 12). Since cell homogenates were utilized and not purified enzymes, killing resulting from exposure of Candida to homogenates may be due in part to non-oxidative candidal killing by components derived from phagocyte lysosomal compartments (i.e., cationic proteins) or other toxic agents, and is
therefore an artifact of this system. NADPH-dependent killing is presumed to represent oxidative cytocidal mechanisms. Therefore, an examination of the NADPH-independent and NADPH-dependent killing of *Candida* revealed that the majority of candidal killing by control cell homogenates was NADPH-independent (non-oxidative), while the greater portion of candidal killing by drug-treated cell homogenates was NADPH-dependent (oxidative) (Figure 12). In whole PEC-O, however, the proportion of oxidative to non-oxidative candidal killing may be much greater since, in cell homogenates, a bolus of non-oxidative hydrolytic and degradative enzymes, as well as candidacidal cationic proteins (66), may be released upon phagocyte lysis.

In conclusion, SM-1213 treatment of PEC-O was observed to inhibit the growth and reduce the viability of intracellular *Candida* cells, as well as to enhance the production of PEC-O oxidative metabolites, hydrogen peroxide and superoxide anion. The enhanced NADPH-dependent O$_2^-$ production by phagocytes treated with SM-1213 and by cell lysates treated with SM-1213 at the time of assay indicate that the observed enhanced production of oxidative metabolites results from an increased activation of PEC-O NAD(P)H oxidase, the enzyme responsible for the production of reactive oxygen species by phagocytes (8,91). The role of oxygen metabolites in the candidacidal activity of phago-
cytes has been established by others (62,118), and the modulation of NAD(P)H oxidase activity by SM-1213, resulting in a concomitant increased production of these agents, was supported by the enhanced candidacidal action of the NADPH-NAD(P)H oxidase system in the presence of SM-1213.
SUMMARY

SM-1213 [1,2-O-isopropylidene-3-O-3'(N',N'-dimethylamino-n-propyl)-D-glucofuranose] is a synthetic, substituted monosaccharide which exerts immunomodulatory actions. In the absence of PEC, SM-1213 had no direct fungistatic or fungicidal effect against the yeast or hyphal form of Candida albicans, a clinically important opportunistic fungus. However, SM-1213 was effective in inducing PEC-0 to inhibit the intracellular formation and elongation of C. albicans hyphae and to reduce the percentage of viable Candida remaining after phagocytosis. Significant drug-induced candidastatic and candidacidal effects were also observed when PEC-0 were pre-incubated in the presence of SM-1213 and washed prior to infection with Candida. These results indicate that SM-1213 was able to potentiate one or more candidacidal processes expressed by PEC-0. Pre-incubation of PEC-0 with SM-1213 prior to introduction of a phagocytic stimulus resulted in an enhanced production of both hydrogen peroxide and superoxide anion. In addition, incubation of PEC-0 with SM-1213 for 6 h resulted in a selective reduction in adherent cell catalase activity relative to control PEC-0. NADPH-dependent production of superoxide anion was enhanced in cell lysates from PEC-0.
pre-incubated in the presence of SM-1213, as well as in cell lysates from PEC-O pre-incubated in the absence of drug and exposed to SM-1213 during the time of assay. NADPH-dependent killing of \textit{C. albicans} by PEC-O homogenates was also increased by SM-1213. These data suggest that the observed increased candidacidal effects for drug-treated PEC-O result in part from an enhancement of the NADPH-dependent oxidative killing mechanisms of phagocytes and, more specifically, from drug enhancement of phagocyte NAD(P)H oxidase activity by mechanisms yet to be determined.
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