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

Christine Joy Morrison
Loyola University Chicago

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ENHANCEMENT OF THE CANDIDACIDAL ACTIVITY OF CULTURED

PERITONEAL EXUDATE CELLS BY SM-1213,

A SYNTHETIC IMMUNOMODULATOR

by

Christine Joy Morrison

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The author, Christine Joy Morrison, is the daughter of Kenneth Alfred Morrison and Emily (Russell) Morrison. She was born January 29, 1954, in Oak Park, Illinois.

Her elementary education was obtained in the public schools of Berwyn, Illinois, and secondary education at the J. S. Morton High School, West, Berwyn, Illinois, where she was an officer in the Junior Honor Society and a member of the Senior Honor Society. She was graduated in 1972.

In September, 1972, she entered the College of DuPage and, in June, 1974, received an Associate of Arts degree and was graduated with high honors. In September, 1974, she entered Northwestern University and, in June, 1976, received the Bachelor of Arts degree in Biology. While attending Northwestern University, Christine was a member of Beta Beta Beta, the national honorary biology fraternity.

In July, 1977, she entered the graduate program at Loyola University Stritch School of Medicine and, in May, 1980, received the Master of Science degree in Microbiology. Christine is a member of Sigma Xi, the Chicago Association of Immunologists, the American Association of Immunologists, the Illinois Society for Microbiology and the American Society for Microbiology.

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LIST OF ABBREVIATIONS AND SYMBOLS

BCG   Bacillus Calmette-Guérin

BL   Boiled homogenate

BSA   Bovine serum albumin

cfu   Colony forming units

CGD   Chronic granulomatous disease

CMC   Chronic mucocutaneous candidiasis

CMI   Cell-mediated immunity

Con A   Concanavalin A

EBSS   Earle's balanced salt solution (phenol red free)

EPO   Eosinophil peroxidase

EDTA   Ethylenediamine tetraacetic acid

FCC   Ferricytochrome c

HBSS   Hank's balanced salt solution (phenol red free)

HMP   Hexose monophosphate

H_2O_2 Hydrogen peroxide

HPO   Horseradish peroxidase

LDH   Lactate dehydrogenase

LPS   Lipopolysaccharide

MDP   Muramyl dipeptide (N-acetyl-muramyl-L-alanyl-
             D-isoglutamine)

MPO   Myeloperoxidase

NAD(H)   Nicotinamide adenine dinucleotide (reduced)

NADP(H)   Nicotinamide adenine dinucleotide phosphate
             (reduced)

NAD(P)H	Nicotinamide adenine dinucleotide (phosphate), reduced
NAGA	β-N-Acetylglucosaminidase
NBT	Nitroblue tetrazolium salt
ND	Not determined
NI	Non-idet detergent
$^1O_2$	Singlet oxygen
O_2^-	Superoxide anion
·OH	Hydroxyl radical
PBS	Phosphate buffered saline
PEC	Peritoneal exudate cell(s)
PEC-O	Oil-elicited PEC
PEC-r	Resident PEC
PHA	Phytohemagglutinin
PMA	Phorbol myristate acetate
PMN	Polymorphonuclear leukocyte(s)
PRS	Phenol red solution
SBA	Soybean agglutinin
SDA	Sabouraud's dextrose agar
SDB	Sabouraud's dextrose broth
SEM	Standard error of the mean
SM-1213	1,2-O-Isopropylidene-3-O-3'-(N',N'-dimethyl-amino-n-propyl)-D-glucofuranose
SOD	Superoxide dismutase
SWA	Waymouth's medium supplemented with 2 mg/ml BSA and 5 µg/ml oleic acid

TX-100   Triton X-100 detergent

UND   Undetectable

CHAPTER I

INTRODUCTION AND REVIEW OF RELATED LITERATURE

Research interest has recently developed for a
heterogeneous group of chemical compounds which have poten-
tial 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 resis-
tance 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 pa-
tients with chronic granulomatous disease (CGD) or myelo-
peroxidase (MPO) deficiency, where the antimicrobial ca-
pacities of phagocytic cells are impaired (60,64), implies
that neutrophils or activated macrophages play the most
important role in host defense against systemic candidi-
asis (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 can-
didiasis, 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, pro-
teose 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 capac-
ity to kill the same or unrelated organisms (72), and
display such characteristics as an increase in size, en-
hanced 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-asso-
ciated oxidative burst is increased in elicited and acti-
vated macrophages, see Johnston (48).]

Production of reactive oxygen metabolites by macro-
phages mediates much of their microbicidal (50,118,127) and
tumoricidal (92,97) activity, although some killing by oxy-
gen-independent mechanisms also occurs (66,67,86). Oxygen-
dependent systems are classified as such because of a de-
crease in microbicidal or tumoricidal activity under anaer-
obic conditions (63,81,97). However, some microbes (76),
such as Bacteroides fragilis, Staphylococcus epidermidis
and Clostridium perfringens, are normally killed by phago-
cytes in the absence of air. Therefore, oxygen-independent
antimicrobial mechanisms must also exist. Possible media-
tors of macrophage oxygen-independent cytocidal 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 (H_2O_2) and superoxide (O_2^-) (48). The superoxide anion, formed by the addition of an electron to molecular oxygen, can gain an additional electron to produce H_2O_2 or lose an electron to produce molecular oxygen (4). Two superoxide radicals can be spontaneously dismutated to H_2O_2 and oxygen or enzymatically converted to these products by the action of superoxide dismutase (SOD) (79):

$$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$
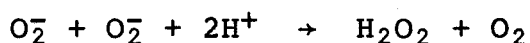
The resulting H_2O_2 can also be removed enzymatically to produce H_2O and oxygen by the action of catalase.

The toxicity of O_2^- and H_2O_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 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_2O_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_2O_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 ($\cdot OH$) and singlet oxygen ($^1O_2$), which may be produced as a result of the interaction of O_2^- and H_2O_2 in the following manner:

$$O_2^- + H_2O_2 \rightarrow {}^1O_2 + OH^- + \cdot OH$$
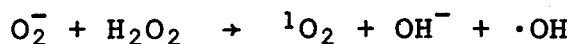
The observed microbicidal activity of phagocyte-produced H_2O_2 and O_2^- may therefore result from the formation of other highly reactive oxygen metabolites, as well (56).

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 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 Candida cells via interaction with other cells or factors (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 Listeria or Mycobacterium has been demonstrated by Mackaness and others (72,98). The lymphokine responsible for the priming of macrophages for an enhanced oxidative and antimicrobial response to Toxoplasma gondii has recently been identified as interferon-γ (94). Maiti et al (73) demonstrated that mouse peritoneal macrophages activated in vivo by BCG or in vitro by exposure to phytohemagglutinin-induced lympho-kines exhibited greater phagocytic and candidacidal activity than resident peritoneal macrophages. The highest phagocytic and candidacidal activity, however, was produced by exposure of macrophages to Candida-induced lymphokines. Immune mouse serum also enhanced the phagocytic and candi-dacidal activity of resident, but not activated, macro-phages. Therefore, humoral and cell-mediated mechanisms may be required for an effective macrophage-mediated con-trol 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 in vivo directly correlates to the augmentation of
candidal killing by macrophages in vitro (23,118). In
addition, administration of MDP prior to or immediately
following infection with C. albicans prolongs the survival
of intravenously inoculated mice (23).

The reactive oxygen metabolites hydrogen peroxide
(H_2O_2), superoxide (O_2^-), hydroxyl radical ($\cdot OH$) and singlet
oxygen ($^1O_2$) have all been implicated in macrophage can-
didacidal activity (118). Candida parapsilosis, a less
pathogenic species of fungi, elicited a greater oxidative
response and was killed more efficiently by mouse peri-
toneal macrophages than was C. albicans (118). Killing of
both candidal species was inhibited by the exogenous addi-
tion of enzymes which remove H_2O_2 (catalase) or O_2^- (super-
oxide dismutase; SOD) or scavenge $\cdot OH$ (benzoate) or singlet
oxygen (azide) (118). Other data to support the require-
ment of oxygen metabolites in macrophage-mediated candida-
cidal activity include: 1) the macrophage-like cell line
J774.1 produced less than 10% as much O_2^- as did resident
mouse peritoneal macrophages and, although candidal in-
gestion 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 <u>Candida</u> (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 cytocidal 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 cytocidal 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 H_2O_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 O_2^- and H_2O_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 O_2^- and H_2O_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, H_2O_2-mediated killing by these cells is enhanced by a myeloperoxidase-H_2O_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 peri-nuclear cisternae, the Golgi vesicles and in some membrane-bound granules (22,25), the functional relevance of this enzymatic activity in relation to H_2O_2-mediated killing mechanisms remains to be established.

The enzyme primarily responsible for the respira-tory burst in guinea pig macrophages has recently been identified as a membrane-bound oxidase that preferentially uses NADPH as substrate (8). Evidence supporting a mem-brane-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 activ-
ity 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 macro-
phages (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 di-
rectly 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'-di-
methylamino-n-propyl)-D-glucofuranose, monohydrochloride]
was obtained from Greenwich Pharmaceuticals, Inc., Green-
wich, CT. Muramyl dipeptide (MDP, N-acetyl-muramyl-L-
alanyl-D-isoglutamine·¼ H$_2$O, grade A; Calbiochem-Behring
Corp. La Jolla, CA) was used in some experiments in par-
allel 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 Micro-
biology, Roslyn, NY)], was grown aerobically on Sabouraud's
dextrose agar (SDA; Difco, Detroit, MI) for 24 h at 37°C

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Figure 1.   A.   The chemical structure of SM-1213 [1,2-O-
                 isopropylidene-3-O-3'-(N',N'-dimethylamino-
                 n-propyl)-D-glucofuranose, monohydrochloride]

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

HO—CH₂
HO—CH
H
O
O
H
H
C—CH₃
CH₃
O
CH₂CH₂CH₂N(CH₃)₂ · HCl

A. SM-1213

CH₂OH
H
O
H
OH
HO
O
H
H
H
NHCOCH₃
H
CH₃CHCO
(αNH)
L-ALANINE
D-ISOGLUTAMINE
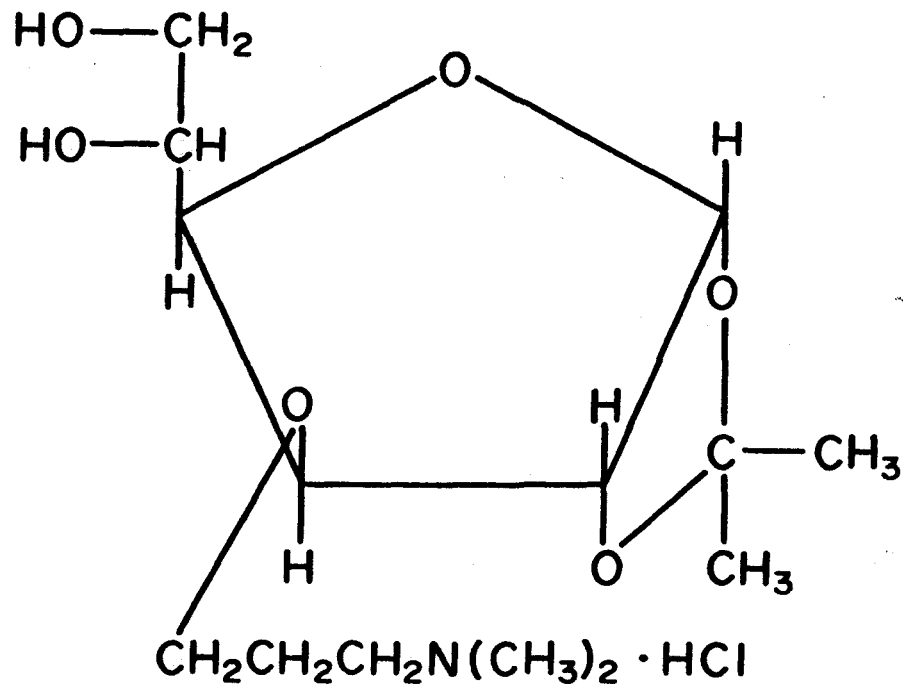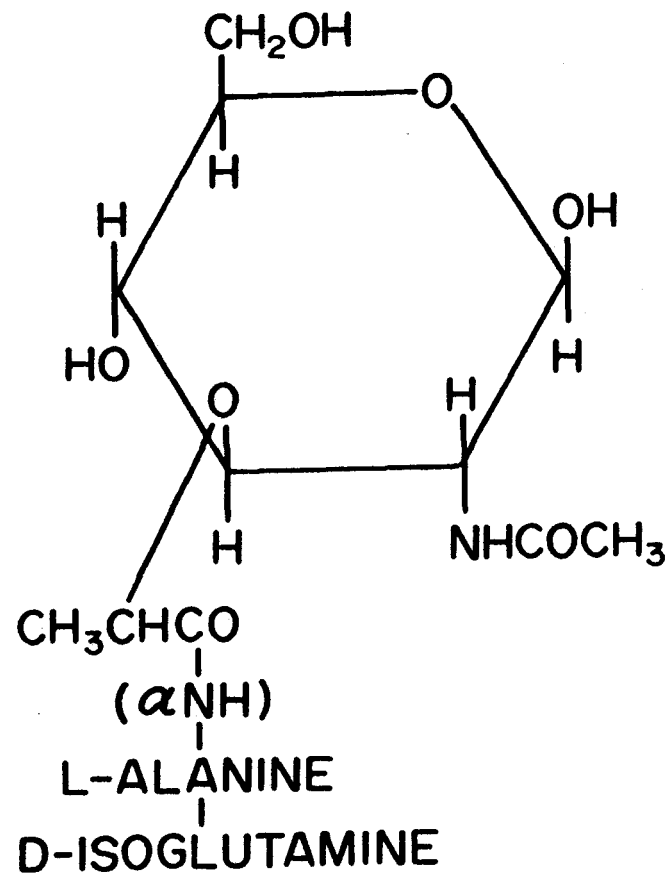
B. MDP

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 1×10^5 cfu. Flasks were incubated aerobically at 37^OC 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^OC. 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-O), 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-O 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 (Schering) 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, KH_2PO_4 0.012 and Na_2HPO_4 0.091), quantified in
a hemacytometer, and diluted to the appropriate concen-
tration in SWA medium. Differential counts of at least
200 PEC-O were performed on Wright-Giemsa stained smears.
The average cellular profile derived from multiple experi-
ments was as follows: 77.2 ± 0.8% macrophages and mono-
cytes, 2.6% PMN and 20.2% lymphocytes, mast cells and
eosinophils. Total cellular harvest per animal averaged
1.17 (±0.14) X 10^8 PEC-O.

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 fol-
lows: 93.0 ± 3.3% macrophages and monocytes, 0% PMN and
7.0% lymphocytes, mast cells and eosinophils. Total cellu-
lar harvest per animal averaged 3.07 (±0.61) X 10^7 PEC-r.
After 2 h in culture, greater than 95% of resident or oil-
elicited adherent cells consisted of macrophages, as deter-
mined 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^OC 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 <u>Candida</u>
was determined by counting 100 to 200 <u>Candida</u>-containing
peritoneal cells:

$$\frac{\%\ \underline{Candida}}{\underline{killed}} = \frac{\text{Number of } \underline{Candida}\text{ unable to form microcolonies}}{\substack{\text{Number of } \underline{Candida} \\ \text{able to form} \\ \text{microcolonies}} + \substack{\text{Number of } \underline{Candida} \\ \text{unable to form} \\ \text{microcolonies}}} \times 100$$

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 inocula-
tion with yeast cells. Hyphal elongation was determined
microscopically by eyepiece micrometer and candidal via-
bility 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 sam-
ples 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-idet 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 determina-
tions 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 H_2O and 1.0 ml
0.059 M H_2O_2 in 0.05 M KH_2PO_4 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 H_2O_2 per min at 25°C, pH 7.0, as deter-
mined from absorbance measurements at 240 nm using a molar
extinction coefficient of 70 $M^{-1}cm^{-1}$ for H_2O_2 (7).

2. Peroxidase (EC 1.11.1.7)

Peroxidase activity was measured as the decrease
in sample fluorescence following the oxidation of scopo-
letin (7-hydroxy-6-methoxy coumarin; Sigma) by peroxidase
and H_2O_2 (114). Fluorescence was monitored for a period
of 5 min on an Aminco-Bowman spectrophotofluorometer (Amer-
ican 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_2PO_4 buffer (pH 7.0),
0.1 ml 0.1 M H_2O_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 oxi-
dized 1 μmole of o-dianisidine per min at $25^{\circ}C$, 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.   β-N-acetylglucosaminidase (EC 3.2.1.30)

The method of Woollen et al (129) was used to
measure β-N-acetylglucosaminidase (NAGA) activity in bio-
logical samples. The formation of p-nitrophenol from p-
nitrophenyl-N-acetyl-β-D-glucosaminide (Sigma) was deter-
mined 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-
N-acetyl-β-D-glucosaminide in citrate buffer, and 0.5 ml of
the biological sample and were incubated for 2 h at $37^{\circ}C$ 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 H_2O, 1.0 ml 1% w/v copper sulfate in H_2O, 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 H_2O. Biological sample (0.1 ml) was diluted with 0.4 ml H_2O 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 H_2O_2-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 H_2O_2 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 H_2O_2 generation by the cell lysate was determined spectrophotometrically after centrifugation and alkalinization of the supernatants as described above. Hydrogen peroxide concentration was determined using a standard curve which utilized PRS and a range of H_2O_2 concentrations up to 200 μM.

Superoxide anion generation was measured by one of two methods. Whole cell O_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 $37^{O}C$, 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 X 10^3 M^{-1}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 X 10^{-4}M xanthine (Calbiochem), and 1.8 ml of 2.22 X 10^{-8}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 X 10^{-4}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 X 10^4 M^{-1}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-

natants at 550 nm was read. The concentration of ferri-
cytochrome c reduced was calculated using the molar extinc-
tion coefficient of $2.1 \times 10^4 \ M^{-1}cm^{-1}$ (49).

Ferricytochrome c or NBT reduction was used to
quantitate NADPH-dependent (50,51) superoxide anion gener-
ation 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 stimu-
lant 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^OC 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 reac-
tion mixture for 15 min at 37^OC 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 sam-
ples initially incubated in SWA medium alone and not pre-
viously 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_2O_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 X 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 X 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 agglutin-

ation 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 follow-

ing 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 seri-

ally 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 $37^{O}C$ 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 (χ^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 phago-cytosed 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

SM-1213 concentration (ng/ml)	Candidal growth (% of control)	
	Yeast form[a]	Hyphal form[b]
0	100	100
1	101	105
10	101	105
100	100	103
1000	101	98

a) 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 repre-
   sentative of the absence of drug effect at earlier
   intervals.

b) 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-O. PEC-O 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-O 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.
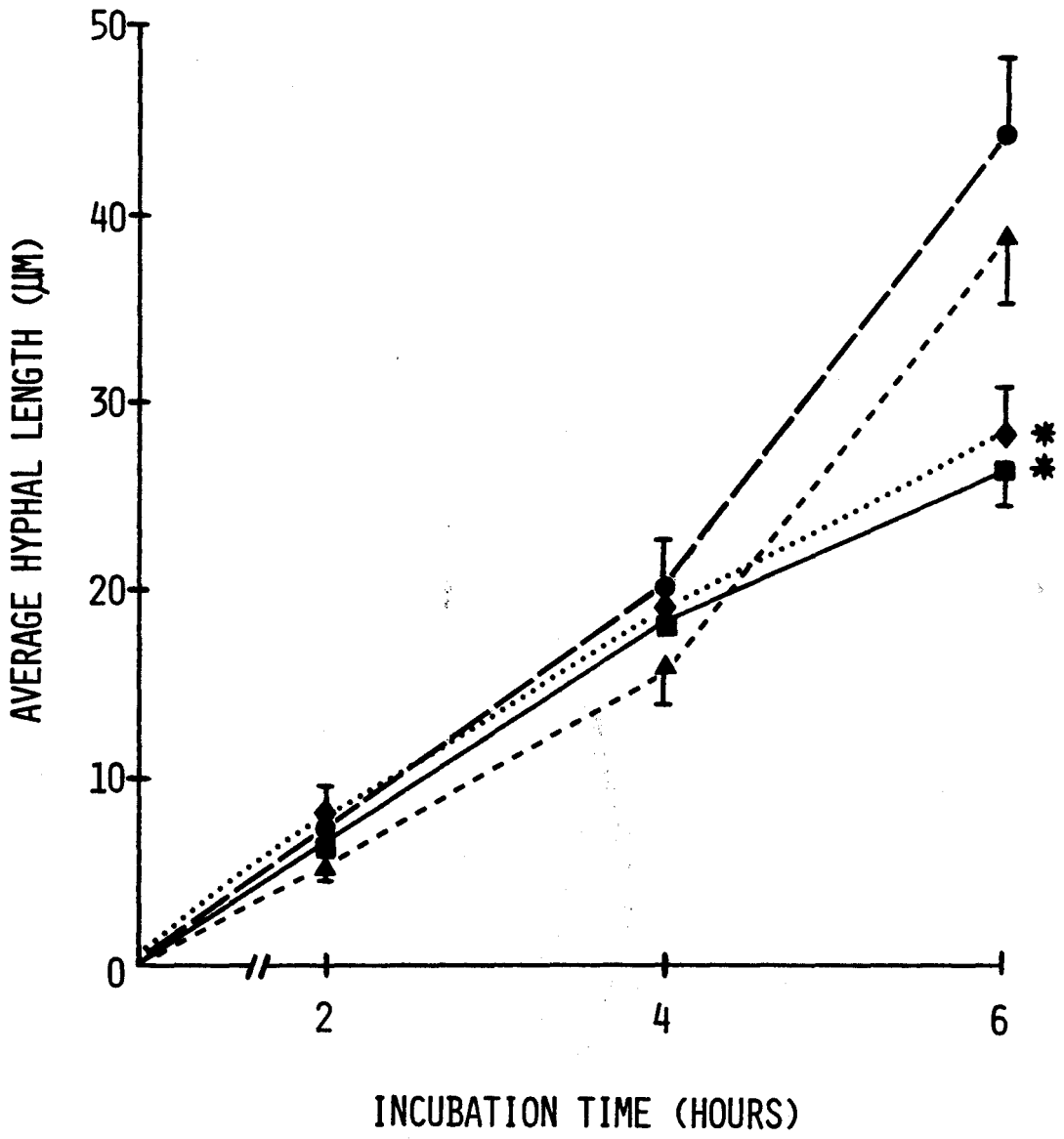
drug level, a drug concentration at which no direct anti-
candidal effects were observed in the absence of PEC (Table
I). No significant drug-induced differences in hyphal
length were observed at any drug concentration tested after
incubation for 2 or 4 h (Figure 2).

Although hyphal formation was initiated intra-
cellularly, the majority of viable hyphae within control
and drug-treated PEC-O 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-O could initiate hyphal forma-
tion, some hyphae were eventually killed by the microbi-
cidal action of the phagocytic cells. As shown in Table
II, after incubation with Candida for 6 h, untreated PEC-O
(0 ng/ml) killed 22% of intracellular Candida while PEC-O
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

SM-1213 concentration (ng/ml)	% Candida killed	
	PEC-O incubated with drug[a]	PEC-O pre-incubated with drug[b]
Medium control[c]	2.5 ± 0.5[d]	2.5 ± 0.5
0	22.0 ± 5.6	16.5 ± 2.5
0.1	35.5 ± 2.5*[e]	36.5 ± 2.5*
1.0	46.0 ± 9.6*	30.0 ± 1.0*
10.0	25.0 ± 3.0	34.0 ± 1.0*

a) PEC-O were infected and incubated with drug for 6 h.
   The percent intracellular Candida killed was deter-
   mined 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 des-
   cribed 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 (*).

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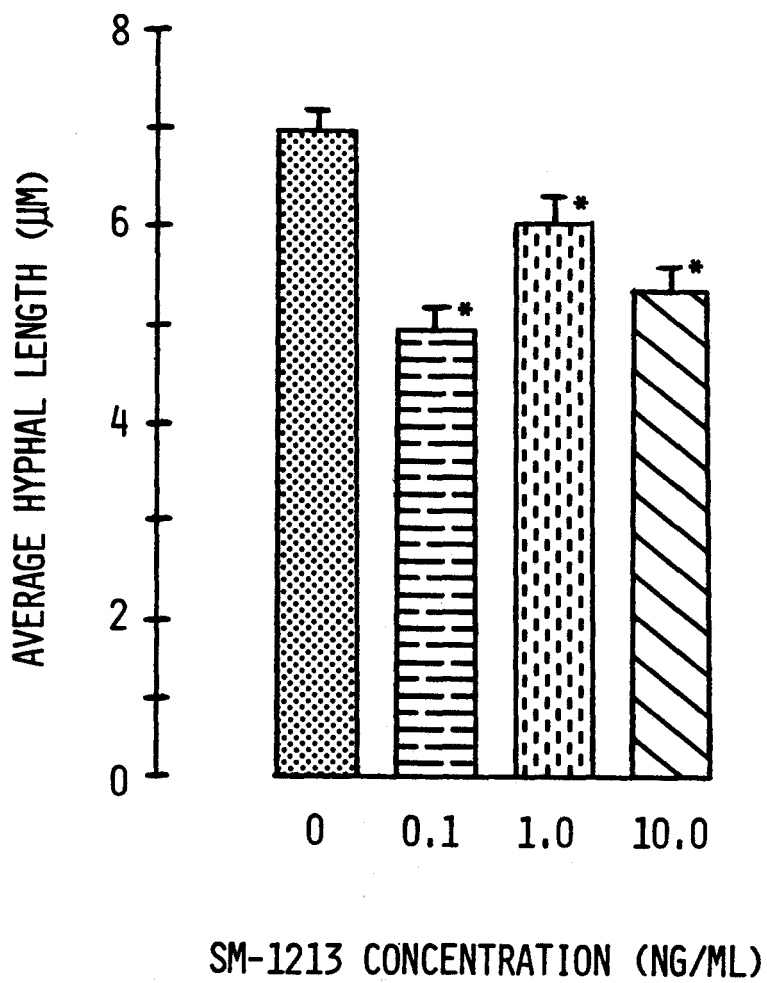
induced retardation of hyphal growth occurred when PEC-O were pre-incubated with drug for less than 6 h prior to candidal infection (data not shown).

E.   Effect of SM-1213 Pre-incubation of PEC-O on the Kill-
     ing of Phagocytosed C. albicans

PEC-O were pre-incubated in culture for 6 h in the presence or absence of drug. Adherent PEC-O were washed, infected with yeast cells, incubated for 2 h in the absence of drug, and assessed for candidal killing. PEC-O pre-incubated in the absence of drug killed 16.5% of intra-cellular Candida during the subsequent 2 h infection period. In comparison, PEC-O 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-O killed an equal or somewhat lower percentage of intracellular Candida than did PEC-O incubated with drug from the time of infection (Table II). However, killing of Candida within pre-incu-bated PEC-O was substantial, since Candida were exposed to PEC-O for only a 2 h infection period relative to a 6 h infection period for PEC-O 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 H_2O_2 and 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 effi-
cacy, utilization and catabolism of H_2O_2. This might
result, for example, from a drug-induced modification of
the activity of peroxidase, an enzyme associated with an
H_2O_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 ad-
herent 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 reduc-
tions in catalase activity occurred in adherent cell popu-
lations 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

PEC type[b]	SM-1213 concentration (ng/ml)	Adherent cell enzyme activity (units/mg protein)[a]		
		Catalase	Peroxidase	N-Acetyl-glucosaminidase
Resident	0	31.85 ± 0.69[c]	10.23 ± 0.22	1.86 ± 0.21
	1.0	32.34 ± 0.46	11.00 ± 0.33	2.14 ± 0.09
Oil-elicited	0	40.76 ± 2.64	0.55 ± 0.07	1.99 ± 0.11
	0.1	25.97 ± 0.83*[d]	0.45 ± 0.08	1.40 ± 0.14
	1.0	27.79 ± 1.95*	0.64 ± 0.07	1.62 ± 0.08
Oil-elicited infected	0	61.97 ± 1.35		1.76 ± 0.25
	0.1	36.37 ± 1.95*	N.D.[e]	1.96 ± 0.14
	1.0	51.02 ± 1.25*		1.96 ± 0.08

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-O 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-O on adherent cell, non-adherent cell, and extra-
cellular catalase activities in vitro.   PEC-O 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 (*).
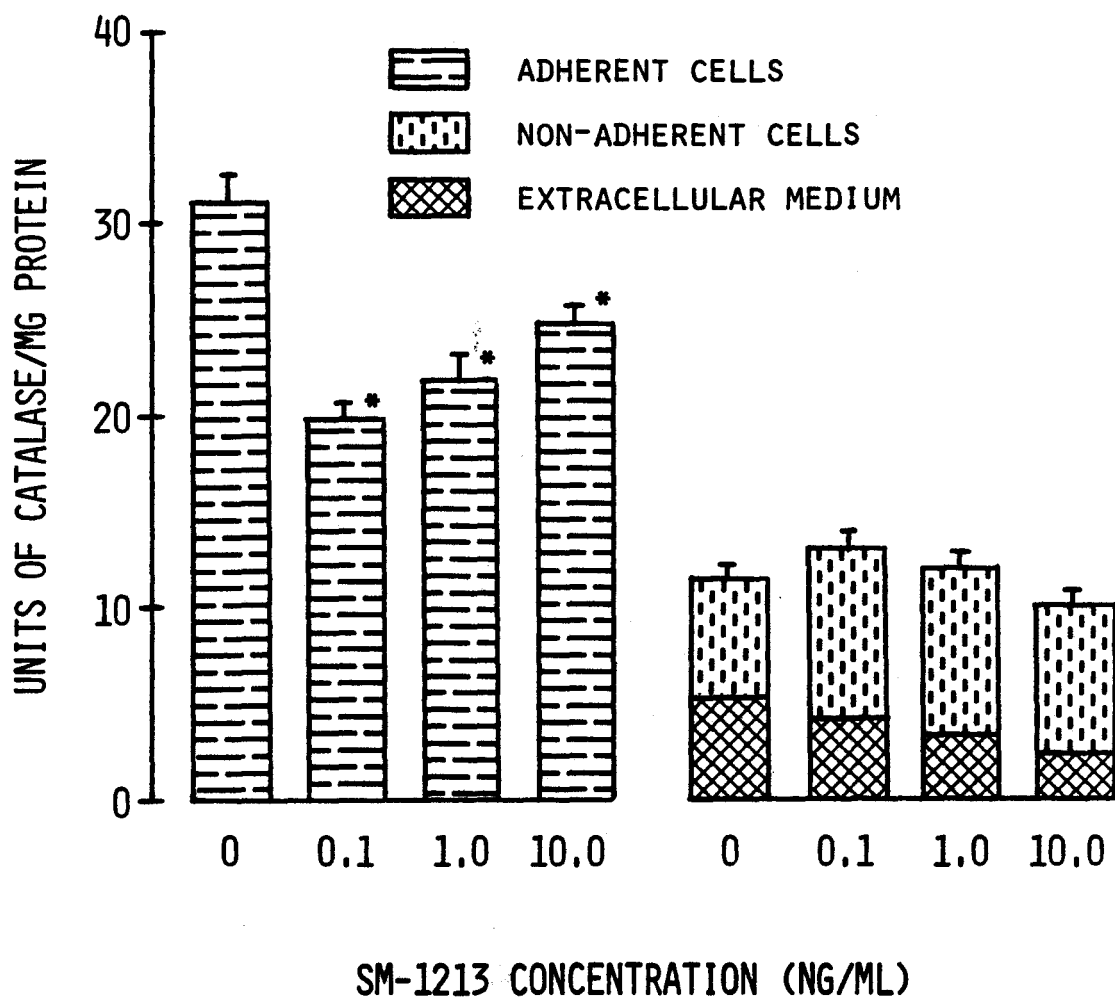
direct drug effect on standard bovine liver catalase activity was observed when drug was added during enzyme assay.

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

An explanation for the reduction of catalase activity observed in drug-treated PEC-O 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-O pre-incubated with SM-1213, as assessed by NBT reduction during phagocytosis of opsonized

Figure 5. The effect of SM-1213 pre-incuba-
tion of PEC-O on NBT reduction by phagocytes during
ingestion of opsonized C. albicans. PEC-O were pre-
incubated for 2 or 6 h with the designated concentra-
tions of drug, washed, and adherent PEC-O 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 repre-
sentative experiment. Significant drug-induced en-
hancement of SOD-inhibitable NBT reduction was ob-
served, 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.

SM-1213 CONCENTRATION (NG/ML)
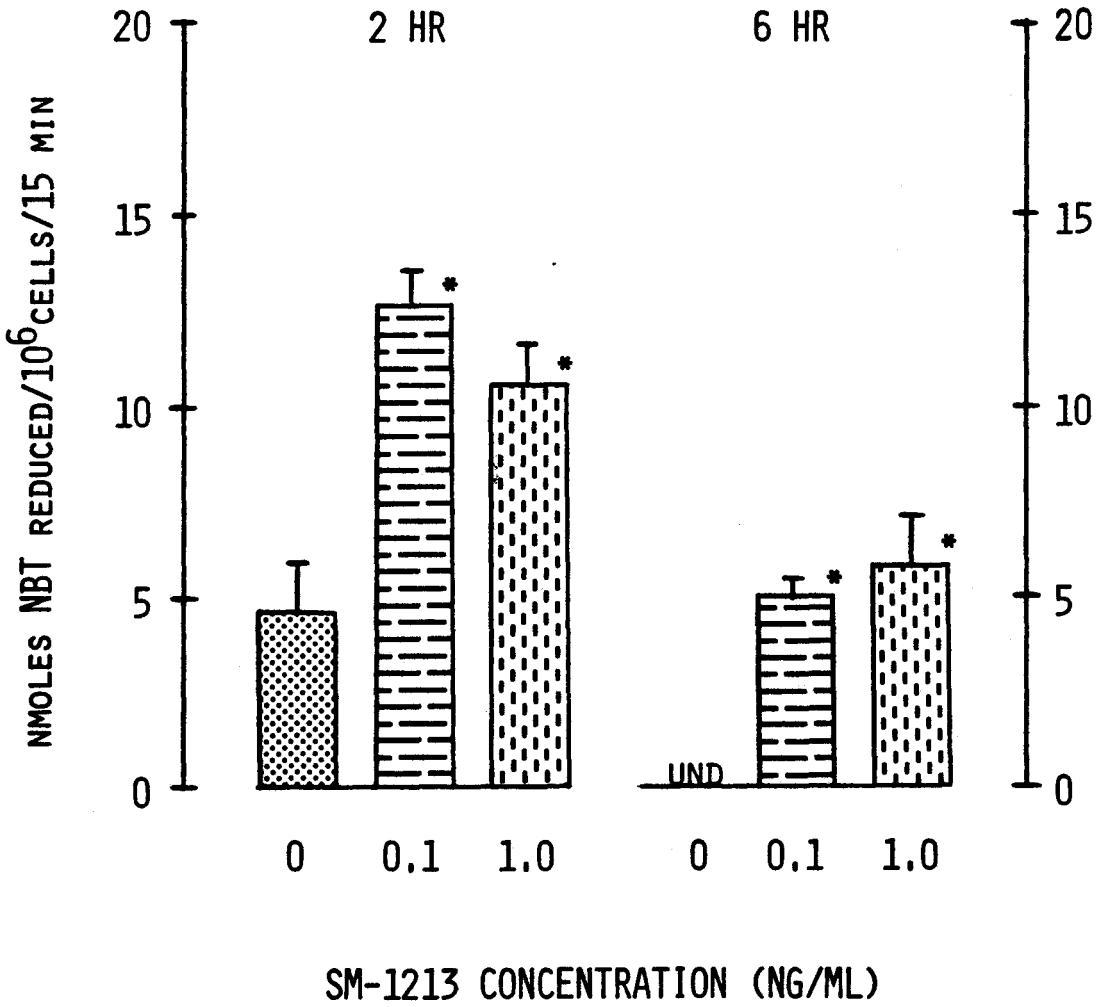
Candida. Although O_2^- production was reduced at 6 h relative to 2 h, SM-1213 treatment enhanced O_2^- production by PEC-O at both time intervals (Figure 5). The reduction in O_2^- generation at 6 h was not due to a loss of PEC-O viability as assessed by trypan blue dye exclusion or due to a reduced 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-O kill Candida, it was of interest to examine the location of O_2^- production by PEC-O, 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-O on NBT and FCC Reduction by Phagocytes after Ingestion of Opsonized Zymosan

PEC-O 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-O pre-incubated with SM-1213 was enhanced relative to PEC-O 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-O 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-O, 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-O 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-incuba-
tion of PEC-O on NBT reduction by phagocytes during
ingestion of opsonized zymosan particles. PEC-O were
pre-incubated for 2 or 6 h with the designated con-
centrations of drug, washed, and adherent PEC-O 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 (*).
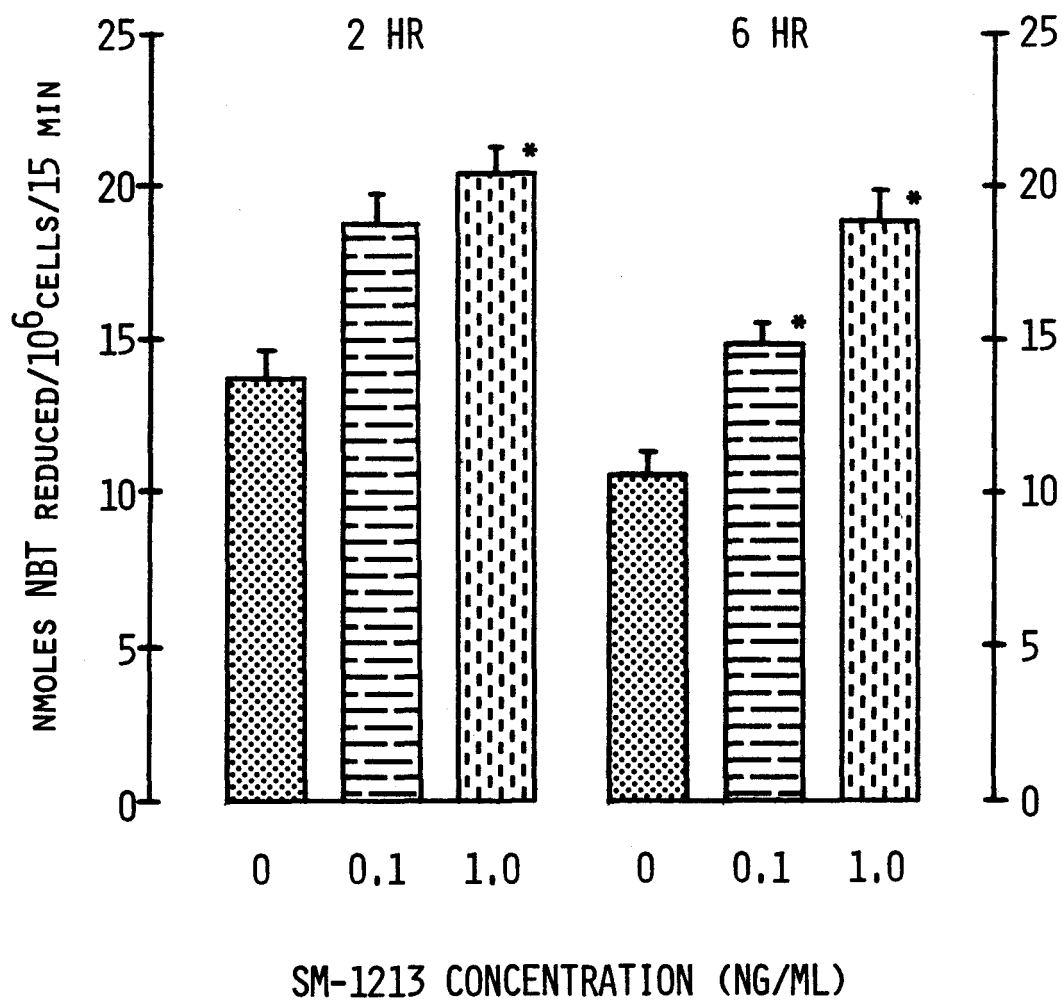
Figure 7.   The effect of SM-1213 pre-incubation of PEC-O on ferricyto-
chrome c reduction by phagocytes during ingestion of opsonized zymosan particles.
PEC-O were pre-incubated for 2 or 6 h in the presence or absence of SM-1213,
washed, and adherent PEC-O were exposed to the FCC assay system with opsonized
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 (*).

SM-1213 CONCENTRATION (NG/ML)
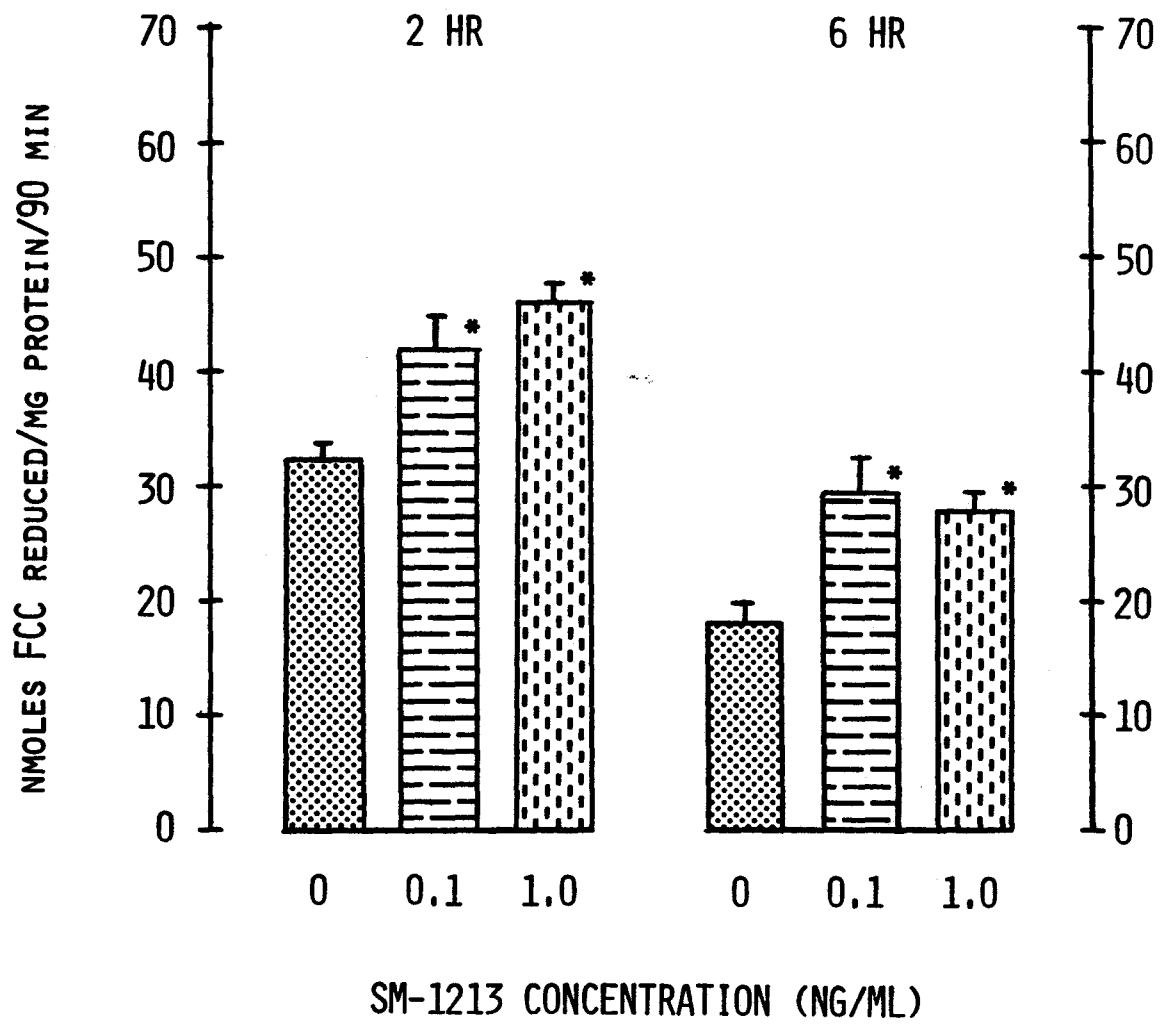
Figures 5 and 6, although O_2^- production was reduced at 6 h relative to 2 h, SM-1213 treatment enhanced O_2^- production by PEC-O at both time intervals (Figure 7).

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

63

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 (*).
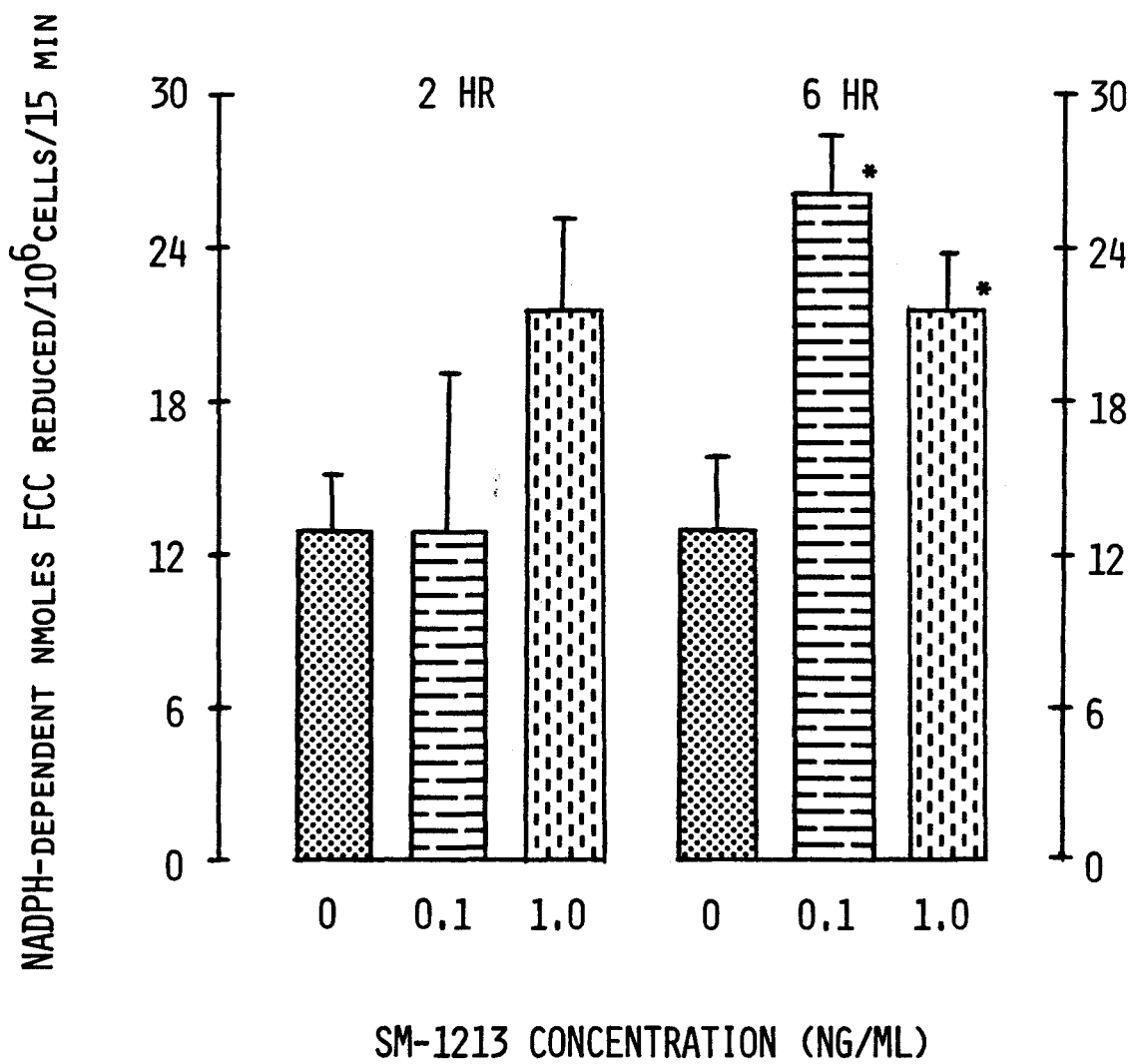
Figure 9. Time-dependent drug enhancement of NADPH-dependent NBT reduc-
tion by cell lysates from PEC-O pre-incubated with SM-1213. PEC-O were pre-
incubated in the presence or absence of SM-1213 for 2, 4 or 6 h and adherent
PEC-O were washed prior to stimulation with opsonized zymosan and cell lysis.
Cell lysates were assayed for NBT reduction as described in Methods section. At
0 h, PEC-O in suspension (consisting of 77% macrophages, 3% PMN and 20% lympho-
cytes) were incubated with the opsonized zymosan stimulant mixture, washed by
centrifugation in PBS and lysed. Lysates were assayed for NBT reduction as des-
cribed in Methods section. Cell lysates from PEC-O 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-O pre-incubated in the absence of drug and those from PEC-O
pre-incubated in the presence of drug. Data represent means ± SEM of the number
of experiments denoted in parentheses ( ).
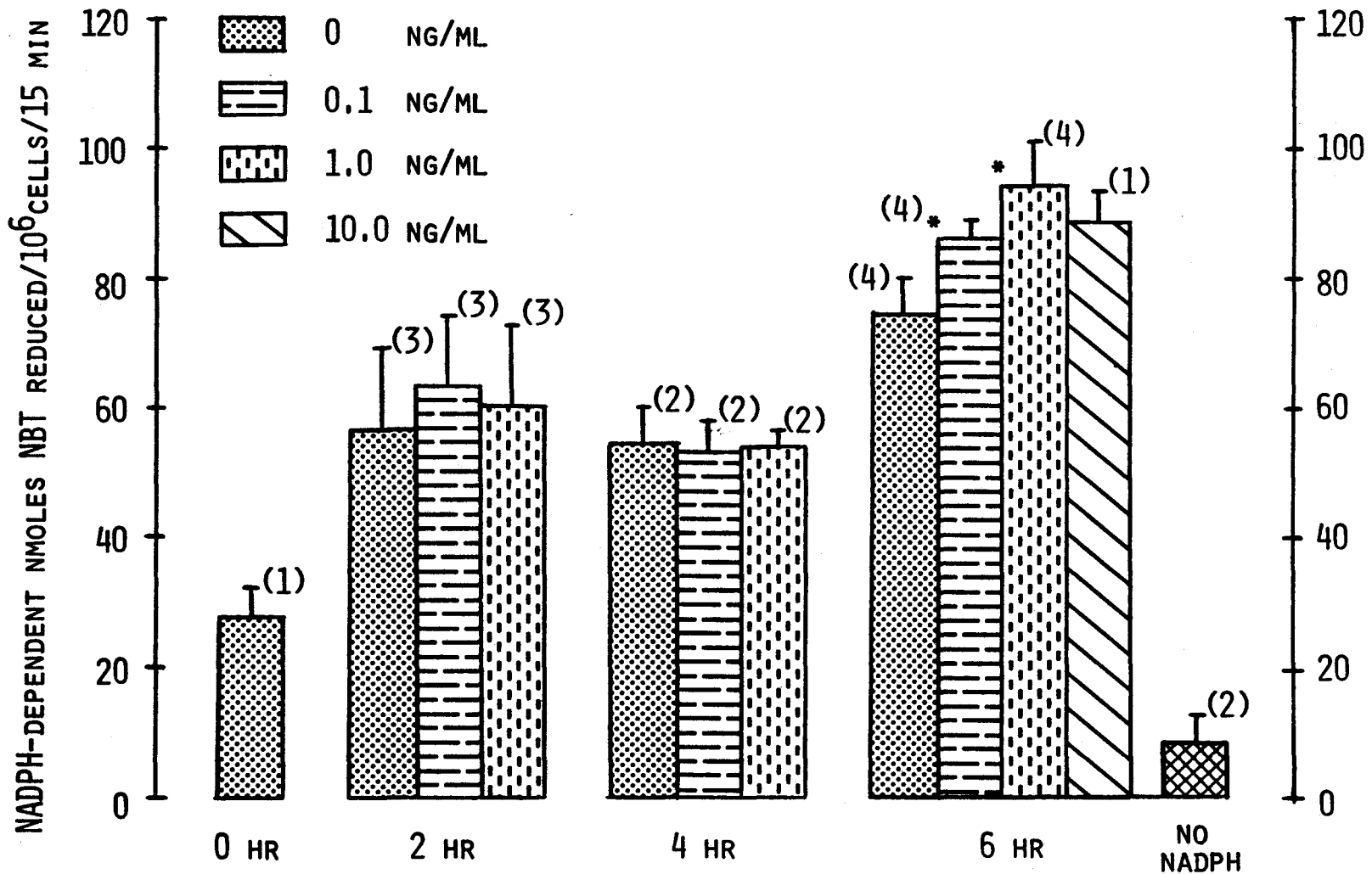
PRE-INCUBATION TIME WITH SM-1213

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-O were exposed to SM-1213 or whether it was a result of the time PEC-O were held in culture, PEC-O 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-O pre-incubated with SM-1213 to cell lysates from PEC-O pre-incubated without SM-1213 but receiving drug during NBT assay. When PEC-O 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-O with drug resulted in an average percent increase in NBT reduction by cell lysates of 50.2% relative to cell lysates from PEC-O pre-incubated in the absence of drug. Pre-incubation of PEC-O 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-O pre-incubated in the absence of drug. When comparing NBT reduction by cell lysates from PEC-O pre-incubated with SM-1213 to cell

Table IV. NADPH-dependent reduction of NBT by cell lysates from oil-elicited PEC (PEC-O)

| SM-1213 concentration (ng/ml) | nmoles NBT reduced/10^6 cells/15 min | | | | (% increase with vs. w/o drug during pre-incubation)[d] |
| | PEC-O pre-incubated for: | | | | |
	6 h with drug[a]	(% drug effect)[b]	6 h w/o drug[c]	(% drug effect)[b]	
0	58.59 ± 1.12[e]	--	55.80 ± 2.79	--	(5.0)
0.1	87.05 ± 3.91*[f]	(48.6)	69.75 ± 3.91*	(25.0)	(24.8)
1.0	88.72 ± 3.91*	(51.4)	75.33 ± 6.70*	(35.0)	(17.8)
10.0	88.16 ± 5.02*	(50.5)	70.31 ± 3.91*	(26.0)	(25.4)
Average		(50.2)		(28.7)	(22.7)

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 = $\dfrac{\text{nmoles NBT reduced by:drug-treated} - \text{control(0 ng/ml) samples}}{\text{nmoles NBT reduced by control (0 ng/ml) samples}}$ X 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 = $\dfrac{\text{nmoles NBT reduced by: a) - c)}}{\text{nmoles NBT reduced by c)}}$ X 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-O 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-O 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-O pre-incubated in the presence of drug were increased an average of 82.9%).

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

The results presented thus far suggest that SM-1213 treatment of PEC-O or of PEC-O lysates induces an increase

Table V. NADPH-dependent reduction of NBT by cell lysates from resident PEC (PEC-r)

SM-1213 concentration (ng/ml)	nmoles NBT reduced/10^6 cells/15 min		
	PEC-r pre-incubated for:		
	2 h with drug[a]	6 h with drug[a]	6 h w/o drug[b]
	Experiment #1		Experiment #2
0	59.86 ± 1.55[c]	55.21 ± 3.10	41.85 ± 2.79
0.1	54.70 ± 0.52	47.99 ± 3.10	45.20 ± 1.67
1.0	53.15 ± 2.06	47.99 ± 1.55	35.71 ± 2.23

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-O, 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-O, 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-O 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-O 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-O on extracellular H$_2$O$_2$ production during phagocytosis of opsonized zymosan. PEC-O were pre-incubated for 2 or 6 h with the designated concentrations of SM-1213, washed to remove residual drug, and adherent PEC-O 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-O pre-incubated in the absence of drug and those from PEC-O pre-incubated in the presence of drug.

EXTRACELLULAR
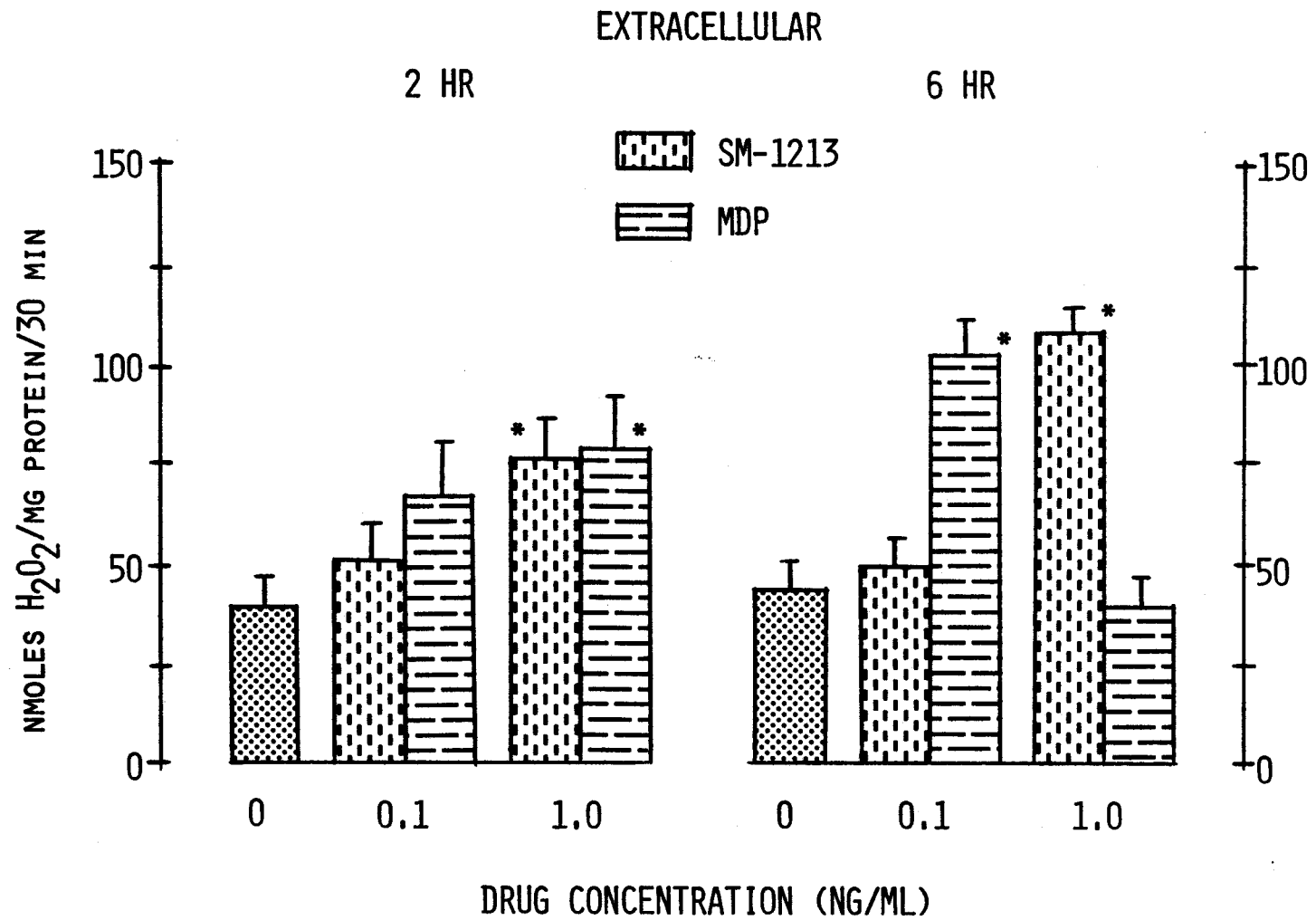
Figure 11. The effect of SM-1213 pre-incubation of PEC-O on cell lysate H_2O_2 production following phagocytosis of opsonized zymosan. Experimental conditions were as in Figure 10, except PEC-O were lysed with detergent following ingestion of opsonized zymosan and exposed to the phenol red assay system. Cell lysates were assessed for H_2O_2 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 H_2O_2/MG PROTEIN/30 MIN

DRUG CONCENTRATION (NG/ML)

75
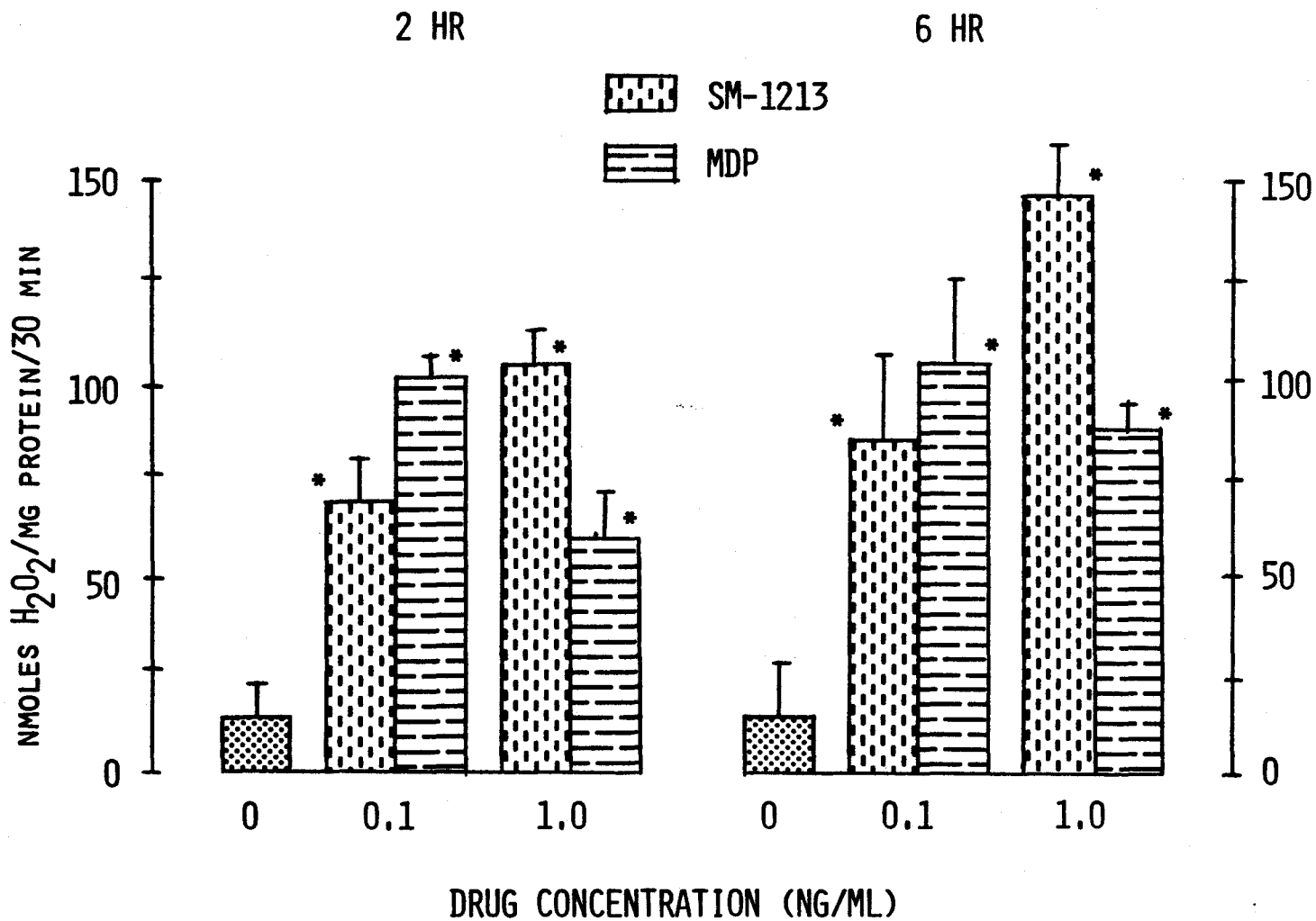
In samples from PEC-O pre-incubated for 6 h in the absence of drug, 85.7% of the total H_2O_2 produced by phagocytes was extracellular, while 14.3% was produced by cell lysates. In contrast, in all samples from PEC-O pre-incubated for 6 h in the presence of drug (MDP or SM-1213), an average of 56.4% of the total H_2O_2 produced was extracellular (range 47.1-66.1%), while 43.6% was produced by cell lysates (range 33.9-52.9%). While extracellular H_2O_2 production either increased or decreased between 2 and 6 h, H_2O_2 production by cell lysates increased in all drug-treated samples an average of 30% during this interval (Figures 10 and 11). H_2O_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 H_2O_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 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 in vitro for 2 or 6 h in

the absence of drug, stimulated with opsonized zymosan and homogenized. Cell homogenates were tested for SOD-inhibitable, 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-O 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-O pre-incubated for 6 h in the absence of drug and subsequently 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 microscopy.

Candidal killing by cell homogenates in the presence or absence of SM-1213 was also assessed by the methylene 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-O 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-O 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)
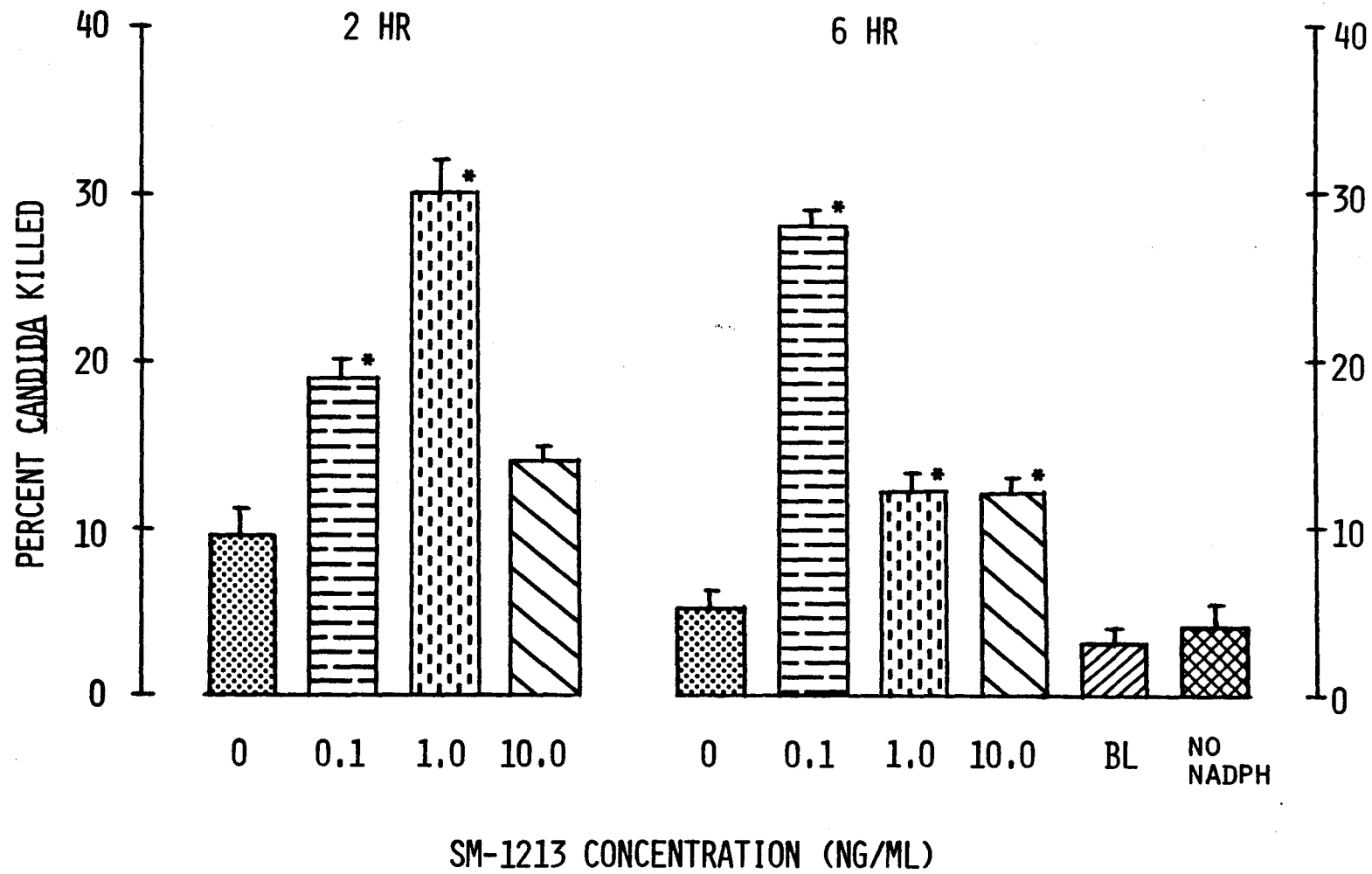
dead), the enhancement of candidal killing by drug-treated cell homogenates or cell lysates (lysed with 0.5% NI) was evident using this assay system (P < 0.05). The methylene blue assay system may, however, have given artificially high values for the number of apparently viable Candida. For example, 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 fungi-
static 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-O 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). Signif-
icant drug-induced candidastatic and candidacidal effects
were also observed when PEC-O 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-O.

When PEC-O 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)

81

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 intra-
cellular Listeria monocytogenes within oil-elicited guinea
pig macrophages treated with the lowest SM-1213 concen-
tration 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 concen-
trations (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-O 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-O
after only 2 h of drug treatment and that sustained oxi-
dative injury may be responsible for the observed candida-
static and candidacidal effects at 6 h. However, the
effect of culturing PEC-O over time may also influence
candidacidal mechanisms and the modulation of these mech-
anisms by SM-1213.

When PEC-O 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-O 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-O exposed to drug at the
time of infection (Figure 2, Table II). Other immuno-
modulatory agents, such as MDP and LPS, are totally in-
effective 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-O 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 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-O pre-incubated in the presence of drug, as opposed to PEC-O exposed to drug during infection, may provide an explanation for the observed differences in PEC-O candidacidal effectiveness at the 10 ng/ml drug level. For example, PEC-O pre-incubated in the presence of SM-1213 may use pinocytotic or sugar transfer mechanisms to transport SM-1213 into PEC-O. PEC-O exposed to SM-1213 at the time of infection would also take drug into the phagocytic vacuole along with 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-γ, 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-O 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-O were
composed of an entirely adherent cell population, while non-
pre-incubated PEC-O were composed of freshly plated adher-
ent and non-adherent cells. It was therefore conceivable
that differences in PEC-O 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-O. However,
this was probably not the case, since PEC-O allowed to ad-
here to culture dishes for 2 h and washed prior to infection
gave virtually identical results to those obtained for un-
separated PEC-O (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-O to kill percentages of Candida almost as great or
greater than PEC-O incubated with Candida for 6 h (Table
II). This suggests that, once PEC-O are primed by pre-
incubation in culture for 6 h, they become more effectively

candidacidal upon presentation of the infecting micro-
organism. 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 opson-
ized 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^- pro-
duction 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 H_2O_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 H_2O_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 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 Leishmania donovani by CGD monocytes and, upon exposure to lymphokines, CGD macrophages (86). The observed killing of Candida at 6 h by PEC-O pre-incubated in the absence of drug (Table II) without detectable 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 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 mechan-
isms whereby they "turn off" the anticandidal systems of
macrophages. Ozato and Uesaka (99) observed that intra-
cellular 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 in-
gesting Candida can inhibit phagocyte candidacidal activity
(118) and, in conjunction with PMN (77) or macrophages
(88), microbial or phagocyte catalase may antagonize phago-
cyte-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-O 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-O demonstrated a 1.5-fold
greater catalase activity when infected relative to un-
infected PEC-O (Table III). This suggests that, after
phagocytes produce reactive oxygen metabolites in response
to an infectious agent, PEC-O initiate an enhanced endo-
genous production of catalase to protect against auto-
oxidative 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, infec-
tions may be resolved as a result of the sequential rein-
forcement 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 unin-
fected PEC-O may be due to a contribution of catalase
activity from C. albicans cells. Sasada and Johnston (118)
suggested that intracandidal SOD or catalase did not affect
the level of detectable O_2^- produced by macrophages, since
intact, viable 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 there-

fore plausible that killing of candidal cells by PEC-O

could result in some leakage of intrafungal catalase.

However, it is unlikely that this is the case, since: 1)

SM-1213-treated PEC-O, which killed more Candida, did not

demonstrate greater catalase activity after cell lysis

than cell lysates from control PEC-O (Table III); 2) the

increased catalase activity of infected PEC-O 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-O lysates did not contain more protein

than cell lysates from uninfected PEC-O.

Since an increase in phagocyte catalase activity

has been correlated to a reduced microbicidal capacity

(88), the observed reduced capacity of control PEC-O to

kill C. albicans cells (Table II) relative to drug-treated

PEC-O 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-O. It has been re-

ported 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-O (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-O, 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-O 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-O 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-O. 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-O relative to control PEC-O. In control PEC-O, only 0 to 14% of total oxidative metabolites were cell-associated, while, in drug-treated PEC-O, 34 to 44% were cell-associated. Therefore, if oxidative candidacidal mechanisms are initiated intraphagosomally or intracellularly, drug-treated PEC-O would be expected to kill a greater percentage of phagocytosed Candida than untreated PEC-O. Also, since 100% of O_2^- and 86% of H_2O_2 produced by control PEC-O 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 O_2^- may be an especially important reactive oxygen metabolite in phagocyte anticandidal activity (118). However, several researchers have suggested that H_2O_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 H_2O_2 production by PEC-O (Figures 10 and 11). This enhancement was evident at 2 and at 6 h. H_2O_2 production increased between 2 and 6 h by an average of 30% for PEC-O pre-incubated in the presence of drug, while H_2O_2 production remained unchanged over time for PEC-O pre-incubated in the absence of drug (Figures 10 and 11). The increase in H_2O_2 production between 2 and 6 h correlates with the decrease in detectable O_2^- produced during this time interval (Figures 5,6,7). This suggests that a greater quantity of O_2^- is being converted into H_2O_2 (reducing detectable 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 O_2^- over time in culture while PEC-O viability was intact. However, this explana-

tion is relevant to drug-treated PEC-O only, since no increase in H_2O_2 production over time was observed for control PEC-O (Figures 10 and 11). Not all O_2^- produced was spontaneously dismutated to H_2O_2, however, since the addition of SOD to the assay system increased detectable H_2O_2 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) O_2^--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, O_2^- and H_2O_2 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 O_2^--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 cere-

visiae 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 repor-

ted to enhance H_2O_2 production by PMN, while mannose in-

hibited 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. albi-

cans 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-O with SM-1213 enhances the subsequent stimulation of PEC-O 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-O 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 O_2^- by cell lysates revealed that, in the absence of Candida, SM-1213 could enhance NADPH-dependent 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 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-O, 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-O with drug prior to cell lysis in-
creased 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-O with drug, as well as
incubation of PEC-O in culture, primes phagocytes to pro-
duce greater quantities of O_2^-.

The significantly higher quantities of O_2^- produced
by cell lysates (Figure 9) relative to whole PEC-O (Figure
6) may relate to the availability of the O_2^--producing
enzyme to activation. Although located in the plasma mem-
brane (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 marked-
ly deficient in O_2^- production, while cell lysates from CGD
phagocytes were only mildly deficient (50). It was sug-
gested 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 basis 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 sh own by others (6) to differ from newly recruited inflammatory cell types, which are primarily monocytes. The matu ration of mononuclear phagocytes into macrophages is assoc iated 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 r. elative O_2^--producing capacities of PEC-r and PEC-O. Th. erefore, 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'-dimeth-
ylamino-n-propyl)-D-glucofuranose] is a synthetic, substi-
tuted 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-O
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-O 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-O. Pre-incu-
bation of PEC-O 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-O with SM-1213 for 6 h resulted in a
selective reduction in adherent cell catalase activity
relative to control PEC-O. NADPH-dependent production of
superoxide anion was enhanced in cell lysates from PEC-O

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 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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APPROVAL SHEET

The dissertation submitted by Christine Joy Morrison has
been read and approved by the following committee:

Dr. Paul Gordon, Co-Director
Adjunct Professor, Microbiology
Loyola University of Chicago

Dr. Tadayo Hashimoto, Co-Director
Professor, Microbiology
Loyola University of Chicago

Dr. Charles F. Lange
Professor, Microbiology
Loyola University of Chicago

Dr. Allen Frankfater
Associate Professor, Biochemistry
Loyola University of Chicago

Dr. Raoul Fresco
Professor, Pathology
Loyola University of Chicago

The final copies have been examined by the directors of the
dissertation and the signatures which appear below verify
the fact that any necessary changes have been incorporated
and that the dissertation is now given final approval by
the Committee with reference to content and form.

The dissertation is therefore accepted in partial fulfill-
ment of the requirements for the degree of Doctor of
Philosophy.

2-17-84
Date

Co-Director (s) Signature

Co-Director's Signature

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