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The in Vitro and in Vivo Effects of SM-1213 Drug Therapy on Pathogenesis and Host Defense in Experimentally Induced Candidiasis

Christine Joy Morrison
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THE IN VITRO AND IN VIVO EFFECTS OF SM-1213 DRUG THERAPY
ON PATHOGENESIS AND HOST DEFENSE IN EXPERIMENTALLY
INDUCED CANDIDIASIS

by

Christine Morrison

A Thesis Submitted to the Faculty of the Graduate School
of Loyola University of Chicago in Partial Fulfillment
of the Requirements for the Degree of
Master of Science

May

1980

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VITA

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.

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LIST OF ABBREVIATIONS AND SYMBOLS

Avg	Average
BME	Eagle's basal medium
°C	Degrees centigrade
cfu	Colony forming units
cm	Centimeter(s)
Δ	Delta or change
EBSS	Earle's balanced salt solution
EMB	Eosin methylene blue agar
G	Gravity
g	Gram(s)
Gm	Gentamicin
HBSS	Hank's balanced salt solution
H&E	Hematoxylin-eosin
hr	Hour(s)
i.m.	Intramuscularly
i.p.	Intraperitoneally
i.v.	Intravenously
kg	Kilogram(s)
μ	Micron(s) (10^{-6} meters)
μ g	Microgram(s) (10^{-6} grams)
ml	Milliliter(s) (10^{-3} liters)
mm	Millimeter(s) (10^{-3} meters)

N	Total population number
#	Number
N.A.	Not applicable
N.D.	Not done
N.S.	Not significant (P < 0.05 by the χ^2 analysis or by the Student's t test)
P	Probability
PAS	Periodic acid-Schiff
PASH	Periodic acid-Schiff-hematoxylin
PBS	Na-K phosphate buffered saline
PMN	Polymorphonuclear (leukocyte)
R _x	Drug treatment
SDA	Sabouraud's dextrose agar
SDB	Sabouraud's dextrose broth
± S.E.	Plus or minus the standard error of the mean
SM-1213	1,2-O-isopropylidene-3-O-3'-(N',N'-dimethyl- amino-n-propyl)-D-glucofuranose, monohydro- chloride
TC medium	Tissue culture medium
v/v	Volume per unit volume
w/v	Weight per unit volume
X	Times or power
χ^2	Chi-square

CHAPTER I

INTRODUCTION AND REVIEW OF RELATED LITERATURE

Research interest has recently developed for a group of chemical compounds which have immunomodulatory properties. These compounds exert anti-microbial actions which have been termed "prohost" (20) because, rather than being directly bactericidal or fungicidal, they either restore the host's deficient immune response or augment a normal response. Under specified conditions, these drugs could become highly important in treating immunodeficiency diseases, as well as viral, fungal, and bacterial infections. With the increasingly frequent development of single and multiple resistance to conventional antibiotic therapy (4,6,10,21), immunopotentiators become increasingly more important therapeutic alternatives. Also, in therapy for atopic individuals who experience undesirable side effects from traditional treatment (1,27,61), or in cases where adverse effects such as oto- or nephrotoxicity accompany the use of antibiotics (5,11), immunomodulators may provide successful treatment with fewer serious complications as a result of therapy.

Immunomodulatory substances consist of a wide variety of agents including micro-organisms and their subcellular fractions (mycobacteria, Corynebacterium parvum, LPS, zymosan), macromolecules such as nucleic acids or polynucleotides, small inorganic or organic molecules (silica, alum, fatty acids), or pharmacologic agents such as levamisole (1,2,3,5 tetrahydro-6-phenyl-amidazo (2,1- β)-thiazol, monohydrochloride). These agents effect their actions in a number of ways: by prolonging antigen release, by recruitment of macrophages and lymphocytes, by stimulating proliferation and differentiation of immunologically competent cells or by the enhancement of cellularly mediated immune responses (64).

Since complex immune mechanisms are affected by such immunomodulation, it could be expected that specific drug regimens may be more effective when immune cells are most sensitive to regulation. This has been demonstrated to occur in levamisole therapy where pre-infection drug treatment was effective against a bacterial agent (12) while simultaneous or post-infection therapy was ineffective. In particular, synthetic, non-polymer pharmacologic agents have been found to enhance the cellular immune response. A principle action of these agents involves the potentiation of the thymus-derived T-lymphocytes and the proliferation and/or activation of the monocyte-derived tissue macrophage (20).

One such immunomodulatory agent is SM-1213 (1,2-O-isopropylidene-3-O-3'-(N',N'-dimethylamino-n-propyl)-D-glucofuranose), a synthetic, substituted monosaccharide. The possible enhancement of host defenses by such a drug during active infection has prompted a careful examination of the in vitro and in vivo effects of this substance.

Recent studies have elucidated the anti-viral or immunomodulatory properties of SM-1213 in rhinovirus (15, 16), influenza and vaccinia viruses (17), and in herpes simplex virus infections in mice (39) and hamsters (18). Oral SM-1213 therapy has been demonstrated to delay death in B16 melanoma-bearing C57BL mice, as well as enhance delayed-type immune responses in Ha/ICR mice challenged with human type O red blood cells (38). In addition, SM-1213 has been shown to significantly increase intracellular killing of bacteria (20) in studies concerning infection of a macrophage monolayer with Listeria monocytogenes. No studies, however, have examined the effects of SM-1213 upon in vitro or in vivo fungal infection.

Candida albicans is a fungus commonly found as part of the normal flora of many mammals, including man. It is considered an opportunistic fungus, since individuals who carry the organism do not display symptoms of infection unless injury or metabolic disorders reduce resistance to microbial invasion. Diabetes, indwelling catheters, and broad-spectrum antibiotic or immunosuppressive therapy

are all factors which favor the development of candidiasis. In addition, patients with various neoplasms, immunological defects, or serious burns are highly susceptible to candidal infection (48).

The wide range of factors contributing to susceptibility to candidiasis, the poor and even damaging chemotherapy available, and the increased incidence of systemic disease (22) have made candidiasis a serious clinical problem. Therefore, studies of the causative organism were undertaken to examine the mechanisms of pathogenesis and immunity in candidiasis and the effects of SM-1213 therapy on host defense.

A. In Vitro Studies

Several researchers have examined the ability of polymorphonuclear leukocytes (PMN) or macrophages to phagocytize and kill intracellular Candida albicans (2, 36,60,63). In general, the PMN leukocyte has been demonstrated to be more efficient in destroying Candida than the macrophage (2).

Stanley and Hurley (60) observed that cultured mouse peritoneal macrophages were destroyed by the elongation of candidal germ tubes and that escaping Candida survived ingestion. In addition, Louria and Brayton (37) suggested that the pathogenicity of various strains of Candida albicans in mice is roughly correlated to the ability of Candida to form germ tubes and survive ingestion

by leukocytes derived from human blood. These studies demonstrate the invasiveness of Candida to be related to the hyphal rather than to the yeast form of this micro-organism.

Lehrer and Cline (36), however, have shown that a significant fraction of Candida phagocytized by PMN's in vitro are destroyed. Arai et al (2) also found the guinea pig peritoneal neutrophil capable of killing intracellular Candida in the presence of normal serum. Rabbit alveolar macrophages, on the other hand, whether in the presence of normal or immune serum, showed no Candida dead after one hour of incubation. No significant improvement of candidacidal activity was observed when macrophages derived from immune or normal rabbits were exposed to immune serum, but an enhancement of phagocytic ability of these cells was evident. It was suggested that immune serum enhanced opsonization. However, Venkataraman et al (63), working with immune rabbit neutrophils, demonstrated no significant enhancement of phagocytosis, but rather an enhancement of candidacidal properties in combination with immune serum.

Studies were therefore conducted in our laboratory to determine whether glycogen induced guinea pig peritoneal neutrophils and paraffin oil induced peritoneal macrophages could inhibit intracellular Candida in vitro with or without the addition of whole, normal guinea pig serum.

B. In Vivo Studies

1. Intravenous Inoculations

Conventional therapy for a systemic candidal infection involves the use of a polyene antifungal agent such as amphotericin B. While being relatively effective in the treatment of such infections, amphotericin B has concomitant deleterious side effects, particularly renal damage (62). Since the kidney has also been demonstrated to be a primary organ damaged in systemic candidiasis (26, 37,51,66), treatment of these patients with amphotericin B may compound the problem. In addition, individuals receiving immunosuppressive cortisone therapy often become infected with Candida (13) and are subsequently treated with amphotericin B; recent evidence indicates that amphotericin B can be synergistically nephrotoxic in mice receiving cortisone therapy (30).

Unlike amphotericin B, SM-1213 is a drug of low toxicity. The acute oral LD₅₀ of SM-1213 in mice (17), rats, and rhesus monkeys has been determined to be greater than 10 g/kg, or greater than 10⁴ the usual therapeutic dose. In tissue culture studies, anti-viral and anti-bacterial effects were demonstrated while toxicity to tissue culture cells was not evident at SM-1213 concentrations 1000 times higher than the effective dosage (17,20).

In addition, unlike other immunomodulatory substances (12), SM-1213 does not require administration prior to infection (15,17,39). This aspect of SM-1213 treatment could therefore prove useful in the therapy of disease states upon the appearance of symptoms (38). Therefore, if SM-1213 could be proven to be effective against a systemic candidal infection, it might provide a viable alternative to classical polyene antibiotic therapy.

The pathogenesis of systemic candidal infection has been examined by several researchers; the kidney has been demonstrated to be a primary organ damaged by such infection.

Hurley and Winner (26) described an acute and a chronic type of kidney involvement in Swiss mice infected intravenously with 1.5×10^4 to 4×10^6 candidal cells. Doses of 2×10^6 or more candidal cells per mouse caused animals to develop acute diseases and to succumb within one to ten days with histologically distinct lesions in the heart, brain, and kidneys. Chronic kidney disease occurred in mice dying in six to sixty days with lesions confined to the kidneys.

In a similar study, Winbald (66) also showed that the kidney was an organ of major involvement. In mice infected with 8×10^4 Candida cells intravenously, histology and scanning electron microscopy of the infected

kidneys demonstrated two types of involvement: acute (occurring within ten days of challenge) and chronic (occurring in those mice surviving longer than ten days post-infection).

Studies conducted by Rogers and Balish (50) on clearance of Candida from liver, lungs, kidneys, and spleens of male Swiss-Webster mice infected with various doses of Candida intravenously have shown that 60-120 minutes after injection, the kidney, on a weight basis, was the most efficient organ for clearing Candida from the bloodstream. The kidneys became chronically infected 17-24 days post-challenge with $1-3 \times 10^4$ viable units of Candida injected. Increasing numbers of Candida were found in the kidneys and, by day 24 post-infection, the number of viable units was as high as 1×10^6 per two kidneys.

Louria and Brayton (37) studied Swiss mice infected intravenously with various strains and inocula of Candida. Tissue homogenates were performed on kidney, spleen, liver, heart, brain, and lung tissues after intravenous challenge with 1×10^5 Candida cells of the PR strain. Progressive infection occurred only in the kidneys by day 7 post-infection. Populations in brain, heart, liver, spleen, and lung samples remained unchanged or fell by day 7, while that of the kidney increased. The authors suggested such an increase was related to a four hour

delay in mobilization of neutrophils to this organ and that, once Candida were able to germinate within the kidney tubule lumens, they were able to evade the host's inflammatory response.

2. Intramuscular Inoculations

Mice injected intravenously with Candida albicans frequently succumb to infection within two weeks, which hinders the study of the course of an immune response. By using a model developed by Selbie and O'Grady (57) and modified by Pearsall and Lagunoff (44), it became possible to study the mechanisms of candidal pathogenesis and immunity in mice under the influence of drug therapy.

Selbie and O'Grady (57) developed a model whereby mice were injected intramuscularly in the thigh so as to produce a measurable tuberculous lesion. Pearsall and Lagunoff (44) used such a model to examine experimental candidiasis in mice. An inoculum of 5×10^8 Candida cells was injected into the thigh muscle of C57BL/Ks mice and the development of candidal abscesses was monitored by periodic measurement of the infected leg with the aid of a caliper. Histological examination revealed a predominantly granulocytic infiltrate of cells. Lesions were self-limiting within four to six weeks post-infection.

Immunity to abscess formation could be passively transferred by the use of immune serum, but not by the administration of lymphoid cells obtained from convalescing

mice (43).

Secondary leg inoculations of Candida into the uninfected contralateral leg of recovered mice were also conducted by Pearsall and Lagunoff (43). Such inoculations were used by Pearsall and Lagunoff to determine the acquisition of immunity to abscess formation following an initial infection.

While Pearsall and Lagunoff (44) examined the histology of the leg lesions, no culturing of the abscesses or visceral organs was conducted.

C. Purpose of the Present Work

The purpose of this thesis is to examine the possible enhancement of host defenses by SM-1213 during an infection produced by experimental injection of Candida albicans. Initially, in order to determine whether or not there is a direct fungicidal or fungistatic activity associated with SM-1213 treatment, an examination of the effect of SM-1213 concentration on the growth of the yeast and hyphal forms of Candida albicans will be undertaken. In addition, an examination of the effect of SM-1213 concentration upon leukocytes infected in vitro with C. albicans will be discussed. In particular, SM-1213 treatment will be investigated for its effect on the ability of peritoneal exudate cells in tissue culture to inhibit intracellular germ tube elongation and to reduce candidal viability. The effect of whole, normal guinea pig serum on germ tube

elongation and candidal viability within phagocytic leukocytes in the presence and absence of SM-1213 will also be examined.

In vivo, studies will be conducted to examine the effects of oral SM-1213 drug therapy upon systemic infection in mice injected intravenously with C. albicans. Since host defense mechanisms evolve during the interval following the initiation of infection, and since immunomodulatory substances may prove most effective when host defense mechanisms are sensitive to regulation (12), various regimens of drug treatment will be tested. In addition, since the kidney is a primary organ in the pathogenesis of candidiasis (26,66), tissue homogenizations of kidneys from intravenously infected mice will be performed to assess renal involvement in control and drug treated mice.

The effects of SM-1213 treatment on the course of leg lesion development in intramuscularly infected mice will also be investigated. Leg lesion development will be monitored periodically with the aid of adjustable calipers and leg swelling will be used as an indicator of the pathogenesis of candidal disease. Recovered mice will be reinoculated in the contralateral leg to assess the effect of SM-1213 on lesion development during a second infection when drug is administered during initial infection only. Also, leg lesions will be cultured or homo-

genized to detect the presence of C. albicans and kidneys and/or spleens of intramuscularly infected mice will be examined for the metastatic spread of fungus. It is hypothesized that SM-1213, via its immunomodulatory properties, will promote the fungistatic or fungicidal capabilities of leukocytes in vitro and will promote an enhanced host defense in vivo against experimentally induced infections of Candida albicans.

CHAPTER II

MATERIALS AND METHODS

A. Experimental Drug

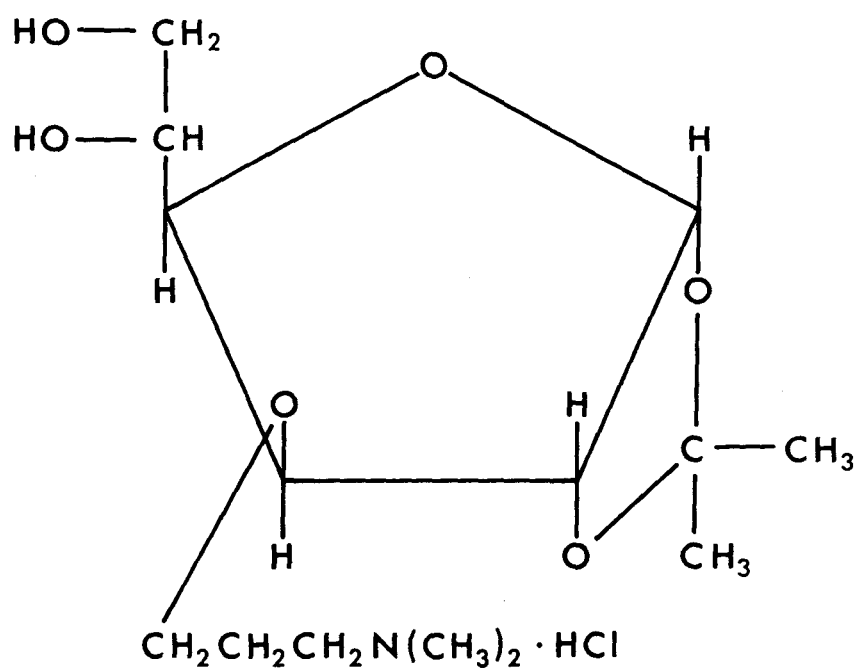
SM-1213 (1,2-O-isopropylidene-3-O-3'-(N',N'-di-methylamino-n-propyl)-D-glucofuranose, monohydrochloride) was supplied by Dr. Paul Gordon, Greenwich Pharmaceuticals, Inc., Greenwich, CT. and Department of Microbiology, Loyola University, Stritch School of Medicine, Maywood, IL. The chemical structure of the drug is represented in Figure 1. SM-1213, lot #13/24-31, hydrated from the powdered form with distilled water, was stored at -20°C in 5 ml stock solutions (50 mg/100 ml) and diluted as needed.

B. Micro-organism

Candida albicans originally isolated from a human patient was kindly supplied by Dr. T. Hashimoto of this department. Permanent stocks were lyophilized in Sabouraud's dextrose broth (Difco) and rehydrated as needed. Working stocks were kept on Sabouraud's dextrose agar (Difco) slants at room temperature and at 4°C and transferred at monthly intervals.

This strain showed typical features of Candida albicans when tested in a Uni-Yeast-Tek diagnostic system

Figure 1. The chemical structure of SM-1213 (1,2-0-isopropylidene-3-0-3'-(N', N'-dimethylamino-n-propyl)-D-glucofuranose, monohydrochloride).



SM - 1213

(Corning Medical Microbiology, Roslyn, NY). Assimilation of sucrose and maltose with latent assimilation of trehalose and soluble starch and lack of lactose, raffinose, and cellobiose utilization as well as negative urea and nitrate tests were identifying characteristics. In addition, characteristic germ tube formation in the presence of serum, characteristic filamentous growth on EMB agar, and typical chlamydospore formation on corn meal-tween agar were confirmatory.

Cultures used for injection were grown for 24 to 72 hours at 37°C on Sabouraud's dextrose agar (SDA) or in Sabouraud's dextrose broth (SDB) to which 50 µg gentamicin/ml (Garamycin, Schering Pharmaceutical Corp., P.R.) had been added to retard bacterial growth.

Final suspensions of Candida to be used for injection were made in sterile Na-K phosphate buffered saline (PBS, pH 7.3, % w/v in distilled water: NaCl 0.8, KCl 0.02, KH₂PO₄ 0.012, and Na₂HPO₄ 0.091). Candida were quantified prior to infection in a hemocytometer (Bright-Line, American Optical Co.) and serially diluted and plated on SDA for the determination of colony forming units.

Day 1 has been designated as the day of infection in all of the following experiments.

C. Experimental Animals

Intravenous inoculations were carried out in male,

randomly-bred, Ha/ICR mice (Sprague-Dawley, division of Mogul Corp., Madison, Wisc.). Several strains of mice were tested for use in leg inoculation studies (Ha/ICR, C3H, Balb/c, and CBA/J). The CBA/J strain of mouse was determined to be most suitable for these studies for reasons which will be detailed later. CBA/J mice were numbered by ear punch and randomized for each experiment. Ha/ICR mice were numbered with 0.1% w/v picric acid-ethanol dye and randomized for each experiment. Mice were primarily 4 to 6 week old males, weighing 20 to 25 grams at the time of experimental initiation.

Mice were fed (Purina Rat Chow #5012) and watered ad libitum. Animals were housed at this laboratory's isolation facility (Building 188) in plastic cages in groups of 5 to 10 per cage. Twelve hour diurnal rhythms were maintained with automatically timed lighting. Temperatures were controlled to comfortably maintain the animals at 22-24°C.

Male guinea pigs, Hartley strain, weighing approximately 500 grams, were housed on the second floor of the Stritch School of Medicine in metal cages in groups of three. The guinea pigs were fed (Purina Guinea Pig Chow) and watered ad libitum. Guinea pigs were used for peritoneal exudate cell harvests, since sufficient numbers of leukocytes could be obtained from one animal for each experiment.

D. Drug Administration

Drug was administered ad libitum in the drinking water of animals receiving therapy. Water consumption of drug treated animals was similar to control animals receiving water without an additive. Drug concentrations were prepared so as to allow the desired consumption over each 24-hour interval based on the observed drinking habits of the experimental animals. Thus, for normal mice early in infection, 4 ml of water was consumed per day per mouse. Alternatively, drug was administered by gavage. Gavage doses were administered according to the average cage weight and were given as $\mu\text{g/kg/day}$. Stainless steel gavage tubes (Popper and Sons, Inc., New Hyde Park, NY) were placed on 1 ml tuberculin syringes (Becton-Dickinson and Co., NJ) and drug was thereby given orally by placement of the tube into the esophagus.

E. Statistical Analysis

In mortality studies, tissue homogenization studies, and viability studies, the χ^2 analysis was used; for germ tube elongation averages and average animal weight and abscess sizes, the Student's t test was utilized. The Pearson's r was used to determine correlations. Averages were represented plus or minus (\pm) the standard error of the mean. P values were considered significant when they were less than 0.05.

F. In Vitro Studies

1. Methods for Determining the Effects of SM-1213 on the Growth of Candida albicans

In order to determine whether SM-1213 had direct inhibitory effects on the growth of Candida, 100 ml of Sabouraud's dextrose broth (SDB) in 500 ml side-arm Erlenmeyer flasks were inoculated with 1×10^5 Candida cells. Candida had been grown for 24 hours on SDA at 37°C prior to inoculation. The increase in turbidity in each of five flasks with different concentrations of SM-1213 were monitored. Concentrations of SM-1213 tested were: 0 (Control), 1.0, 0.1, 0.01, and 0.001 µg/ml. Also, a control blank (no drug, no Candida) was examined. Hourly turbidimetric readings were taken at 660 nm on a Bausch and Lomb Spectronic 70 spectrophotometer over a period of ten hours. Since greater than 98% of Candida grew in the yeast form in SDB, such readings demonstrated what effect, if any, SM-1213 had on this morphologic form of Candida. The normal growth of this particular strain of Candida albicans in SDB was also determined from this data.

To determine whether SM-1213 had a direct effect upon the hyphal form of Candida, 250 ml flasks containing 50 ml of HI-WO₅/BA₂₀₀₀ tissue culture medium with the above concentrations of SM-1213 were inoculated with 10^5 Candida cells and incubated for 24 hours on a rotary shaker

at 37°C. The growth was determined in each of these flasks by dry weight analysis. Dry weight determinations were made after Millipore filtration of the cultures. Three washes of the Candida were performed with distilled water while on the filtration system, followed by drying of the filters in a Stabil-Therm oven (Model #OV-12) at 60°C for 48 hours.

2. Methods for Peritoneal Cell Harvest, Infection and Tissue Culture

One white guinea pig (Hartley strain, male, 500 g) was used in each of the in vitro experiments as a source of peritoneal exudate cells. Either 20 ml of paraffin oil (mineral oil) or glycogen (0.1% w/v in Earle's balanced salt solution) was used as an inducing agent. Harvesting procedures were identical for both types of inducing agents except that the paraffin oil was injected into the peritoneal cavity 72 hours prior to animal sacrifice while the glycogen was injected 24 hours prior to sacrifice. The abdomen of the guinea pig was shaved with an electric shaver (Oster) and cleansed with 70% isopropyl alcohol (Alco-Wipes, Will Ross, Inc., Milwaukee, WI) prior to injection of the inducing agent.

On the day of sacrifice, peritoneal cells were harvested from the guinea pig by five serial washes of 20 ml volumes of heparinized Eagle's basal medium (BME)

containing 50 μg gentamicin/ml. Five units of heparin/ml was included in the medium to inhibit aggregation of exudate cells. A 20 ml plastic syringe and 19 gauge needle was used to deliver the BME into the peritoneum for each wash. Cell harvests were facilitated by a 5 cm longitudinal incision in the abdominal wall to allow access to the peritoneum. A 5 ml polystyrene disposable pipet (Scientific Products, Division of American Hospital Supply) could then be used to collect the wash fluid from the cavity. Polystyrene equipment was used to lessen the adherence and subsequent loss of peritoneal cells during harvesting procedures. The washes were delivered into sterile, 40 ml screw-cap plastic centrifuge tubes and the cells were pelleted by centrifugation at 250 X G for 5 minutes (20°C) in an International PR-6 centrifuge. The pellet was resuspended and washed three times in HI-WO₅/BA₂₀₀₀ serum-free medium (International Scientific).

After washing, the cells were counted in a hemocytometer (Bright-Line, American Optical). A total leukocyte count was made and a differential count was then performed on Wright-Giemsa (Harleco, Division of American Hospital Supply) stained smears.

Total harvests ranged from 5.8×10^7 cells from paraffin oil induction (65% macrophages) to 1.1×10^7 cells from glycogen induction (54% polymorphonuclear cells;

remainder macrophages, monocytes, and lymphocytes). Cells were then seeded onto 60 mm plastic tissue culture Petri plates containing two 2.2 cm square glass cover slips (#1 thickness). It was found that the most workable number of cells was 2×10^6 per Petri plate. These cells were then incubated in HI-WO₅/BA₂₀₀₀ medium with or without the addition of SM-1213 for two hours at 37°C, 5% CO₂ atmosphere, to allow attachment of the cells to the cover slips. Concentrations of SM-1213 tested were 0.1, 0.01, 0.001, and 0.0 (control) µg/ml.

In cell cultures containing 2×10^6 peritoneal cells per plate, 2 to 4×10^5 Candida cells were inoculated into the culture medium directly following the addition of peritoneal cells. After two hours, non-adherent cells were removed by washing once with HI-WO₅/BA₂₀₀₀ medium and appropriate medium (with or without SM-1213) was replaced for those samples requiring further incubation. In this way, any extracellular Candida was removed after the initial two hours of incubation by the medium wash described above. A Candida to peritoneal cell ratio of 1:5 to 1:10 permitted ideal measurements, since only one Candida would be contained in any one peritoneal cell. In studies employing serum, whole normal guinea pig serum obtained from cardiac puncture and stored frozen at -20°C was added to each sample to a final concentration of 2%

v/v. Serum was added to the cultures following the addition of cells.

Candida albicans was grown on Sabouraud's dextrose agar for 24 hours at 37°C. Under these conditions, greater than 95% of the Candida cells were in the yeast phase. Suspensions of Candida were made in phosphate buffered saline (PBS, pH 7.3). A Klett colorimeter reading (Filter #66) of 100 was determined by colony count enumeration to be equivalent to 1×10^7 Candida cells/ml.

a. Determination of Candidal Germ Tube Elongation

At intervals of 2, 4, and 6 hours, one cover slip was removed from the Petri plate and placed in a staining dish containing 95% methanol for 5 minutes. Such treatment caused the cells to become fixed and allowed for microscopic examination. Upon removal from the methanol bath, the cover slip was air dried and inverted onto a drop of distilled water on a glass microscope slide. The peritoneal cells on the cover slip were then examined by phase contrast microscopy at 1000X for intracellular germ tube elongation. The germ tube lengths of Candida contained in 50 peritoneal cells were determined through the use of a micrometer in the eyepiece of the microscope. Intracellular germ tube elongation was monitored at the 2, 4, and 6 hour intervals in this manner.

b. Determination of Candidal Viability

The second, remaining cover slip was removed from the Petri plate with forceps and rinsed in successive baths of PBS and SDB. Such baths were instituted to remove any residual tissue culture medium from the cover slip. Candida develop hyphae in tissue culture medium and the yeast form, which develops on SDA, was required for this section of the assay. (Viability of Candida was determined by their ability to grow out of peritoneal cells via hyphae and form colonies of yeast on SDA. Morphology was therefore highly important in this assay.) Washed cover slips were then drained by touching one corner of the cover slip to a piece of filter paper. The cover slip was then inverted onto a Petri plate containing a thin layer (3 mm) of SDA and incubated for 2½ hours at 37°C (normal atmosphere). If not properly drained, air would be occluded between the cover slip and the agar and hyphae would develop. After incubation, the Petri plates were refrigerated overnight at 4°C and examined microscopically at 1000X the next day for Candida colony formation. Four adjacent yeast cells were designated as one colony unit. The percentage of viable Candida cells could then be determined by counting 100 peritoneal cells which contained Candida:

$$\frac{\text{Number of } \underline{\text{Candida}} \text{ forming colonies}}{\text{Number of } \underline{\text{Candida}} \text{ forming colonies} + \text{Number of } \underline{\text{Candida}} \text{ unable to form colonies}} \times 100 = \% \text{ Viable cells}$$

G. In Vivo Studies

1. Intravenous Inoculations

The object of this series of studies was to determine the effects of SM-1213 treatment on the survival rate of mice with experimentally induced systemic infections of Candida albicans. In particular, the effects of various regimens of drug therapy were investigated.

a. Computation of the 50% Lethal Dose (LD₅₀)

The method of Reed and Muench (47) was used to determine the LD₅₀ for the strain of Candida albicans used in the following experiments. Candida doses used for these calculations were 10⁸, 1.5 X 10⁷, 10⁶, and 10⁵ per animal injected intravenously into the left lateral tail vein of male, Ha/ICR mice; a 26-gauge needle and a 1 ml tuberculin syringe were utilized. Candida were suspended in sterile PBS and 0.1 ml volumes were injected.

b. Methods for Mouse Mortality Studies

Yeast cell suspensions of Candida albicans were made in PBS and counted in a hemocytometer. Candida were grown for 48 hours on SDA at 37°C. Viability of candidal suspensions was determined by plating serial dilutions by the spread plate method and counting colony formation 48 hours later.

Male, Ha/ICR mice were injected with 2×10^5 Candida cells in the lateral tail vein with a 26-gauge needle and 1 ml tuberculin syringe; volumes injected were 0.1 ml per mouse.

SM-1213 was administered ad libitum in the drinking water of test animals for various 24-hour intervals only. A dosage of 80 $\mu\text{g/kg/day}$ was administered for 24 hours only either beginning on the day of infection (immediately following infection and designated as day 1-2 treatment), or beginning one, two, or three days following infection (designated as day 2-3, day 3-4, and day 4-5 treatment, respectively).

2. Intramuscular Inoculations

In primary leg inoculations, male, CBA/J mice were injected into the right lateral calf muscle with either 7×10^6 , 1.25×10^7 , or 1.25×10^8 Candida cells per mouse, depending upon experimental design. The left leg served as a control for daily measurements, as did uninfected control animals injected with PBS only in the right leg. The diameter (lateral to medial) of the uninfected left leg of each mouse was subtracted from the diameter of the infected right leg to determine the size of the abscess produced. Such methods were undertaken to allow for any increases in leg diameter due to growth of the animal which would occur bilaterally. Corrections for

original differences between the right and left leg were made by measuring both legs before infection, determining the difference, and subtracting this original difference from the post-infection difference for each animal for each measurement day. Such corrections did not significantly alter the average abscess size of any experimental group; however, corrections were made to determine the more precise abscess size for each animal before averages were calculated. The phrase "abscess size" has been used interchangeably with the phrase "leg swelling." The latter is the more correct expression of leg measurement data.

Injectons were made with a 27-gauge needle and 1 ml tuberculin syringe. Leg swelling was measured mechanically in mm using a Schnelltaster numerical dial caliper (H. Kröplein, Hassen, W. Germany). Care was taken to extend the leg thoroughly when measurements were taken.

Secondary leg inoculations in mice which had recovered from a primary leg infection were performed on the uninfected contralateral leg with 1×10^5 Candida cells intramuscularly. No further administration of drug was used beyond that given during the primary infection. Re-inoculation was performed on day 57 of the primary infection; therefore, the long-term effects of SM-1213 treatment given during the initial infection were examined during a second infection.

a. Tissue Homogenization Techniques

In order to obtain a quantitative measurement of candidal infection, tissue homogenization studies were undertaken. Spleens, abscesses, and/or kidneys were removed aseptically and placed into sterile vials containing 2 to 5 ml of PBS. Tissues were homogenized in this solution with Duall 24 ground glass tissue homogenizer tubes and pestles (Kontes Glass Co.) A Tri-R Stir-R (50 to 1100 variable rpm, model K-43) motor was used at setting #7. After homogenization, tubes and pestles were rinsed with 3 to 5 ml of PBS which were then added to the tissue homogenate. Each spleen, abscess, and each pair of kidneys was homogenized separately. Abscesses were obtained by excising the abscess and all surrounding muscle tissue located between the knee and ankle joint with sterile surgical scissors.

Quantitation of viable Candida was obtained by serial dilution of the tissue homogenates and spread plate enumeration. Three dilutions of each sample were plated in duplicate (10^{-1} , 10^{-3} , and 10^{-5}) and colony counts were performed on 48-hour cultures incubated at 37°C.

b. Culturing Techniques

At the time of sacrifice, animals were killed by cervical dislocation and the right leg was skinned and

removed aseptically by cutting with surgical scissors transversely through the femur and the ankle. This portion of the leg, containing the abscess, was placed into 25 ml sterile, cotton-plugged Erlenmeyer flasks containing 10 ml of SDB + gentamicin (Gm). The specimen was minced 50 times with surgical scissors which had been flame sterilized with ethanol. Flasks were then placed in a shaking water bath incubator (Warner-Chilcott Laboratories, Morris Plains, NJ) at 37°C for 24 hours. Colony count enumerations were carried out on serial dilutions of the tissue suspension plated out on SDA and incubated for 48 hours at 37°C. Similarly, each pair of kidneys and each spleen was aseptically removed and cultured in the same manner following mincing. Such a technique was developed so that qualitative differences could be determined between samples. This was particularly important in detecting small quantities of micro-organisms which may not have been detected using classical tissue homogenization techniques.

c. Weight Gain Determinations

In weight gain studies, correction for abscess weight was determined by subtracting the weight of a sphere from the animal weight. The volume of the sphere was determined by using one-half the abscess size as the radius in the equation $V = \frac{4}{3}\pi r^3$. The density was designated as approximately equal to 1 g/ml and the mass could

thereby be determined mathematically (mass = density X volume) and subtracted from the animal weight.

CHAPTER III

RESULTS

A. In Vitro Studies

1. Effects of SM-1213 on the Growth of Candida albicans

As shown in Tables 1 and 2, SM-1213 had no direct fungicidal or fungistatic activity against Candida when tested in Sabouraud's dextrose broth (yeast form of Candida) or HI-WO₅/BA₂₀₀₀ tissue culture medium (hyphal form of Candida). Turbidity was virtually identical in all samples tested regardless of drug concentration (see Table 1). The higher turbidometric reading obtained for the control sample at the 5 hour interval was attributed to a procedural artifact resulting from insufficient shaking of the sample flask. Thorough shaking of the sample flask for subsequent readings resulted in a more even distribution of Candida in the SDB and the elimination of aberrant readings.

Dry weights of Candida grown in tissue culture medium for 24 hours with and without SM-1213 present were also very similar, as were the pH readings of the culture supernatants (see Table 2). The pH of the culture supernatant was used as an indication of change in acidity of the medium due to candidal growth.

Table 1. Effect of SM-1213 on the growth of Candida albicans in Sabouraud's dextrose broth (SDB)^a

Incubation time (hrs)	Growth of <u>C.albicans</u> at 37°C. ^b				
	SM-1213 concentration in SDB (µg/ml)				
	0	1.0	0.1	0.01	0.001
1	0.050	0.045	0.035	0.030	0.047
2	0.056	0.038	0.075	0.048	0.060
3	0.095	0.116	0.129	0.101	0.102
4	0.221	0.192	0.167	0.201	0.228
5	0.525	0.303	0.320	0.283	0.333
6	0.300	0.309	0.300	0.300	0.308
7	0.450	0.460	0.460	0.460	0.480
8	0.610	0.620	0.610	0.610	0.620
9	0.740	0.750	0.740	0.740	0.750
9.5	0.840	0.850	0.850	0.840	0.850
10	0.940	0.950	0.940	0.950	0.950

a) 100 ml of SDB was inoculated with 1×10^5 C.albicans in each of five 500 ml side-arm Erlenmeyer flasks containing the specified concentration of SM-1213 at the time of inoculation.

b) Growth of C.albicans was monitored by the increase in turbidity of candidal cultures with time. Spectrophotometric readings were taken at each time interval at 660 nm.

Table 2. Effect of SM-1213 on the growth of Candida albicans in tissue culture medium^a

SM-1213 concentration in TC medium ($\mu\text{g/ml}$)	pH of TC ^b medium	Dry weight ^c (grams)
0	7.35	0.0297
1.0	7.10	0.0291
0.1	7.24	0.0307
0.01	7.35	0.0311
0.001	7.35	0.0311

a) 50 ml of HI-WO₅/BA₂₀₀₀ tissue culture medium was inoculated with 1×10^5 C.albicans in each of five 250 ml Erlenmeyer flasks and incubated for 24 hours on a rotary shaker at 37°C.

b) pH of supernatant TC medium in which C.albicans was grown.

c) Dry weight determinations of candidal growth from (a) above after 24 hours incubation at 37°C in TC medium. Cultures were Millipore filtered, washed, and dried at 60°C for 48 hours.

2. Tissue Culture Studies

a. Effect of SM-1213 on Germ Tube Elongation of Phagocytized *Candida albicans* in the Absence of Serum

In the first experiment employing paraffin oil induced guinea pig peritoneal cells, two drug levels and a control group were tested. As demonstrated in Figure 2, the average germ tube lengths were nearly identical in the control group and the drug treated groups until the 6-hour sampling time. At this point, the average germ tube length in the control group was significantly higher than in the SM-1213 treated samples.

The apparent lag in time before the divergence between the control and drug groups would seem to indicate that there is a mechanism by which SM-1213 induces a change in the ability of the peritoneal exudate cells to inhibit the growth of intracellular Candida, but that there is an interval before the peritoneal cells are activated or before the activation can mediate an effect.

The lower drug concentration of SM-1213 (0.001 $\mu\text{g/ml}$) inhibited germ tube elongation more efficiently than did the higher drug concentration (0.01 $\mu\text{g/ml}$) after 6 hours of incubation, while both drug treated groups were significantly lower than the control.

Drug treated groups also showed the lowest intracellular viability (see Figure 3). The differences in

Figure 2. Effect of SM-1213 on germ tube elongation of phagocytized Candida albicans in mineral oil induced exudate cells in the absence of serum.

Phagocytic leukocytes were induced by i.p. injection of mineral oil into a male, Hartley strain guinea pig 72 hours prior to cell harvest. Germ tube lengths were measured microscopically at 1000X via a micrometer in the microscope eyepiece. Each point represents the average germ tube length (\pm S.E.) of 50 intracellular Candida cells measured at the designated time interval. (●) Control; (▲) 0.01 μ g SM-1213/ml TC medium; (■) 0.001 μ g SM-1213/ml TC medium.

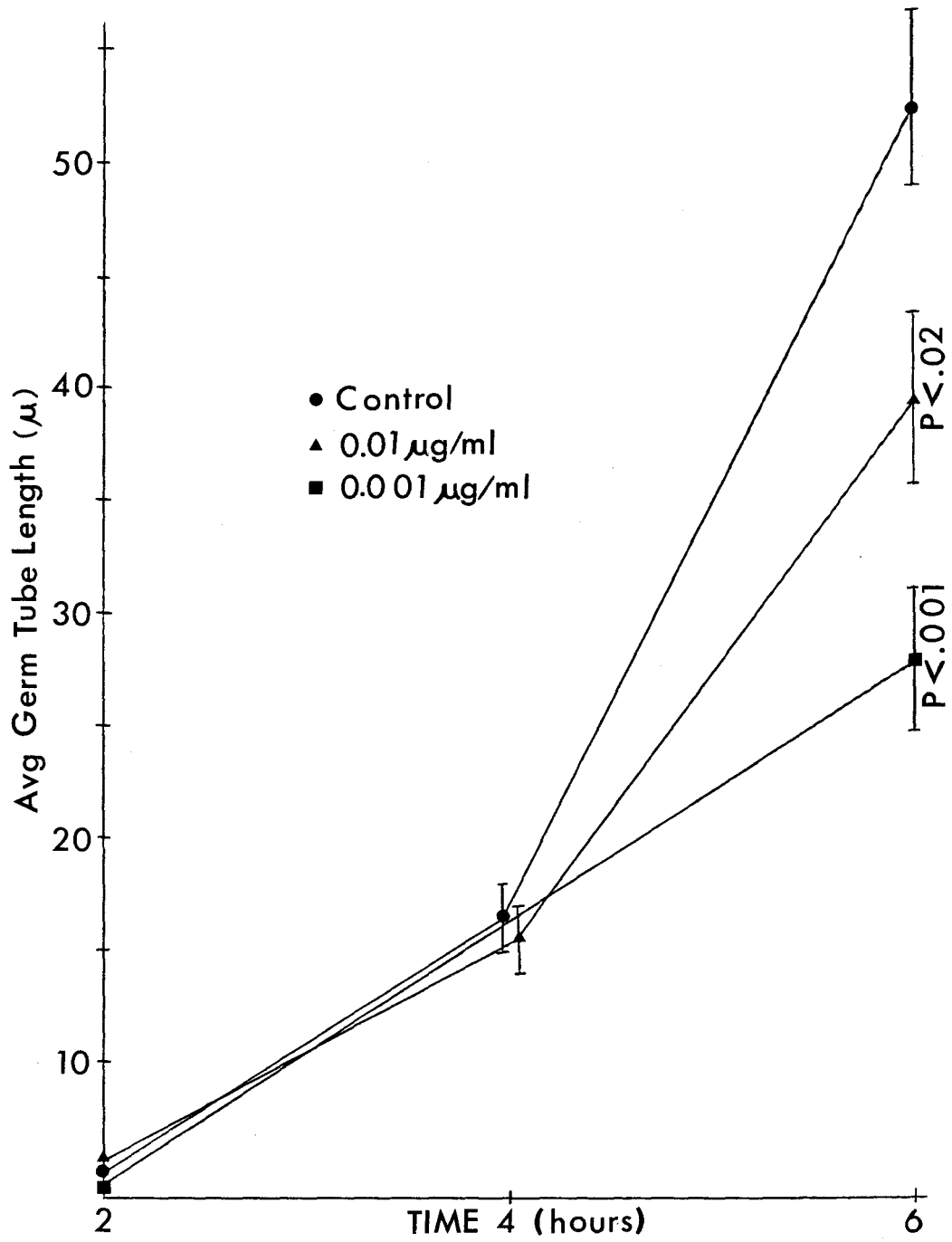
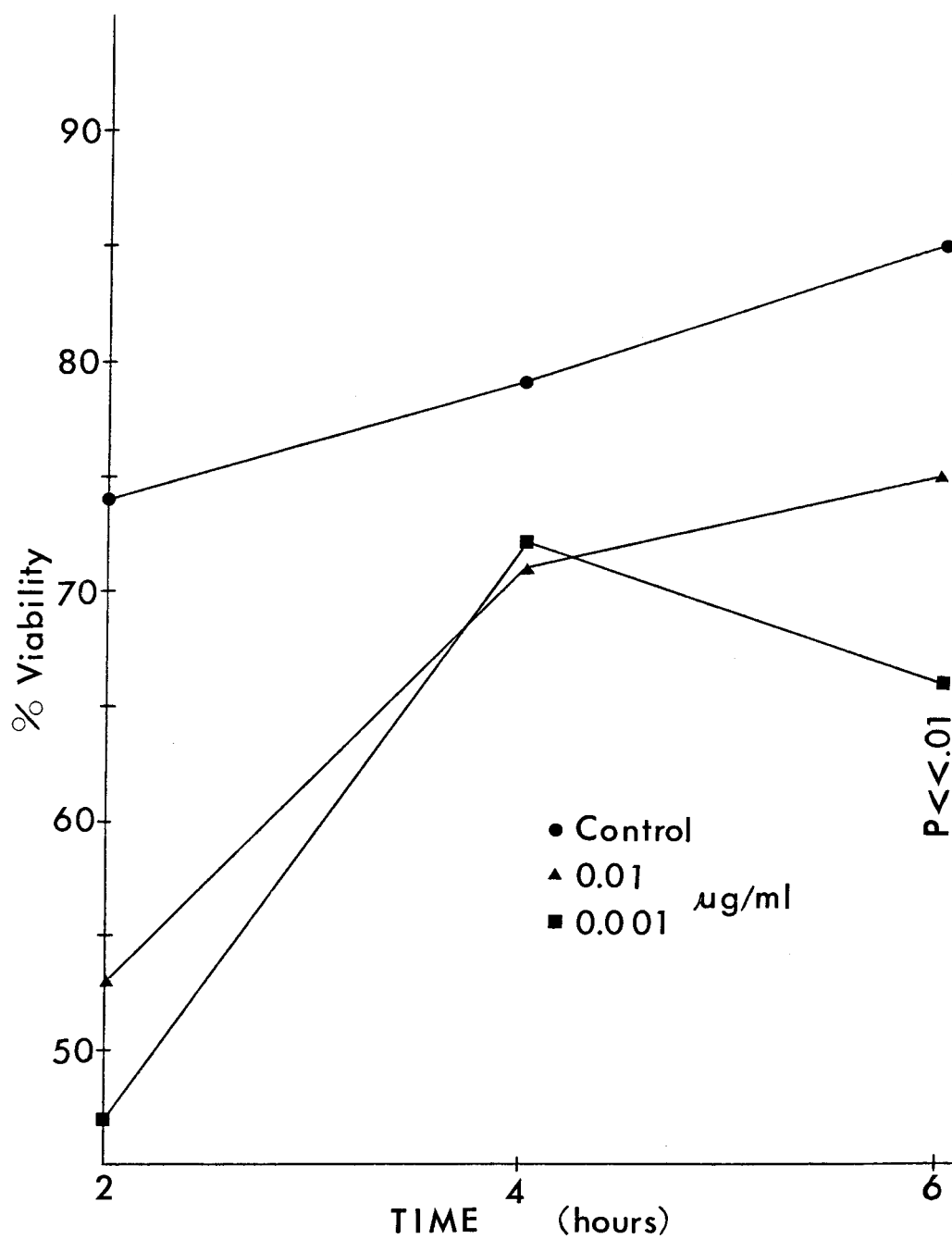


Figure 3. Effect of SM-1213 on the viability of phagocytized Candida albicans in mineral oil induced exudate cells in the absence of serum.

Each data point represents the percentage of viable Candida cells in 100 leukocytes containing fungus.

$$\% \text{ Viability} = \frac{\# \text{ of } \underline{\text{Candida}} \text{ forming colonies}}{\# \text{ of } \underline{\text{Candida}} \text{ forming colonies} + \# \text{ of } \underline{\text{Candida}} \text{ unable to form colonies}} \times 100;$$

a colony was designated as four adjacent C.albicans yeast cells formed on Sabouraud's dextrose agar after two and one-half hours of secondary incubation at 37°C. (●) Control; (▲) 0.01 µg SM-1213/ml TC medium during initial incubation; (■) 0.001 µg SM-1213/ml TC medium during initial incubation.



viability, unlike germ tube length, were apparent after only 2 hours of incubation. Thus, exposure to SM-1213 for as little as 2 hours can mediate an effect on candidal viability.

Since percent viability is a measure of the ability of candidal cells to emerge from within peritoneal cells and form colonies on SDA, germ tube lengths do not strictly correlate with viability results during the first hours of incubation when germ tubes are wholly contained within leukocytes. Apparently, during the subsequent 2½ hours of incubation on SDA, germ tubes in the control sample were better able to escape from within the peritoneal cells and form colonies, even though their average length at the 2- and 4-hour intervals in tissue culture were comparable to drug treated groups.

It was noted also that, after 6 hours of incubation in tissue culture followed by incubation on SDA, the control group formed massive colonies, which were greater in size as well as in number than the drug treated groups. This indicates that the drug may be implicated in the reduction of candidal division and multiplication upon agar after exiting peritoneal cells as a result of its effect on leukocytes while in tissue culture. The correlation between the average germ tube length and the percent viable cells was strong by the 6-hour interval of tissue culture, as will be discussed later.

In an experiment where glycogen was used as an inducing agent, germ tube elongation was more greatly inhibited than in the paraffin oil induced experiment above (see Figure 4). The germ tube lengths in the 0.001 $\mu\text{g/ml}$ treatment group were significantly shorter than in the control. It was speculated that the hyphae were disintegrating rather than elongating due to the observed loss of cell envelope integrity in treated samples.

All drug groups demonstrated shorter germ tube lengths than the control. Here, unlike the previous experiment which used paraffin oil induction, the differences in germ tube lengths were apparent after 4 hours of incubation time.

The effects of SM-1213 on the percent viable cells in this study were also apparent. All drug treated groups showed lower viability than the control. The lowest concentration of drug (0.001 $\mu\text{g/ml}$) showed the lowest Candida viability and was significantly different from the control (see Figure 5).

b. Effect of SM-1213 on Germ Tube Elongation of Phagocytized Candida albicans in the Presence of Serum

Once again, paraffin oil was used as an inducing agent in this study. However, whole, normal guinea pig serum was added to the tissue culture medium to determine what effect it might have on germ tube elongation and

Figure 4. Effect of SM-1213 on germ tube elongation of phagocytized C.albicans in glycogen induced exudate cells in the absence of serum.

Phagocytic leukocytes were induced by i.p. injection of glycogen (0.1 % w/v in EBSS) into a male, Hartley strain guinea pig 24 hours prior to cell harvest. Germ tube lengths were measured microscopically at 1000X via a micrometer in the microscope eyepiece. Each point represents the average germ tube length (\pm S.E.) of 50 intracellular Candida cells at the designated time interval. (●) Control; (◆) 0.1 μ g SM-1213/ml TC medium; (▲) 0.01 μ g SM-1213/ml TC medium; and (▼) 0.001 μ g SM-1213/ml TC medium.

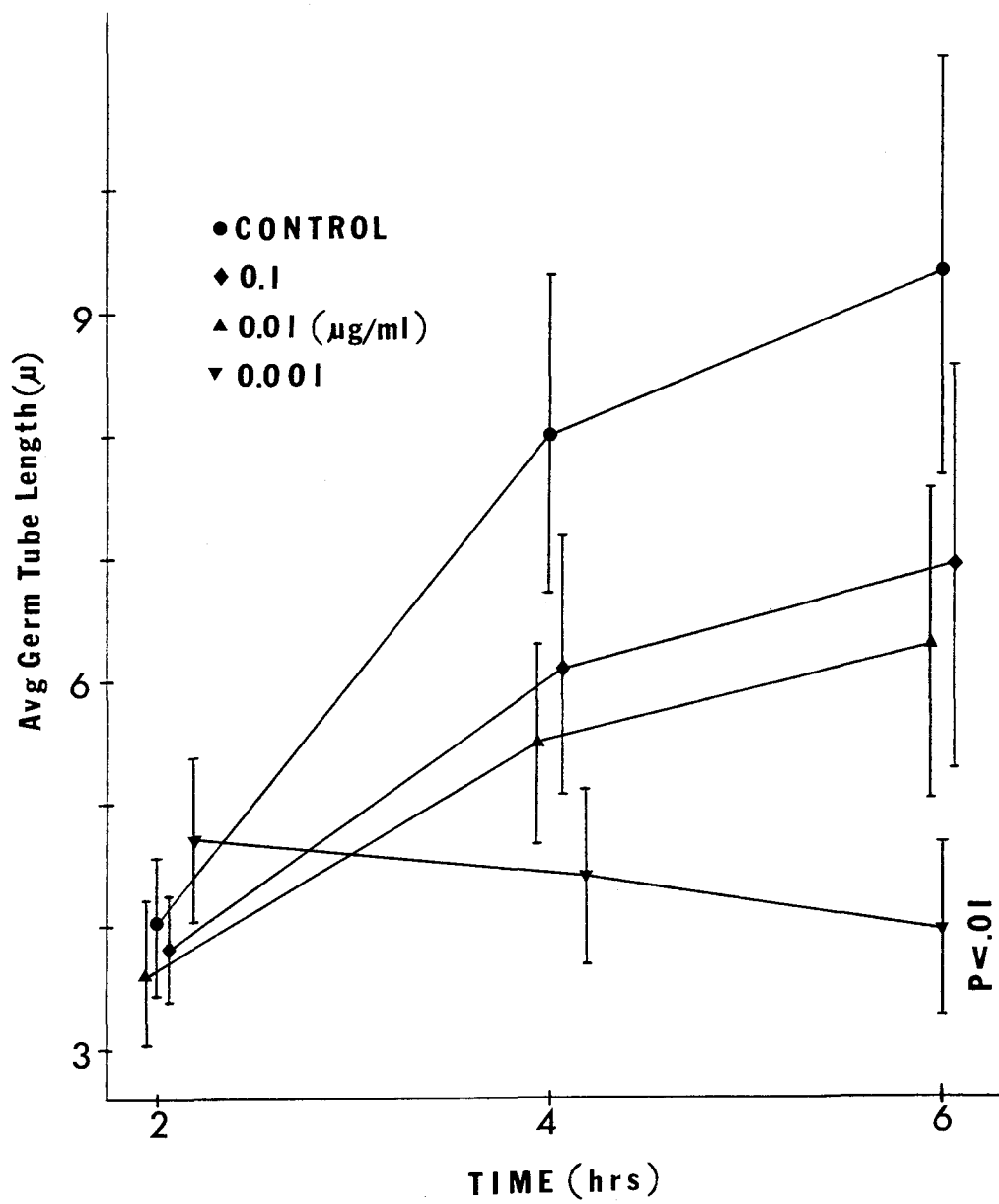
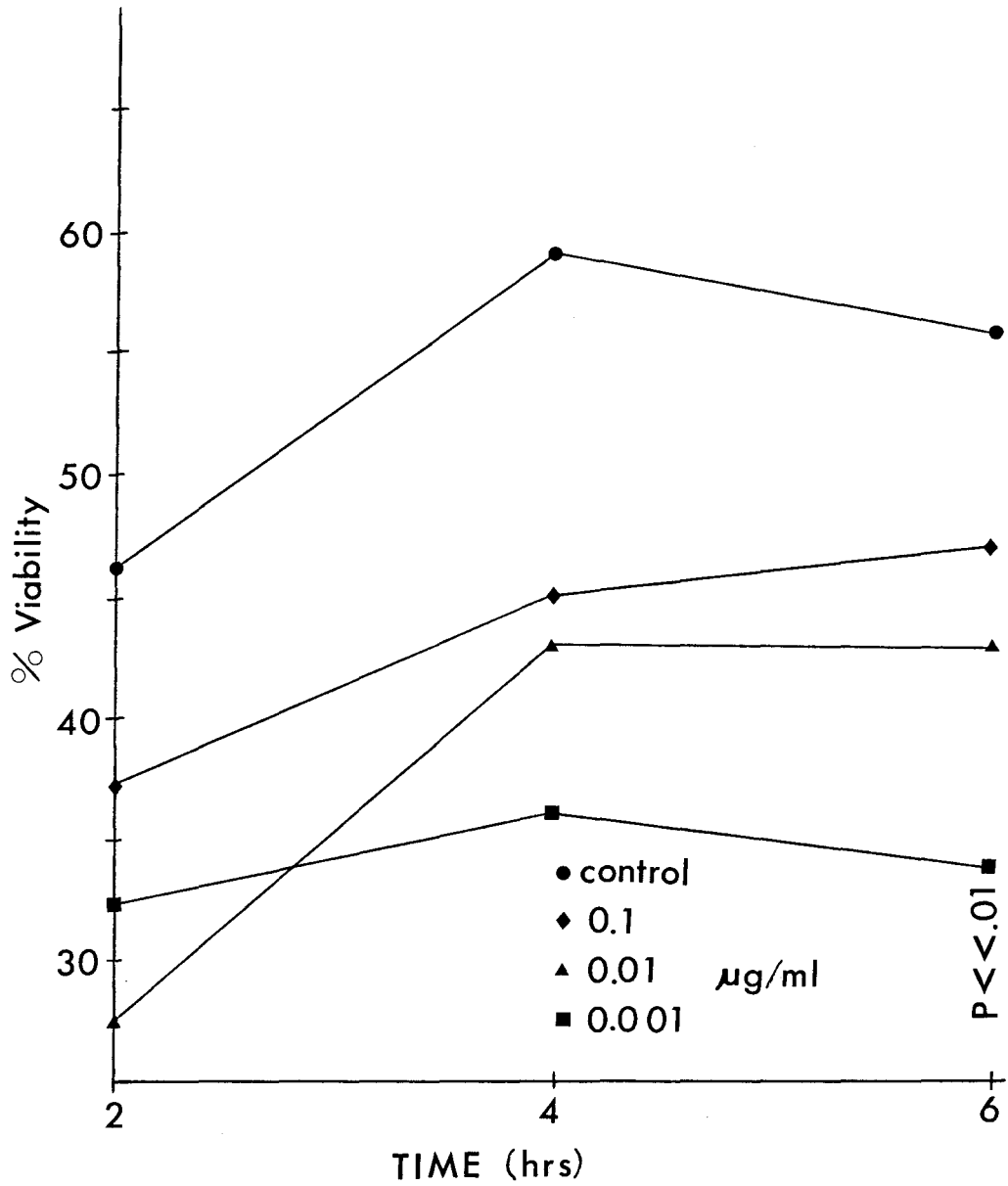


Figure 5. Effect of SM-1213 on the viability of phagocytized Candida albicans in glycogen induced exudate cells in the absence of serum.

Each point represents the percentage of viable Candida cells in 100 leukocytes containing fungus. Percentage viability was determined as in Figure 3. (●) Control; (◆) 0.1 μg SM-1213/ml TC medium during initial incubation; (▲) 0.01 μg SM-1213/ml TC medium during initial incubation; (■) 0.001 μg SM-1213/ml TC medium during initial incubation.



candidal viability. In this study (see Figure 6), significant differences in average germ tube length were not observed.

When compared to Figure 2, where paraffin oil was used but no serum was added, the overall average germ tube length of the control sample at the 6-hour interval was significantly less in the presence of serum. It would appear from this data that the addition of serum to the tissue culture fluid inhibits the rapid elongation observed in the first serum-free experiment between the 4- and 6-hour interval. Serum did not significantly reduce the average germ tube length of drug treated samples, although some reduction was apparent (see upper half of Table 3). It is speculated that SM-1213 may mimic an unknown serum factor which may stimulate peritoneal cells to inhibit germ tube elongation.

Although no significant differences were noted in average germ tube length, the lowest dose of SM-1213 (0.001 $\mu\text{g}/\text{ml}$) was significantly effective in reducing Candida viability at the 6-hour interval (see Table 4).

In addition to germ tube length and viability, the percentage of peritoneal exudate cells containing intracellular Candida was determined for each interval. This was calculated by counting 100 peritoneal cells in each test group. The percentage of these cells which contained Candida was then determined.

Figure 6. Effect of SM-1213 on germ tube elongation of phagocytized Candida albicans in mineral oil induced exudate cells in the presence of serum.

Procedures were identical to those described in Figure 2 except, in this case, 2% v/v whole, normal guinea pig serum was added to the incubation medium. Each point represents the average germ tube length (\pm S.E.) of 50 intracellular Candida cells at the designated time interval. (●) Control; (◆) 0.1 μ g SM-1213/ml TC medium; (▲) 0.01 μ g SM-1213/ml TC medium; (▼) 0.001 μ g SM-1213/ml TC medium.

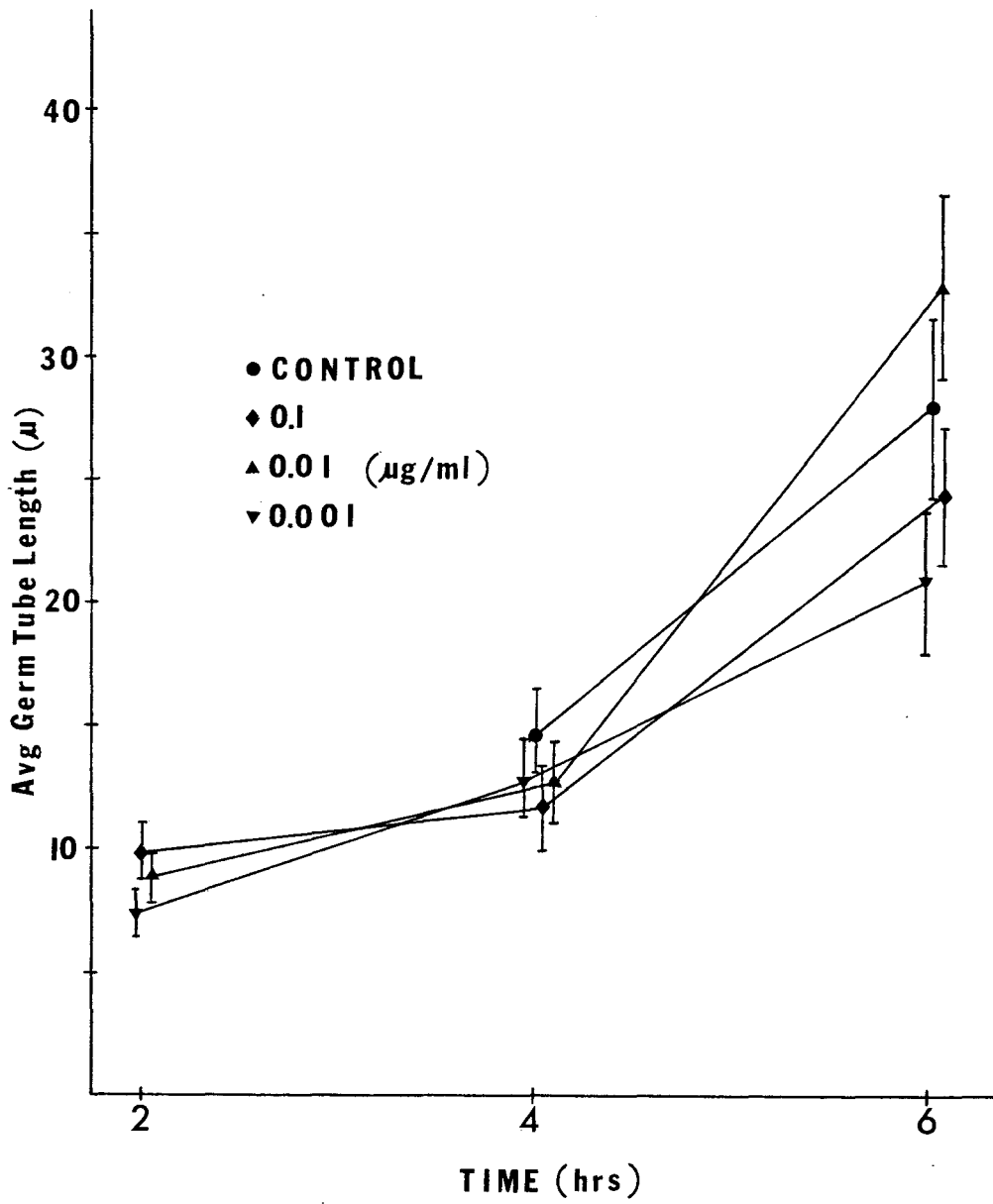


Table 3. Effect of SM-1213 on the germ tube length of Candida albicans in the presence or absence of serum

SM-1213 concentration ($\mu\text{g/ml}$) ^b	Average germ tube length (μ) ^a		Significance level ^d
	Without serum	With serum ^c	
Mineral oil ^e			
0	52.68 \pm 3.83 ^g	27.94 \pm 3.30	P < 0.001
0.001	27.68 \pm 3.30*	20.82 \pm 3.00	N.S.
0.01	39.28 \pm 3.80*	32.82 \pm 3.86	N.S.
Glycogen ^f			
0	9.34 \pm 1.74	6.80 \pm 1.58	N.S.
0.001	3.94 \pm 0.72*	3.80 \pm 0.91	N.S.
0.01	6.28 \pm 1.31	3.12 \pm 1.03*	N.S.

a) 50 C.albicans cells contained within leukocytes were measured for germ tube length after 6 hours of incubation time in HI-WO₅/BA₂₀₀₀ tissue culture medium at 37°C, 5% CO₂. Germ tube lengths were measured by the use of a micrometer in the eyepiece of a microscope at 1000X.

b) Incubation medium contained the specified concentration of SM-1213.

c) Incubation medium contained 2% v/v whole, normal guinea pig serum.

d) N.S. indicates P > 0.05 by Student's t test.

e) Mineral oil was used as an inducing agent for leukocyte harvest; 20 ml was injected i.p. into a male, Hartley strain guinea pig 72 hours prior to cell harvest.

f) Glycogen was used as an inducing agent; 20 ml of a 0.1% w/v solution of glycogen in Earle's balanced salt solution was injected as in (e) above except 24 hours prior to cell harvest.

g) Average germ tube length in microns plus or minus the standard error of the mean (\pm S.E.); an asterisk (*) following the S.E. indicates a significant (P < 0.05) difference in germ tube length between the drug treated and control sample by Student's t test.

Data was compiled from four experiments.

Table 4. Effect of SM-1213 on the viability of phagocytized Candida albicans in mineral oil induced exudate cells in the presence of serum^a

SM-1213 concentration ($\mu\text{g/ml}$) ^b	Percentage viable <u>C.albicans</u> ^c		
	Incubation time (hours) ^d		
	2	4	6
0	N.D. ^e	74	79
0.001	62	64	56* ^f
0.01	70	72	74
0.1	65	60	72

a) Initial tissue culture incubation medium contained 2% v/v whole, normal guinea pig serum.

b) Leukocyte + Candida mixtures were exposed to the specific SM-1213 concentrations noted above contained in HI-WO₅/BA₂₀₀₀ tissue culture medium during the initial incubation time at 37°C, 5% CO₂.

c) Percentage viable cells was determined as in Figure 3.

d) Initial incubation time in TC medium before the additional two and one-half hours of incubation on Sabouraud's dextrose agar at 37°C.

e) N.D. = not done.

f) An asterisk (*) denotes a significantly reduced percentage viability of C.albicans between the control and drug treated samples ($P < 0.001$ by χ^2 analysis).

Theoretically, if all extracellular Candida were removed by the wash with medium at the 2-hour interval, the percentage of leukocytes containing Candida intracellularly should remain constant at each sampling time for any given group. The percentages ranged from as low as 13% at 2 hours after incubation to as high as 20% after 6 hours of incubation. The initial ratio of Candida to peritoneal cell was 1:10 to 1:5 (10-20%) in all experiments. No significant differences were observed over time in the percentage of exudate cells containing intracellular Candida. In addition, virtually all Candida used as an inoculum was apparently phagocytized.

A cell-free sample of tissue culture medium was also inoculated with Candida. This was done to insure that intracellular Candida which remained in the yeast form (hyphal length = 0 μ) did so due to the influences of the peritoneal cells present (see upper half of Table 5). At the 2-hour interval, 88% of all Candida cells had developed into the hyphal form; by 6 hours, 94% produced hyphae. Clearly, the majority of Candida cells, when not influenced by peritoneal cells, develop into the hyphal form in the tissue culture medium tested (HI-WO₅/BA₂₀₀₀ with 2% whole, normal guinea pig serum). The longest hypha observed in this study ranged from 62 μ at the 2-hour time interval to 115 μ at the 6-hour interval. The longest hypha at the 4-hour interval measured 75 μ .

Table 5. Effect of tissue culture medium on candidal morphology in the presence of serum^a

Experiment number ^b	Percentage of <u>Candida</u> in hyphal form ^c		
	Incubation time (hours)		
	2	4	6
1	88	92	94
2	90	95	N.D. ^d

a) 3 ml of HI-WO₅/BA₂₀₀₀ tissue culture medium containing 2% v/v whole, normal guinea pig serum was inoculated with 4×10^5 yeast cells in the absence of leukocytes and incubated at 37°C, 5% CO₂.

b) Experiment #1 was conducted in parallel with an experiment utilizing mineral oil induction and experiment #2 was conducted in parallel with an experiment utilizing glycogen induction.

c) 50 Candida were counted from tissue culture samples at the intervals indicated above and examined for the formation of germ tubes. The number of C.albicans displaying germ tube formation divided by the total number of yeast and hyphal forms present $\times 100 = \%$ of Candida in hyphal form.

d) N.D. = not done.

Lastly, studies were performed in which glycogen induced peritoneal exudate cells were incubated with whole, normal guinea pig serum added to the tissue culture medium (see Figure 7). In this study, which was performed blind, the drug groups once again showed significantly less germ tube elongation than the control. However, unlike the other experiments discussed, the highest drug concentration appeared to be the most efficient in inhibiting intracellular Candida growth.

All drug groups demonstrated significantly fewer viable Candida cells than the controls under the influence of serum (see Figure 8 and Table 6).

The percentage of Candida in hyphal form in samples containing no exudate cells was determined as in the previous experiment to confirm that inhibition of germ tube elongation was truly due to the presence of leukocytes (see lower half of Table 5). Both the glycogen induced and paraffin oil induced studies contained serum in the tissue culture medium and, in both, approximately 90% of the Candida cells developed into the hyphal form by 2 hours of incubation.

The range in hyphal length was marginally greater in this study than in the last when comparing lengths of the longest germ tube observed at a particular time interval. After 2 hours of incubation, the longest hypha

Figure 7. Effect of SM-1213 on germ tube elongation of phagocytized Candida albicans in glycogen induced exudate cells in the presence of serum.

Procedures were identical to those described in Figure 4 except, in this case, 2% v/v whole, normal guinea pig serum was added to the incubation medium. Each point represents the average germ tube length (\pm S.E.) of 50 intracellular Candida cells at the designated time interval. (●) Control; (◆) 0.1 μ g SM-1213/ml TC medium; (▲) 0.01 μ g SM-1213/ml TC medium; (▼) 0.001 μ g SM-1213/ml TC medium.

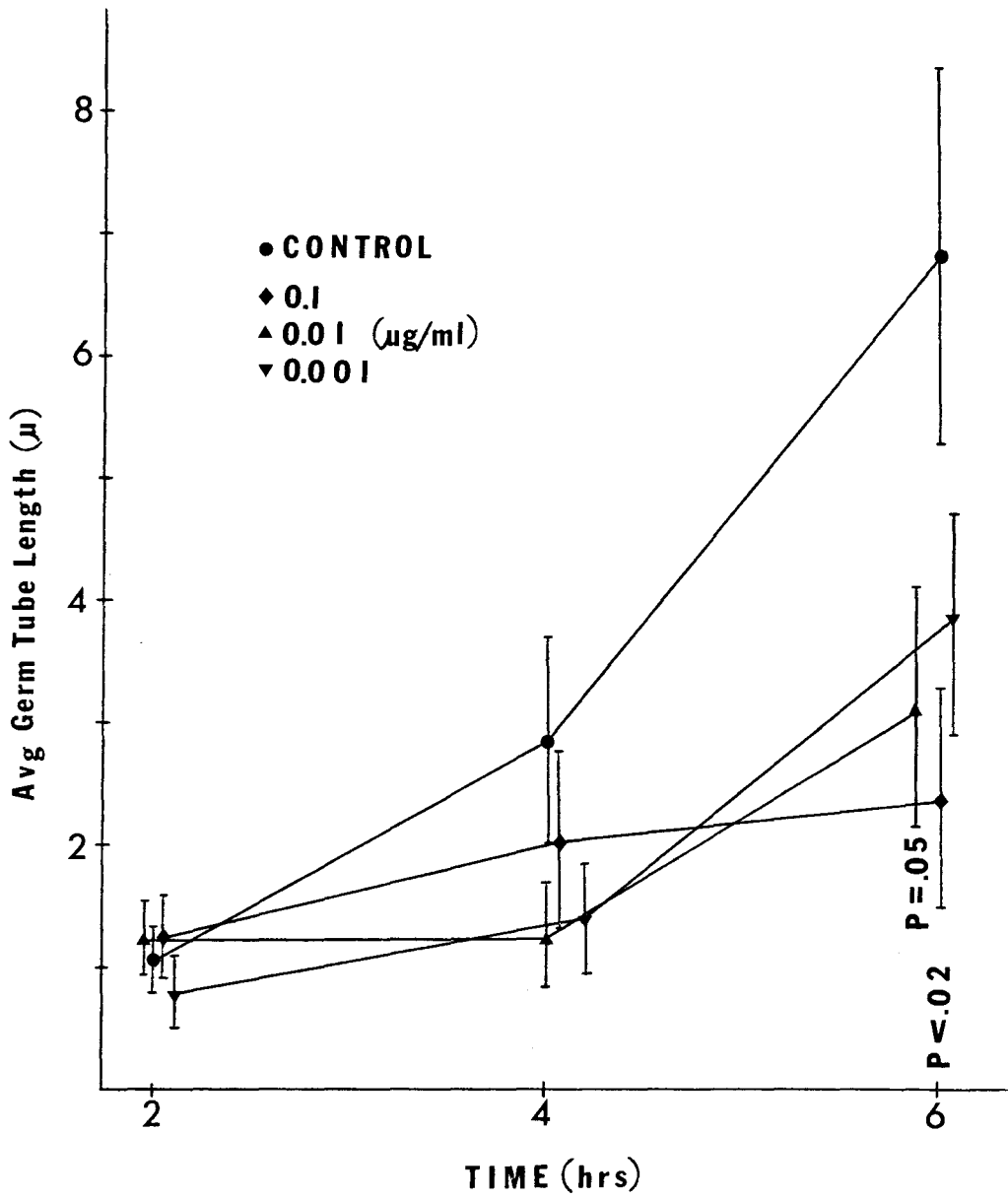


Figure 8. Effect of SM-1213 on the viability of phagocytized Candida albicans in glycogen induced exudate cells in the presence of serum.

Each point represents the percentage of viable Candida cells in 100 leukocytes containing fungus. Percentage viability was determined as in Figure 3. (●) Control; (◆) 0.1 μg SM-1213/ml TC medium during initial incubation; (▲) 0.01 μg SM-1213/ml TC medium during initial incubation; (▼) 0.001 μg SM-1213/ml TC medium during initial incubation.

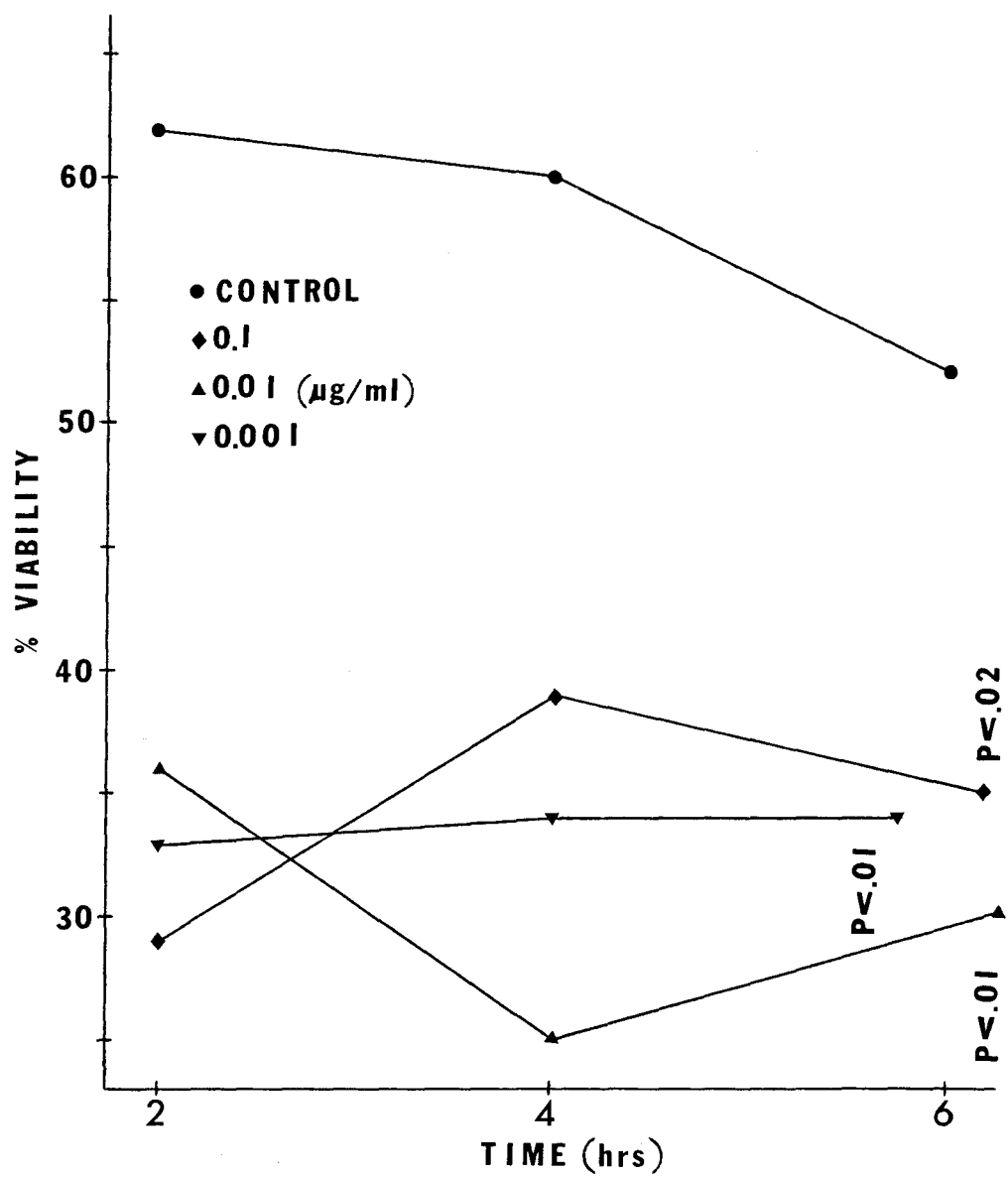


Table 6. Effect of SM-1213 concentration on the viability of intracellular Candida albicans in the presence or absence of serum

SM-1213 concentration (μ g/ml) ^b	Percentage viable <u>Candida albicans</u> ^a			
	Mineral oil ^c		Glycogen ^d	
	No serum	Serum ^e	No serum	Serum ^e
0	85	79	54	52
0.001	66* ^f	56*	34*	34*
0.01	75	74	42	30*
0.1	N.D. ^g	72	47	35*

a) % Viability = $\frac{\# \text{ of } \underline{\text{Candida}} \text{ forming colonies}}{\# \text{ of } \underline{\text{Candida}} \text{ forming colonies} + \# \text{ of } \underline{\text{Candida}} \text{ unable to form colonies}} \times 100$;

a colony was designated as four adjacent C.albicans yeast cells formed on Sabouraud's dextrose agar after two and one-half hours of incubation at 37°C. 100 leukocytes were counted for each percentage.

b) Leukocyte + Candida mixtures were exposed to the specific SM-1213 concentrations noted above contained in HI-WO₅/BA₂₀₀₀ tissue culture medium during the initial 6 hours of incubation at 37°C, 5% CO₂.

c) Mineral oil was injected i.p. into a guinea pig 72 hours prior to leukocyte harvest.

d) Glycogen (0.1% w/v in EBSS) was injected i.p. into a guinea pig 24 hours prior to leukocyte harvest.

e) Initial incubation medium contained 2% v/v whole, normal guinea pig serum.

f) An asterisk (*) indicates a significant difference in percentage viable C.albicans between control and drug treated samples ($P < 0.05$ by the χ^2 analysis). In addition, glycogen induced cells demonstrated a significantly reduced candidal viability relative to mineral oil induced cells in the presence or absence of serum ($P < 0.01$ by χ^2 analysis).

g) N.D. = not done.

Data was compiled from four experiments.

observed measured 37 μ , at 4 hours 94 μ , and at 6 hours no reading was taken.

The average germ tube length in the cell-free sample was significantly greater than any test group containing peritoneal cells and is illustrated in Table 7.

The addition of serum to the tissue culture medium had the effect of reducing the average germ tube length for every group tested which contained cells (see lower half of Table 3). Serum had a similar inhibitory effect on the average germ tube length in the paraffin oil induced experiments, but only in the control group at the 6-hour sampling time was a significant difference noted.

Although the average germ tube length was not significantly affected by serum in comparable glycogen samples, the frequency of Candida remaining in the yeast form after 6 hours incubation was significantly affected (see Table 8). Therefore, there apparently was an effect mediated by the addition of serum which inhibited hyphal formation in glycogen induced cells (control and drug treated) as well as in the control paraffin oil induced cells. Drug treated cells from the paraffin oil induced studies were once again not significantly affected by the addition of serum.

Average germ tube length closely correlated to the percent viability as determined in these studies. The

Table 7. Effect of glycogen induced exudate cells on germ tube elongation of phagocytized Candida albicans in the presence of serum^a

Experimental group	Average germ tube length (μ) ^b		
	Incubation time (hours)		
	2	4	6
Leukocytes + medium ^c	1.04 \pm 0.28* ^e	2.86 \pm 0.89*	6.80 \pm 1.58
Medium ^d	16.10 \pm 1.55	34.66 \pm 3.85	N.D. ^f

a) 2% v/v whole, normal guinea pig serum was added to the incubation medium.

b) 50 candidal cells were measured for germ tube elongation by the use of a micrometer in the eyepiece of a microscope at 1000X at the intervals detailed above. Average germ tube lengths are represented plus or minus the standard error of the mean (\pm S.E.).

c) 3 ml of HI-WO₅/BA₂₀₀₀ tissue culture medium containing serum as detailed in (a) was inoculated with 4×10^5 yeast cells in the presence of 2×10^6 glycogen induced peritoneal cells and incubated at 37°C, 5% CO₂.

d) 3 ml of HI-WO₅/BA₂₀₀₀ tissue culture medium containing serum as detailed in (a) was inoculated with 4×10^5 yeast cells in the absence of exudate cells and incubated at 37°C, 5% CO₂ for the indicated time intervals.

e) An asterisk (*) indicates a significantly reduced average germ tube length of Candida in samples containing exudate cells ($P < 0.01$ by Student's t test).

f) N.D. = not done.

Table 8. Effect of SM-1213 concentration on the morphology of phagocytized Candida albicans in the presence or absence of serum

SM-1213 concentration (µg/ml) ^a	Frequency of <u>Candida albicans</u> in yeast form ^b		Significance level ^f
	No serum	Serum ^c	
Mineral oil ^d			
0	0/50	11/50	P < 0.001
0.001	4/50	9/50	N.S.
0.01	1/50	5/50	N.S.
Glycogen ^e			
0	9/50* ^g	29/50*	P < 0.001
0.001	17/50	30/50	P < 0.01
0.01	17/50	39/50	P < 0.001
0.1	11/50	42/50	P < 0.001

a) Leukocyte + Candida mixtures were exposed to the specific SM-1213 concentrations noted above contained in HI-WO₅/BA₂₀₀₀ tissue culture medium during the initial 6 hours of incubation at 37°C, 5% CO₂.

b) 50 C. albicans cells contained within leukocytes were examined for germ tube formation after 6 hours of incubation time under the conditions described in (a).

c) Incubation medium contained 2% whole, normal guinea pig serum.

d) Mineral oil was used as an inducing agent for leukocyte harvest; 20 ml was injected i.p. into a male, Hartley strain guinea pig 72 hours prior to cell harvest.

e) Glycogen was used as an inducing agent; 20 ml of a 0.1% w/v solution of glycogen in EBSS was injected as in (d) above except 24 hours prior to cell harvest.

f) N.S. indicates $P < 0.05$ by the χ^2 analysis.

g) Glycogen induced leukocytes demonstrated a significantly greater number of Candida cells in the yeast form than the mineral oil induced cells in the presence of serum ($P < 0.05$ by the χ^2 analysis).

correlation was strongest in the absence of serum in both the glycogen and paraffin oil studies (Pearson's $r = 0.99$). The correlation was moderately strong in the paraffin oil study with serum ($r = 0.74$) and strong in the glycogen study with serum added ($r = 0.94$).

As will be discussed later, the differences in the cell type which predominated in the peritoneum as a result of the inducing agent employed may provide an explanation for the differences observed between paraffin oil induced cells and glycogen induced cells. The predominant cell type produced upon 72-hour paraffin oil induction was the macrophage, whereas the predominant cell type upon 24-hour glycogen induction was the polymorphonuclear leukocyte.

As demonstrated in Table 9, not only was the effect of serum on the germ tube length different upon paraffin oil induced cells than glycogen induced cells, but the overall length attained by germ tubes after 6 hours incubation was significantly lower in glycogen induced cells. In addition, glycogen induced cells also demonstrated a lower percentage of hyphal forms of Candida in the presence and absence of serum than did the paraffin oil induced cells. These attributes may reflect the greater candidacidal activity of the polymorphonuclear neutrophil.

Table 9. Effect of serum on germ tube formation in phagocytized Candida albicans in tissue culture

Leukocyte inducing agent ^b	Average germ tube length (μ) ^a		Significance level ^d
	No serum	Serum ^c	
Mineral oil	52.68 \pm 3.83 (100%) ^e	27.94 \pm 3.69 (78%)	P < 0.001
Glycogen	9.34 \pm 1.74* ^f (84%)*	6.80 \pm 1.58* (42%)*	N.S.

- a) 50 C.albicans cells contained within leukocytes were measured for germ tube length after 6 hours of incubation time in HI-WO₅/BA₂₀₀₀ tissue culture medium at 37°C, 5% CO₂. Germ tube lengths were measured by the use of a micrometer in the eyepiece of a microscope at 1000X.
- b) 20 ml of mineral oil or glycogen (0.1% w/v in EBSS) was injected i.p. into a male guinea pig 72 or 24 hours prior to leukocyte harvest respectively.
- c) Incubation medium contained 2% v/v whole, normal guinea pig serum.
- d) N.S. = not significant (P > 0.05 by Student's t test).
- e) Percentages in parentheses indicate the percentage of 50 intracellular Candida cells which developed germ tubes (hyphae) after 6 hours of incubation time as described in (a). Although the addition of serum did not significantly reduce the germ tube length of Candida in the glycogen induced studies, the percentage of Candida in hyphal form was significantly reduced (P < 0.001 by χ^2 analysis) by the addition of serum to the culture medium. In addition, the presence of serum in the culture medium significantly reduced the percentage of hyphae formed in the mineral oil induced studies as well (P < 0.001 by χ^2 analysis).
- f) Glycogen induced leukocytes demonstrated a significantly reduced germ tube length relative to mineral oil induced cells in the presence or absence of serum (P < 0.001 by Student's t test) as well as a significantly reduced percentage of Candida in hyphal form (P < 0.01 by χ^2 analysis) as indicated by asterisks above.

B. In Vivo Studies

1. Intravenous Inoculations

a. Computation of the 50% Lethal Dose (LD₅₀) of *Candida albicans*

The results for the determination of the 50% lethal dose for the strain of *Candida albicans* used in the following experiments is shown in Table 10. By day 11, an intravenous dose of $1 \times 10^{5.88}$ *Candida* cells, grown for 24 hours on SDA at 37°C, will be lethal to 50% of male, Ha/ICR mice used experimentally. Initial intravenous experiments were primarily concerned with early mortality occurring within the first week following infection where high doses of *Candida* were used as inocula. Day 11 was therefore chosen for the termination of the LD₅₀ study. Subsequent experiments in which low inocula were used were conducted for more prolonged periods and mortality was observed for approximately 30 days, although no further LD₅₀ studies were conducted.

b. Effect of SM-1213 on Mortality of Mice Infected Intravenously with *Candida albicans*

A series of studies were undertaken to determine the effect of SM-1213 drug administration on intravenously infected Ha/ICR mice. SM-1213 therapy for 24 hours only beginning either on the day of infection (day 1) or 24, 48, or 72 hours post-infection was examined. SM-1213

Table 10. Computation of the 50% lethal dose (LD₅₀)* for Candida albicans by day eleven post-infection.

<u>Candida</u> dose injected i.v.	Mortality ratio	Died	Survived	Accumulated values			
				Total dead	Total survived	Mortality	
						Ratio	% Dead
1 x 10 ⁸	8/8	8	0	19	0	19/19	100
1.5 x 10 ⁷	7/8	7	1	11	1	11/12	92
1 x 10 ⁶	4/6	4	2	4	3	4/7	57
1 x 10 ⁵	0/6	0	6	0	9	0/9	0

*Calculation of the LD₅₀ from the above data:

$$\frac{\% \text{ Mortality at dilution next above 50\%} - 50\%}{\% \text{ Mortality at dilution next above 50\%} - \% \text{ Mortality at dilution next below 50\%}} = \frac{\text{Proportionate distance between known \% mortality and 50\% mortality}}{57\% - 0\%}$$

Therefore, $\frac{57\% - 50\%}{57\% - 0\%} = 0.12$ Or, the LD₅₀ titer = 10^{5.88}

was administered in the drinking water at a dosage of 80 µg/kg/day based on average consumption per average assessed animal weight.

In male, Ha/ICR mice infected with 2×10^5 Candida cells intravenously and treated 48 hours later with SM-1213 for 24 hours, a significant reduction in mortality was observed when compared to control mice (see Figure 9).

In order to substantiate these results, two additional experiments were conducted under the same experimental protocol as the first. Table 11 illustrates the cumulative mortality for all three experiments conducted utilizing the day 3-4 drug treatment regimen. Significant differences were obtained between the drug treated and control groups in cumulative mortality from day 18 to day 24. Table 12 illustrates the continued significant differences on day 27 and day 30 in two of the three experiments. The third experiment was altered on day 24 and the results beyond this day (and therefore before this day) could not be included in this table. Mortality plateaued in all test groups from day 27 until experimental termination.

In addition, alternate intervals of drug administration were tested (see Table 13). Various intervals of 24-hour drug treatment were tested in these experiments:

- 1) drug treatment begun on the day of infection for 24

Figure 9. Effect of SM-1213 therapy on the cumulative mortality of mice infected intravenously with Candida albicans.

Male, Ha/ICR mice were injected intravenously with 2×10^5 C.albicans cells on day 1. Drug treated mice received 80 μ g SM-1213/kg/day orally in drinking water for day 3 to day 4 only; control mice received no drug. (●) Control, N = 15; (◆) SM-1213 treated, N = 15.

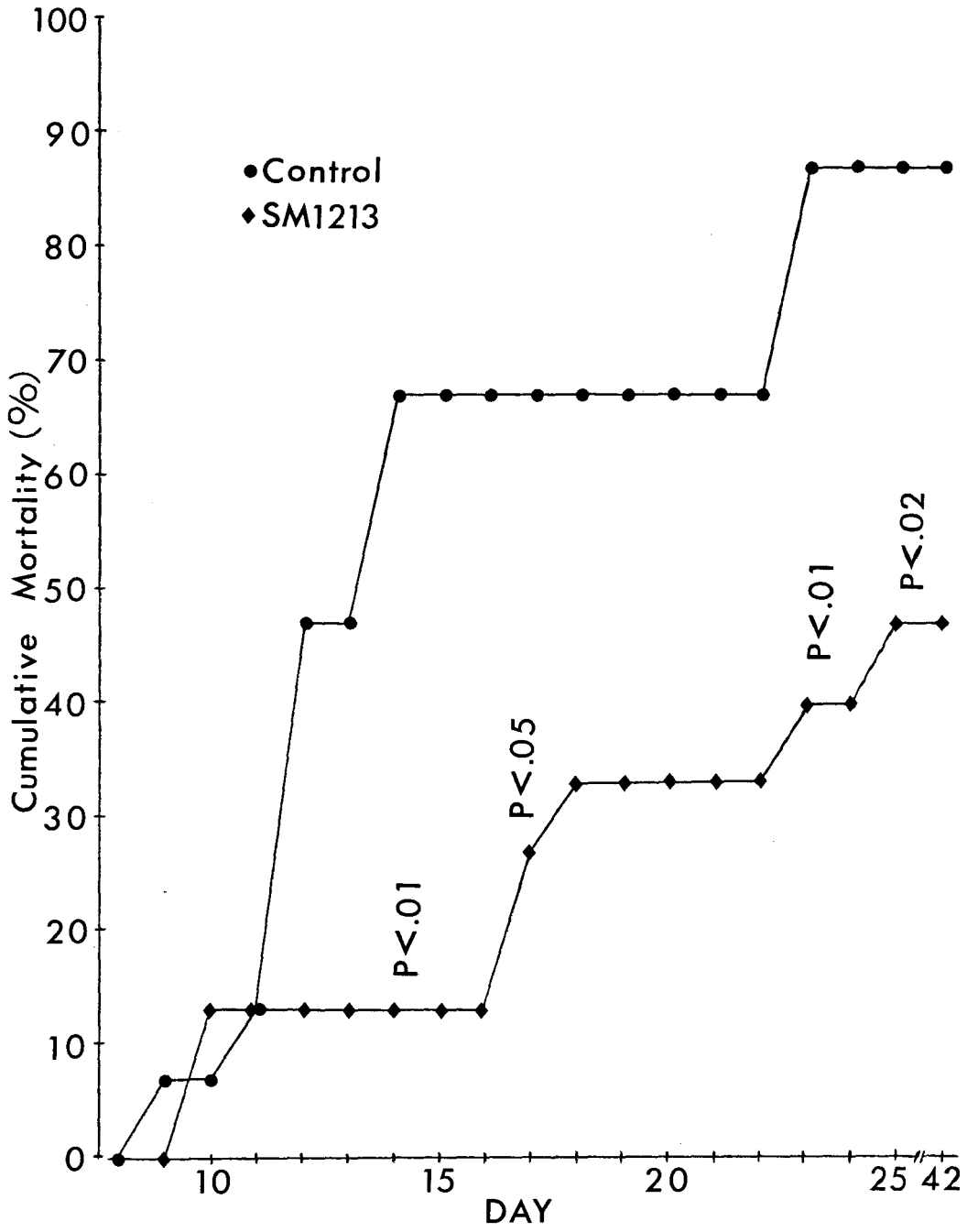


Table 11. Effect of SM-1213 treatment on the cumulative percentage mortality in mice infected intravenously with Candida albicans^a

Treatment group	Cumulative % mortality ^b						(Number of experiments)
	Day 9	Day 12	Day 15	Day 18	Day 21	Day 24	
Control	3	25	41	53	58	64	(3) N=59
SM-1213 ^c	3	18	28	34* ^d	38*	43*	(3) N=61

a) Male, Ha/ICR mice were infected intravenously with 2×10^5 C.albicans cfu on day 1 and observed for the occurrence of death.

b) The cumulative percentage mortality was determined for the days indicated above by determining the total number of mice dead by that day divided by the total number of experimental animals X 100. N = the total number of mice used experimentally.

c) Drug treated mice received 80 µg SM-1213/kg/day orally in the drinking water for 24 hours only from day 3 to day 4 post-infection.

d) An asterisk (*) indicates a significant reduction in cumulative mortality in drug treated mice relative to control mice ($P < 0.05$ by χ^2 analysis).

Table 12. Effect of SM-1213 treatment on the thirty day cumulative mortality in mice infected intravenously with Candida albicans^a

Treatment group	Cumulative % mortality ^b								(Number of experiments)
	Day 9	Day 12	Day 15	Day 18	Day 21	Day 24	Day 27	Day 30	
Control	5	38	57	62	68	78	78	78	(2) N=37
SM-1213 ^c	8	21	32* ^d	42	42*	45*	50*	50*	(2) N=38

a) Male, Ha/ICR mice were infected intravenously with 2×10^5 C.albicans cfu on day 1 and observed for the occurrence of death for thirty days.

b) The cumulative percentage mortality was determined as indicated in Table 11. N = the total number of animals used experimentally.

c) Drug treated mice received 80 µg SM-1213/kg/day orally in drinking water for 24 hours only for day 3 to day 4 post-infection.

d) An asterisk indicates $P < 0.05$ by the χ^2 analysis between control and drug treated group mortality.

Table 13. Effect of SM-1213 treatment on the cumulative mortality of mice infected intravenously with Candida albicans and treated with SM-1213 at various intervals^a

Treatment group	Average cumulative % mortality ^b						(Number of experiments)
	Day 9	Day 12	Day 15	Day 18	Day 21	Day 24	
Control	4 (±2) ^c	28 (±12)	43 (±16)	54 (±9)	59 (±9)	67 (±14)	(3) N=59
**Day 1-2	7 (±7)	22 (±9)	33 (±16)	48 (±18)	61 (±13)	68 (±11)	(2) N=46
**Day 2-3	2 (±2)	9 (±5) ^d	20 (±7)*	34 (±7)*	48 (±7)	61 (±11)	(2) N=44
**Day 3-4	6 (±4)	17 (±4)	26 (±9)	34 (±8)*	37 (±6)*	42 (±3)*	(3) N=61
**Day 4-5	5 (NA) ^e	14 (NA)	23 (NA)	27 (NA)*	27 (NA)*	32 (NA)*	(1) N=22

a) Male, Ha/ICR mice were infected intravenously with 2×10^5 Candida albicans cells on day 1 and observed for the occurrence of death. Drug treated mice received 80 µg SM-1213/kg/day orally in drinking water for 24 hours only for the interval designated by a double asterisk above (**).

b) The cumulative mortality for each designated time interval from day 9 to day 24 was determined by averaging the cumulative mortality of animals from multiple experiments.

c) The average cumulative percentage mortality is expressed plus or minus the standard error of the mean (±S.E.).

d) An asterisk (*) following the S.E. indicates a significant reduction in average cumulative mortality in drug treated mice relative to control mice ($P < 0.05$ by χ^2 analysis).

e) NA = not applicable.

hours only (designated as day 1-2 therapy), 2) treatment begun on the day following infection (day 2-3), 3) treatment begun 2 days following infection (day 3-4), and 4) treatment begun 3 days following infection (day 4-5). Table 13 represents the average cumulative percentage mortality for all three experiments. The earliest significant difference in mortality between drug and control groups occurred in the day 2-3 treated group. However, mortality continued to rise in the day 2-3 treated mice and was not significant from the control group on day 21 or 24. The day 3-4 treated group showed a significantly lower cumulative mortality than controls during experimental continuation from day 18 to day 24. Day 4-5 data was included in this table even though one experiment was conducted using this regimen and was not conclusive evidence that a significant difference between control and drug groups was truly present. The day 4-5 treated group showed a significantly lower cumulative mortality than the average control from day 18 to day 24. However, this treatment group was not significantly different from its own experimental control. There was an indication that further experiments, which would increase experimental numbers, would have shown a significant difference, since multiplication of the data from this one experiment by three produces a significant difference between control

and day 4-5 treated groups from day 20 to day 24. There is strong evidence to support the effectiveness of day 3-4 drug therapy, however, in the reduction of overall cumulative mortality and a suggestion that day 4-5 therapy may be effective as well.

In addition, day 3-4 and day 4-5 drug therapy were significantly more effective in reducing cumulative mortality than day 1-2 drug therapy on day 21 and 24. Day 4-5 drug therapy was also more effective than day 2-3 drug therapy on day 24. Such data supports the suggestion that specific intervals of drug therapy are important in the effectiveness of SM-1213 in reducing mortality.

In order to quantitate the number of yeast cells in the viscera of mice inoculated with Candida albicans, tissue homogenate studies were conducted. Since the kidney is an organ of major involvement in candidal infections (26,66), it was of interest to culture these organs from infected animals. On day 32, surviving mice were killed by cervical dislocation and kidneys were aseptically removed and homogenized.

Significant differences were not observed between any of the test groups at this sacrifice time. The average number of Candida cells per two kidneys homogenized varied from as low as 1×10^4 (± 0.64) for the drug treated animals to 1.7×10^5 (± 1.7) for the control animals.

2. Effect of SM-1213 on the Course of Leg Lesions in Mice Infected Intramuscularly with *Candida albicans*

A series of experiments were conducted utilizing the mouse leg lesion model for experimental candidiasis. Animal weights, as well as abscess sizes, were monitored at intervals throughout infection. In addition, abscesses, kidneys, and/or spleens were either 1) homogenized in order to quantitate Candida present or 2) cultured in order to determine the presence of Candida within these tissues.

The CBA/J strain of mouse was determined to be suitable for these studies, since it consistently produced measurable abscesses with the inocula used in these experiments. The CBA/J inbred strain of mouse provided a more genetically homogeneous population than did the randomly-bred Ha/ICR strain of mouse; this would have been particularly important had lymphoid cell transfer studies been undertaken. In addition, the temperament of the CBA/J mouse was more docile than that of the Ha/ICR mouse, which not only allowed for easier handling of the mice but also eliminated interference with experimental data due to fighting among cohabitating males as observed in the Ha/ICR strain.

The inbred C3H strain proved to be less susceptible than the CBA/J strain to Candida lesion formation and

required twice the inoculum of Candida to produce an abscess equivalent in size to that of the CBA/J strain. Another inbred strain of mouse, the Balb/c strain, was also found to be unsuitable for leg lesion studies, since spontaneous amputation of the infected limbs occurred by day 7 in 10% of the mice inoculated. Such amputation was attributed to a hyperimmune response, since mice immunosuppressed with 300 mg of cyclophosphamide/kg four days prior to infection did not amputate their limbs or develop localized abscesses. Such a dose of cyclophosphamide has been shown to reduce white blood cell counts in mice 100-fold by day 4 (58). However, immunosuppressed mice subsequently died of apparent systemic infection; by day 12, mortality reached 78%. Therefore, the CBA/J strain of mouse was utilized in the following studies.

a. Leg Lesion Study #1: Effect of SM-1213 on the Course of Leg Lesions in Mice Infected Intramuscularly with 1.25×10^8 Candida albicans Cells

In this study, designated as Leg Lesion Study #1, male CBA/J mice were infected with 1.25×10^8 Candida cells per mouse intramuscularly into the right calf. Leg swelling was measured on day 1, 2, 3, 6, 7, 8, 10, 13, 15, 17, 20, 22, and 24. Drug therapy (80 μ g/kg/day) was administered from day 3 to 4 for one treatment group and day 4 to 5 for the other treatment group. As shown

in Figure 10, there were no significant differences in abscess sizes between drug and control groups until day 24.

Animal weights, however, were significantly higher in drug treated animals (see Figure 11), which suggested that these animals were in better overall health (57); SM-1213 has no caloric value due to the substitutions present on the monosaccharide molecule. The average group weight change from prior to infection to various intervals post-infection is shown in Figure 11. Drug treated mice more closely paralleled the weight gain pattern demonstrated by uninfected control mice than did the infected controls.

When differences in animal weights were corrected for any contributions the abscess sizes may have added, the significant differences in animal weight between the drug treated and control mice were actually enhanced moderately ($P < 0.02$ on day 8, $P < 0.01$ on day 15, and P was the same on day 22).

In addition, when convalescing mice were analyzed for Candida content in spleens on day 24, it was discovered that the day 4-5 drug treated groups had a significantly lower incidence as well as number of Candida than controls (see Table 14).

Figure 10. Effect of SM-1213 on the course of leg lesions in mice infected intramuscularly with 1.25×10^8 Candida albicans cells.

Candida cells were injected on day 1. Leg swelling was measured at the designated time intervals with the aid of a Schnelltaster numerical dial caliper. Drug treated groups received 80 μ g SM-1213/kg/day orally in drinking water either for day 3 to day 4 post-infection (R_{x1}) or for day 4 to day 5 post-infection (R_{x2}). The original number of mice in each test group was as follows: (●) Control, N = 14; (■) day 3 to 4 drug treated group, N = 14; and (▲) day 4 to 5 drug treated group, N = 14.

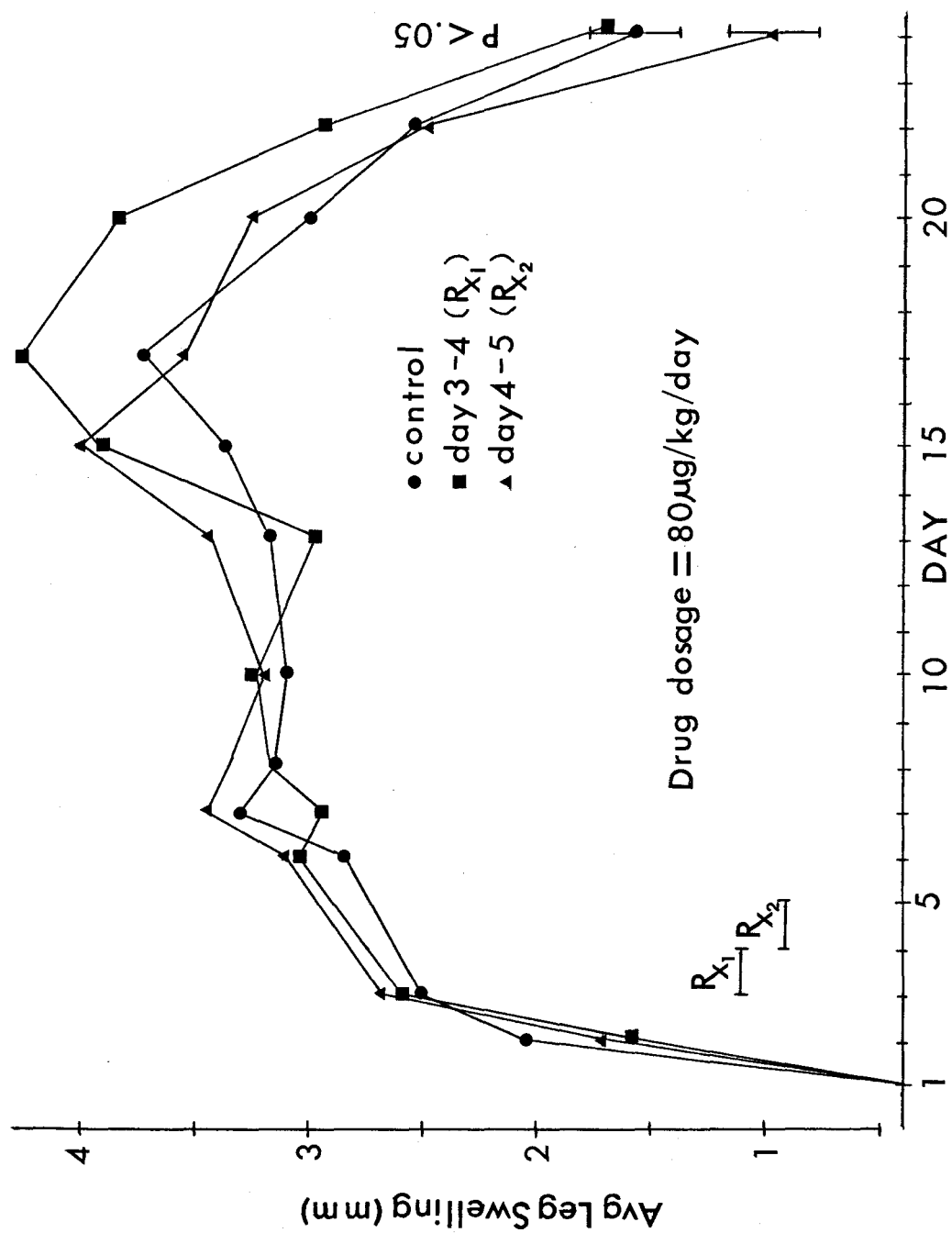


Figure 11. Effect of SM-1213 on the average group weight change from day one in mice infected intramuscularly with 1.25×10^8 Candida albicans cells.

CBA/J mice were weighed prior to infection on day 1 and on days thereafter as indicated at right. The difference between the average group weight on day 1 and on subsequent days was plotted. The original number of test animals was as follows: (●) Control, N = 14; (▼) day 4 to 5 drug treatment group (as described in Figure 10), N = 14; (▲) day 3 to 4 drug treatment group (as described in Figure 10), N = 14; and uninfected control group (injected with PBS only in the right leg), N = 8, represented at right by a star.

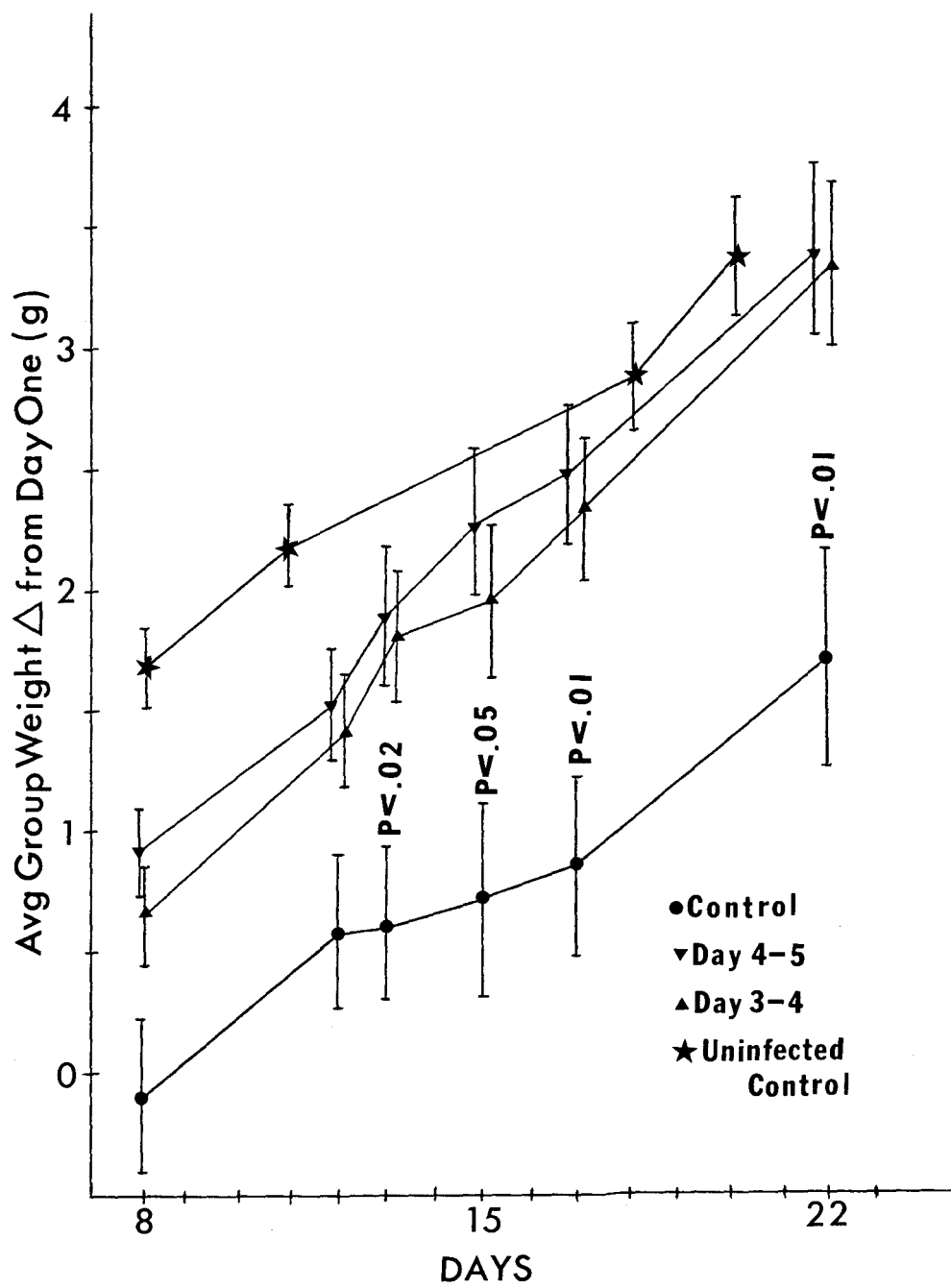


Table 14. Effect of SM-1213 on the number of Candida albicans recovered from spleens of convalescing mice^a

Treatment Interval ^b	Frequency of spleens containing viable <u>C.albicans</u> ^c	Frequency of spleens containing ≥ 50 viable <u>C.albicans</u> ^d
Control	13/14	13/14
Day 4-5	8/14	4/14

a) Male, CBA/J mice were injected intramuscularly in the right calf with 1.25×10^8 C.albicans cells on day 1. On day 24, mice were sacrificed; spleens were excised, homogenized and assessed for colony forming units on SDA at 37°C.

b) Control mice received no drug; drug treated mice received 80 µg SM-1213/kg/day orally in the drinking water for 24 hours only from day 4 to day 5 post-infection.

c) Control mice versus drug treated, $P < 0.05$ by χ^2 analysis.

d) Control mice versus drug treated, $P < 0.001$ by χ^2 analysis.

These results suggest that the drug treated groups were better able to contain the abscess and prevent the systemic spread of the infection. However, no significant differences were obtained between drug and control mice in tissue homogenates of the kidneys ($1.15 \times 10^3 \pm 0.29$ versus $2.81 \times 10^3 \pm 0.68$) when such high levels of Candida were injected. An alternative suggestion would be that drug treated mice were better able to clear Candida from the spleens by day 24.

b. Leg Lesion Studies #2 and #3: Effect of Altered SM-1213 Drug Regimen and Method of Drug Administration on Mice Infected Intramuscularly with Candida albicans

An inoculum similar to that used in Leg Lesion Study #1 was used (1.2×10^8 Candida cells per mouse) and CBA/J mice were inoculated intramuscularly into the right leg. However, in these studies drug regimen was altered. Also, the method of drug administration was altered in one of the following two experiments.

In addition to administering drug by drinking water only, the method of gavaging animals to administer drug was initiated. In Leg Lesion Study #2, day 4-5 drinking water treatment was given as in Leg Lesion Study #1 (80 $\mu\text{g/kg/day}$); however, an additional drinking water dose was also given for day 7-11 (400 $\mu\text{g/kg/day}$). In Leg Lesion Study #3, gavage doses were given for day 1-4 (400 $\mu\text{g/kg/day}$), followed by drinking water administration

of drug for day 7-11 (400 µg/kg/day) and day 17-18 (80 µg/kg/day). See Figures 12 and 13.

Using these drug regimens, significant differences occurred between abscess sizes of drug treated and control groups. Drinking water therapy showed the most significant differences early in the infection, while gavage therapy demonstrated greatest significance later in infection.

Tissue homogenization of kidneys and spleens was carried out on day 46 post-infection. No significant differences were found in the spleens as in Leg Lesion Study #1, where mice were sacrificed on day 24. However, almost twice the number of Candida was found in control kidneys as compared to mice treated with drug by gavage.

c. Sustained Effect of SM-1213 Treatment on Mice from Leg Lesion Study #2 Reinoculated Intramuscularly with Candida albicans

The recovered mice from Leg Lesion Study #2 were reinfected on day 57 in the uninfected leg with 1×10^5 Candida cells per mouse intramuscularly (i.e. left leg was injected). Significant differences were detected between control and previously drug treated groups on day 4 and 5 following reinoculation (see Figure 14). The significant differences observed in the reinfected

Figure 12. Effect of altered SM-1213 drug regimen on mice infected intramuscularly with 1.2×10^8 Candida albicans cells.

Leg swelling was measured at the days indicated above with the aid of a Schnelltaster numerical dial caliper. Drug treated groups received 80 μg SM-1213/kg/day for day 4 to day 5 post-infection as in the initial leg lesion study as well as an additional 400 μg SM-1213/kg/day drug treatment for day 7 to day 11. All drug was administered in the drinking water. (●) Control, N = 12; (○) SM-1213 treated, N = 12. Points of significant differences in leg swelling between control and drug treated groups is indicated below by a star.

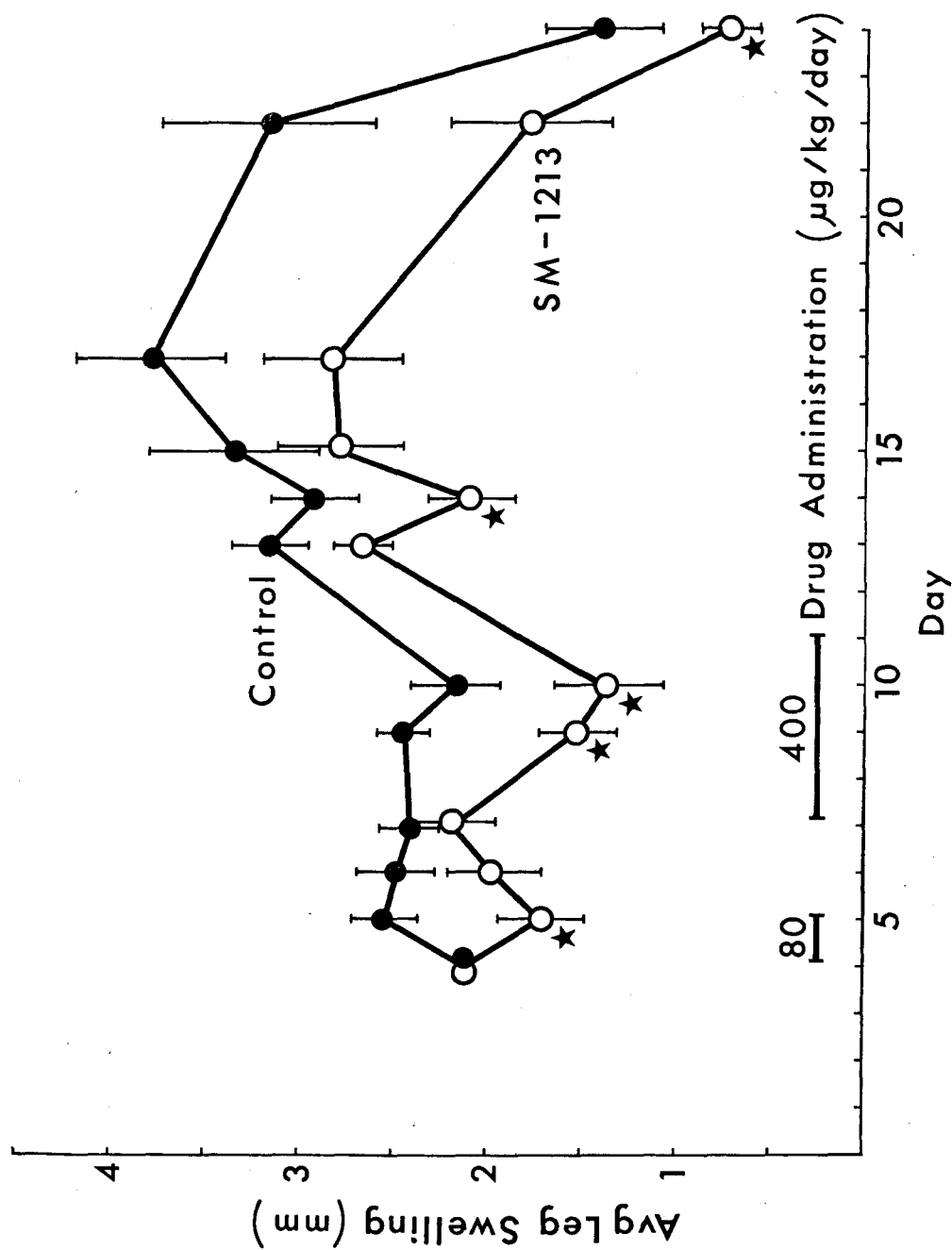


Figure 13. Effect of altered method of SM-1213 administration on mice infected intramuscularly with 1.2×10^8 Candida albicans cells.

Leg swelling was measured at the days indicated above with the aid of a Schnelltaster numerical dial caliper. Drug treated groups received 400 μ g SM-1213/kg/day by gavage for day 1 to day 4, 400 μ g SM-1213/kg/day in drinking water for day 7 to day 11, and 80 μ g SM-1213/kg/day in drinking water for day 17 to day 18. (●) Control, N = 12; (○) SM-1213 treated, N = 14. Points of significant differences in leg swelling between control and drug treated groups is indicated below by a star.

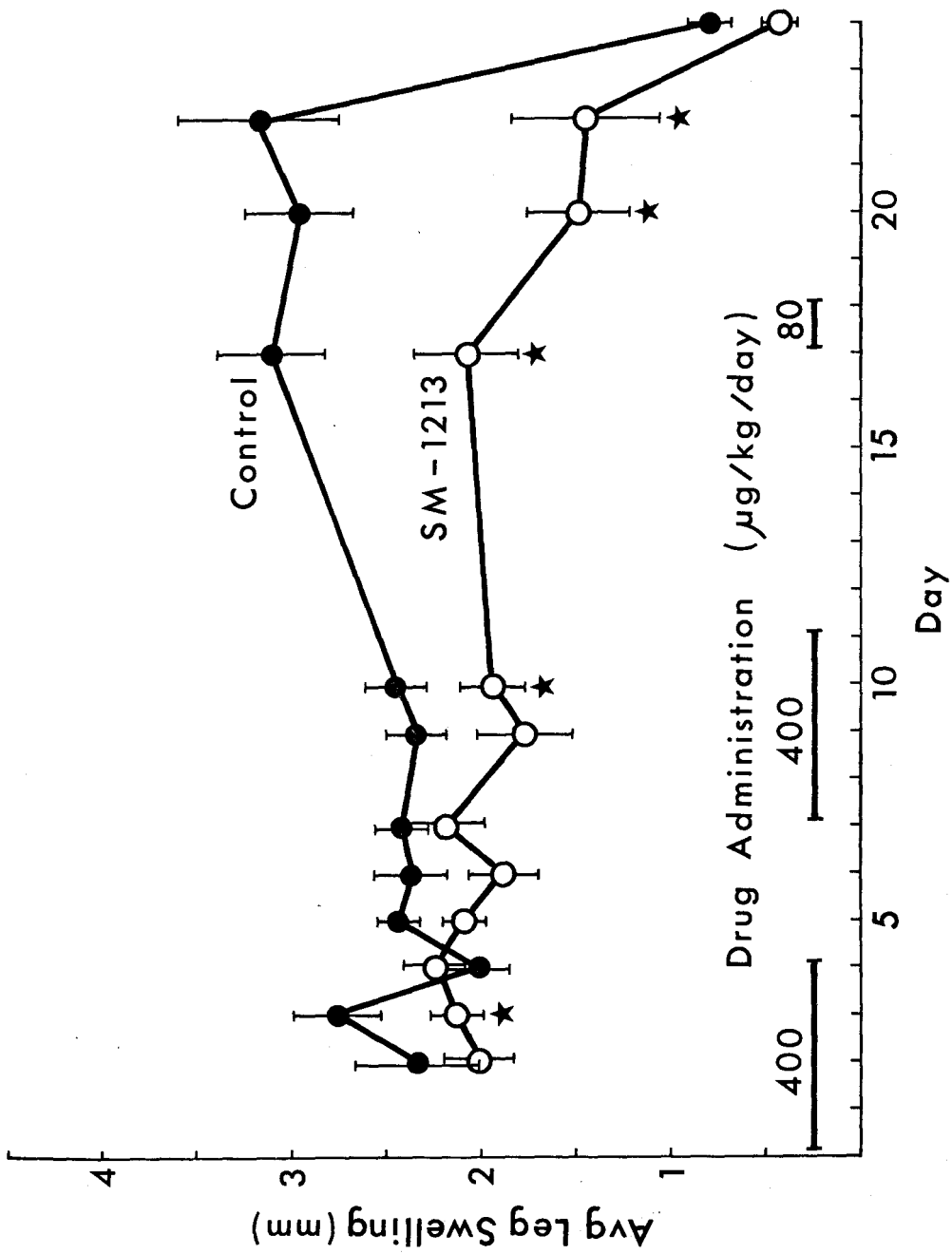
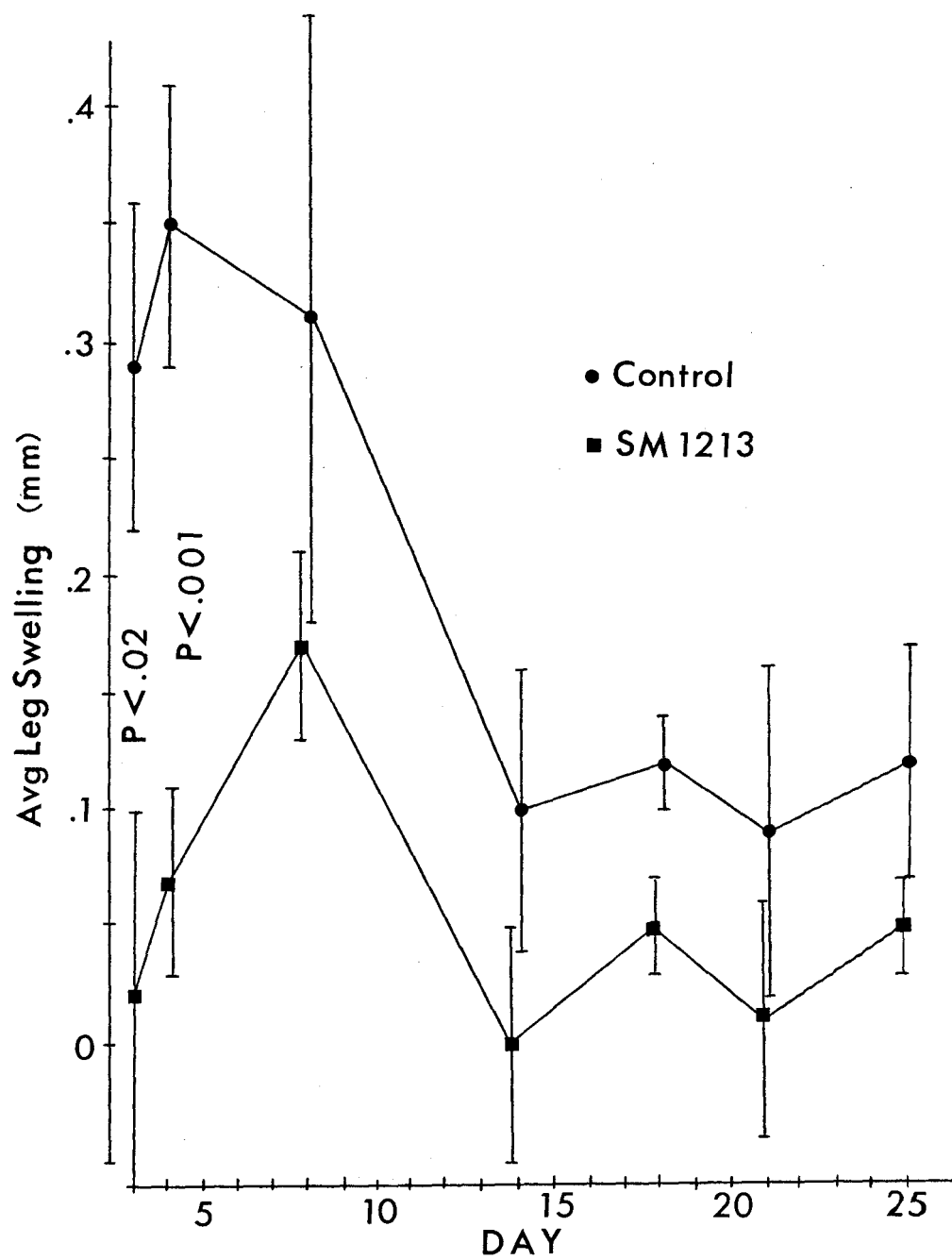


Figure 14. Sustained effect of SM-1213 treatment on mice reinoculated intramuscularly with Candida albicans.

CBA/J mice, originally infected i.m. with 1.2×10^8 Candida albicans cells were reinoculated in the contralateral leg with 1×10^5 Candida cells on day 57 post-initial infection. No additional drug therapy was given. (●) Control, N = 11; (■) SM-1213 treated, N = 10.



mice occurred without additional drug administration. Thus, SM-1213 therapy exerted an effect long after the initial infection had been resolved.

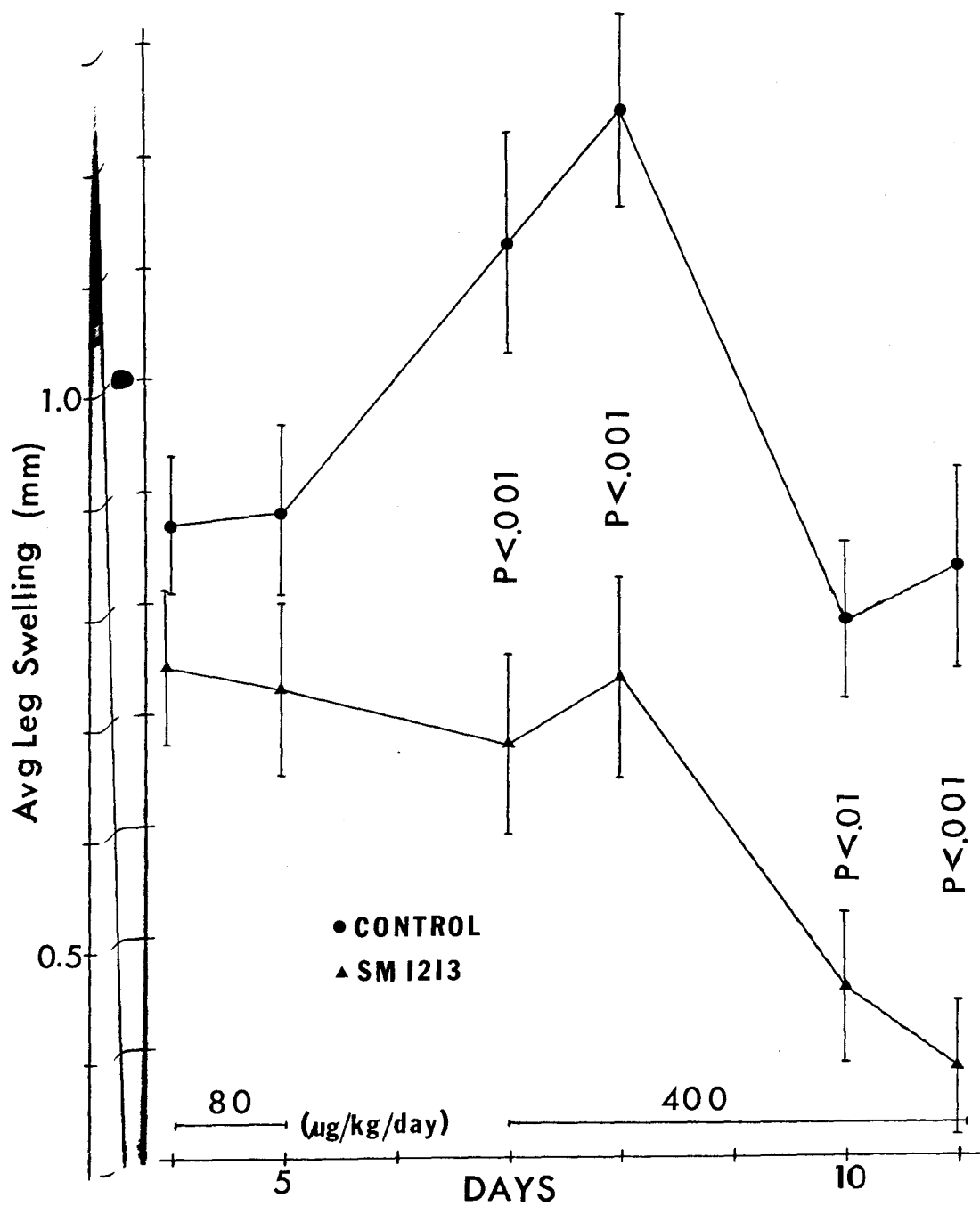
d. Leg Lesion Study #4: Effect of SM-1213 on the Course of Leg Lesions in Mice Infected Intramuscularly with 7×10^6 *Candida albicans* Cells

In an attempt to reduce systemic infection and to produce a localized, limited lesion, the dosage of Candida injected intramuscularly was reduced in the following two experiments. Male, CBA/J mice inoculated with 7×10^6 Candida cells per mouse were monitored regularly for leg swelling, as shown in Figure 15. Drug was administered in the drinking water for day 4 to 5 (80 $\mu\text{g/kg/day}$) and day 7 to 11 (400 $\mu\text{g/kg/day}$). Significant differences between control and drug treated abscess sizes were obtained with this Candida dosage and drug regimen.

Tissue homogenates of spleens and kidneys of both control and drug treated mice were negative for any candidal colony forming units on day 11. Before drug therapy, the abscess sizes of the SM-1213 treated test group were not significantly different from the controls; however, by day 7 the abscesses from the drug treated animals were significantly smaller than those of the controls.

Figure 15. Effect of SM-1213 on the course of leg lesions in mice infected intramuscularly with 7×10^6 Candida albicans cells.

Leg swelling was measured as in Figure 10. Drug treated groups received 80 μg SM-1213/kg/day orally in the drinking water from day 4 to 5 and 400 μg SM-1213/kg/day for day 7 to 11. (●) Control, N = 30; (▲) SM-1213 treated, N = 30.



e. Leg Lesion Study #5: Effect of SM-1213 on the Course of Leg Lesions and Metastasized Fungus in Mice Infected Intramuscularly with 7×10^6 *Candida albicans* Cells

This study repeated the experimental procedure of the last study (Leg Lesion Study #4); however, instead of homogenizing tissues, kidneys, spleens, or abscesses were cultured. This method was adopted in order to determine any presence of *Candida* in these tissues which may not have been detected by ordinary homogenization techniques. Culturing techniques allowed for the determination of candidal incidence or frequency in these tissues even when present in small numbers. Male, CBA/J mice were infected with 7×10^6 *Candida* cells per mouse intramuscularly; drug therapy was as in Leg Lesion Study #4.

Two sets of animals were sacrificed. The first set consisted of ten animals each from the control and drug groups which had the largest abscesses on day 8. The spleens, kidneys, and abscesses were removed and cultured. No *Candida* was found in the spleen, while 2 out of 10 control animals and 3 out of 10 drug treated mice demonstrated *Candida* present in kidney cultures. All mice in both test groups contained *Candida* within cultured abscesses.

The second set of animals which were sacrificed consisted of the next ten largest abscesses in each

experimental group as determined on day 11. Spleens and kidneys were not cultured from these animals, but abscesses were cultured. As above, all test animals demonstrated Candida present in abscess cultures (10 out of 10 each for control and drug treated groups).

Highly significant differences in abscess sizes were obtained between control and drug treated groups utilizing this drug regimen, similar to those obtained in the last leg inoculation study (see Figure 15).

Uninfected control mice (injected with PBS) had an average "abscess" size of 0.0 ± 0.01 over the entire experimental time period ($N = 9$).

Total numbers of mice used in this study to determine average abscess sizes were: Control, $N = 30$; drug treated, $N = 30$.

f. Leg Lesion Study #6: Effect of SM-1213 on the Course of Leg Lesions and Metastasized Fungus in Mice Infected Intramuscularly with 1.25×10^7 Candida albicans Cells

A log less Candida than was utilized in Leg Lesion Study #1 was inoculated into CBA/J mice intramuscularly (1.25×10^7 cfu). Mice were treated from day 4 to 5 with 80 $\mu\text{g/kg/day}$ and from day 7 to 11 with 400 $\mu\text{g/kg/day}$ in drinking water.

Significant differences were observed on day 5, 6, and 8 in average abscess sizes between control and

drug treated mice (see Figure 16). The overall average "abscess" size in uninfected control mice was -0.02 ± 0.02 (N = 8).

No differences in frequency of Candida occurrence in cultured abscesses from control and drug groups were noted on days of sacrifice (day 11 and 18). On day 11, 8 out of 8 control and 8 out of 8 drug treated mice had Candida present within leg abscesses. On day 18, 9 out of 9 control and 8 out of 8 drug treated mice had Candida present in leg lesions. No significant difference was observed between control and drug group kidneys cultured on day 11; however, the difference in frequency of Candida occurrence in the kidneys by day 18 was significant (see Table 15). It was proposed that drug treatment might allow for a more rapid clearance of Candida from the kidney late in infection upon the appearance of specific or opsonic antibody.

The next experiment to be discussed examines the long-term evolution of candidal infection in the kidney.

g. Leg Lesion Study #7: Effect of SM-1213 on the Quantity of Fungus in Leg Lesions and Kidneys of Mice Infected Intramuscularly with 1.25×10^7 Candida albicans Cells

The procedure of the last experiment discussed was repeated in this experiment with the exception of

Figure 16. Effect of SM-1213 on the course of leg lesions in mice infected intramuscularly with 1.25×10^7 Candida albicans cells.

Leg swelling was measured as in Figure 10. Drug treated groups received 80 μg SM-1213/kg/day orally in the drinking water from day 4 to 5 and 400 μg SM-1213/kg/day for day 7 to 11. (●) Control, N = 20; (■) SM-1213 treated, N = 20.

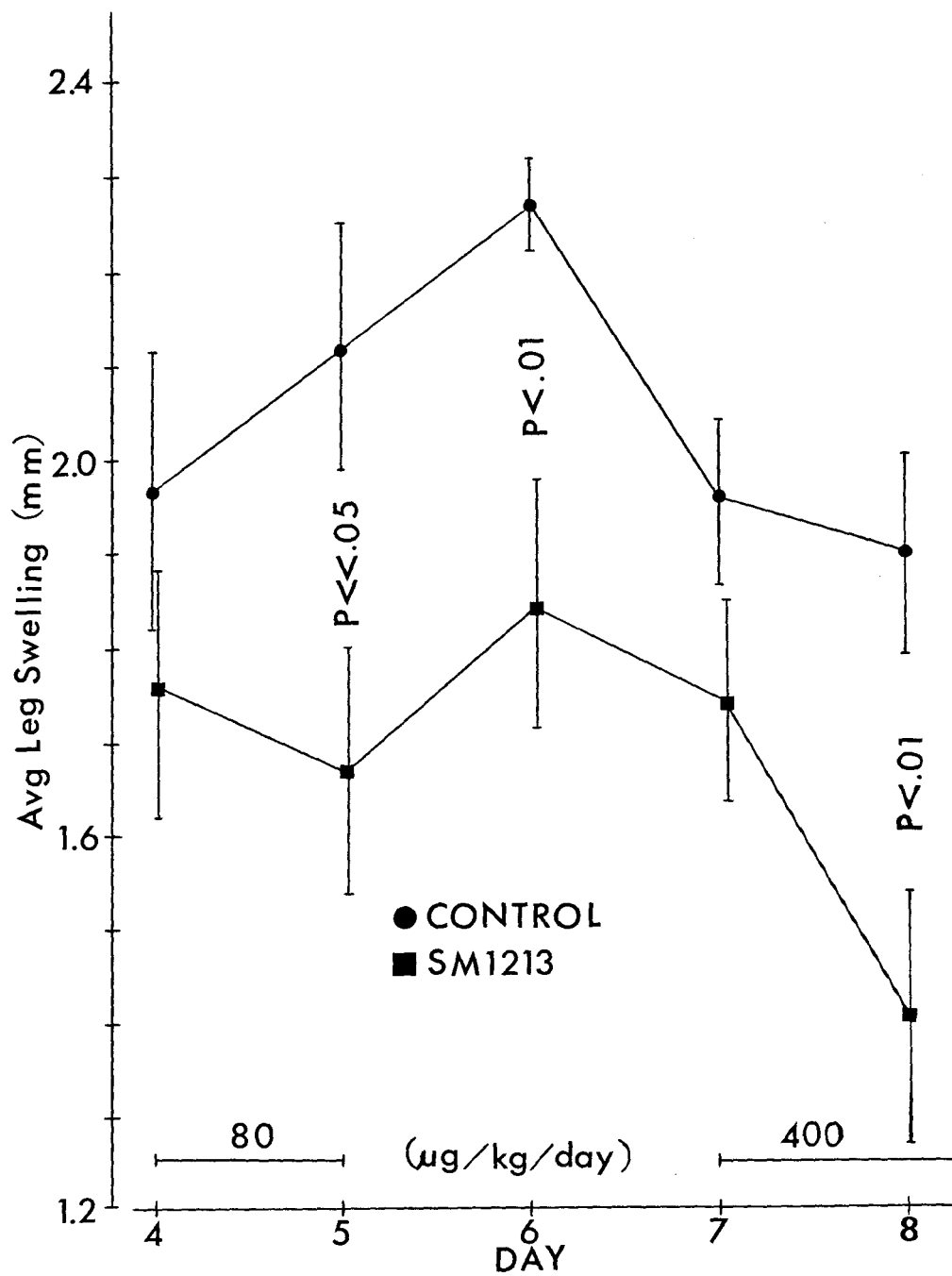


Table 15. Leg lesion study #6: Effect of SM-1213 on the frequency of Candida albicans present in kidneys of intramuscularly infected mice^a

Treatment group ^b	Frequency of kidneys containing viable <u>C.albicans</u> on day 11	Frequency of kidneys containing viable <u>C.albicans</u> on day 18
Control	3/8	4/9
SM-1213	5/8	0/8
Significance level ^c	N.S.	P < 0.05

a) Male, CBA/J mice were injected intramuscularly in the calf with 1.25×10^7 C.albicans cells on day 1. On day 11, and day 18 post-infection, mice were sacrificed; kidneys were excised, cultured, and assessed for the presence of colony forming units on SDA at 37°C.

b) Control mice received no drug; SM-1213 treated mice received 80 µg SM-1213/kg/day for day 4 to day 5 post-infection and 400 µg SM-1213/kg/day for day 7 to day 11 orally in drinking water.

c) N.S. = not significant ($P > 0.05$ by χ^2 analysis).

tissue homogenizations supplementing culturing techniques. A truly quantitative analysis could then be performed in addition to a qualitative analysis. Tissue homogenizations were performed on the kidneys at day 4, 11, and 18. In order to insure that the absence of Candida from the kidneys of drug treated mice on day 18 was not an artifact of homogenization, the kidneys of mice sacrificed on day 27 were cultured.

Kidney homogenates of control and drug treated mice showed no significant difference in Candida occurrence at day 4 (prior to treatment) or at day 11 sacrifices. By day 18, however, significant differences were observed (see Table 16). By day 27, culturing techniques showed that Candida had been cleared from the drug treated kidneys but not from the control kidneys (see Table 16 and Figure 17). Frequency of Candida occurrence is shown in parentheses in Figure 17 and 18.

The course of leg lesion development was much like that observed for the last experiment; however, abscess sizes were significantly different between control and drug treated groups on day 8 and 12 (see Figure 16). Abscess sizes were not significantly different on days of sacrifice and the total number of Candida present in abscesses and the frequency of candidal occurrence were not significantly different (see Figure 18). By day 27,

Table 16. Leg lesion study #7: Effect of SM-1213 on the frequency of Candida albicans present in kidneys of intramuscularly infected mice^a

Frequency of kidneys containing viable <u>C.albicans</u>				
	Day 4 ^b	Day 11	Day 18	Day 27
Control	3/4	3/10	7/10	3/11
SM-1213 ^c	4/4	2/10	0/10	0/10
Significance level ^d	N.S.	N.S.	P < 0.01	N.S.

a) Male, CBA/J mice were injected intramuscularly in the right calf with 1.25×10^7 C.albicans cells on day 1. On day 4, 11, 18, and 27 mice were sacrificed; kidneys were excised and homogenized on day 4, 11, and 18 and assessed for cfu on SDA at 37°C. On day 27, kidneys were excised, minced aseptically, and incubated in SDB for 24 hours at 37°C; cultures were then assessed for the presence or absence of cfu.

b) Day post-infection when mice were sacrificed.

c) Control mice received no drug; SM-1213 treated mice received oral drug therapy as described in Table 15.

d) N.S. = not significant ($P > 0.05$ by χ^2 analysis).

Figure 17. Effect of SM-1213 on the clearance of Candida albicans from the kidneys of mice injected intramuscularly with 1.25×10^7 colony forming units.

Mice were sacrificed at day 4, 11, and 18; kidneys were excised, homogenized, and assessed for cfu by plating serial dilutions of the homogenate on SDA. The ratio of the number of mice found to have Candida present in the kidneys to the total number of mice sacrificed is represented in parentheses. Control mice received no drug; drug treated mice received 80 μ g SM-1213/kg/day for day 4 to 5 post-infection and 400 μ g SM-1213/kg/day for day 7 to 11 post-infection orally in drinking water. The original number of mice in each test group was as follows: (●) Control, N = 40; (◆) SM-1213 treated group, N = 40.

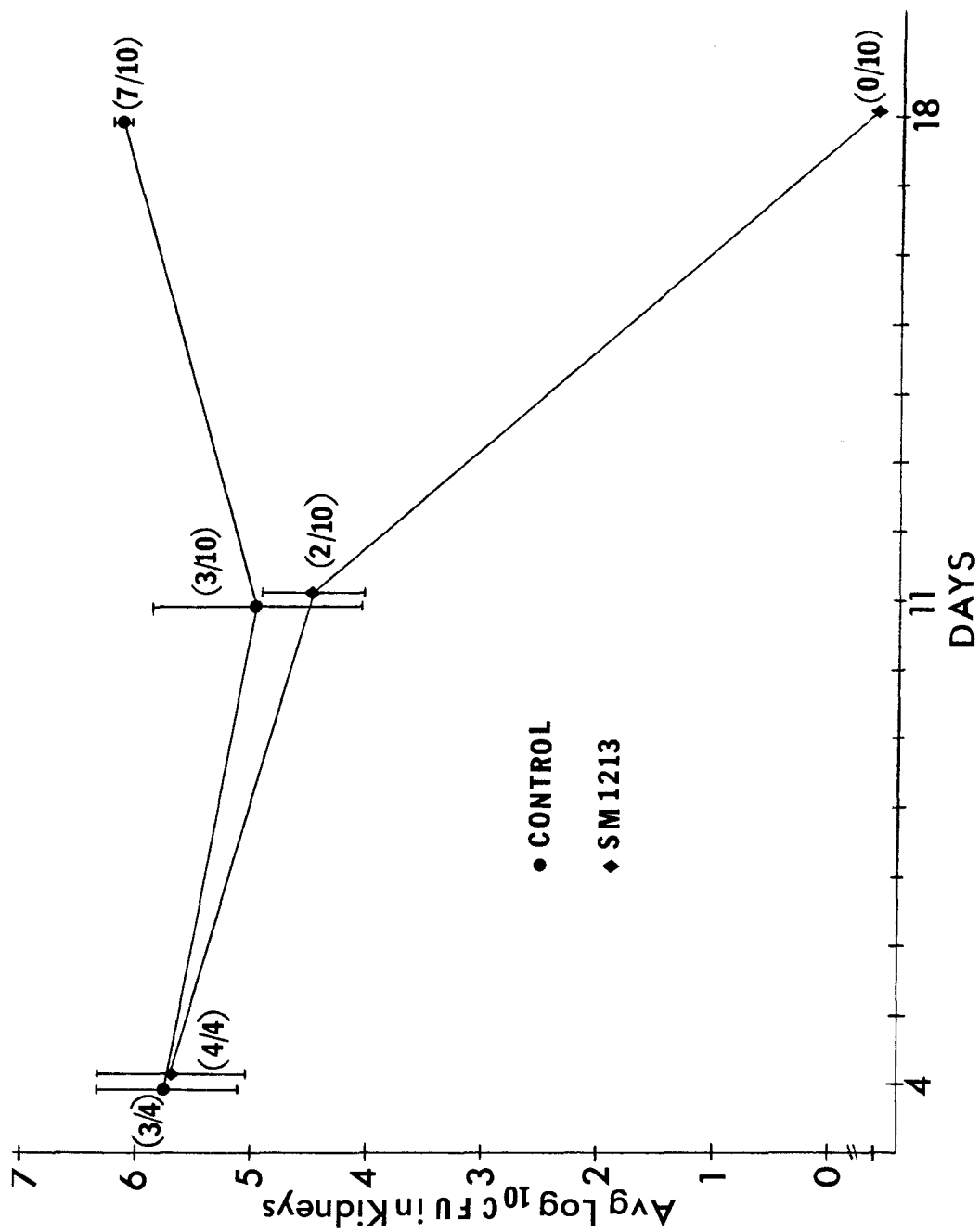
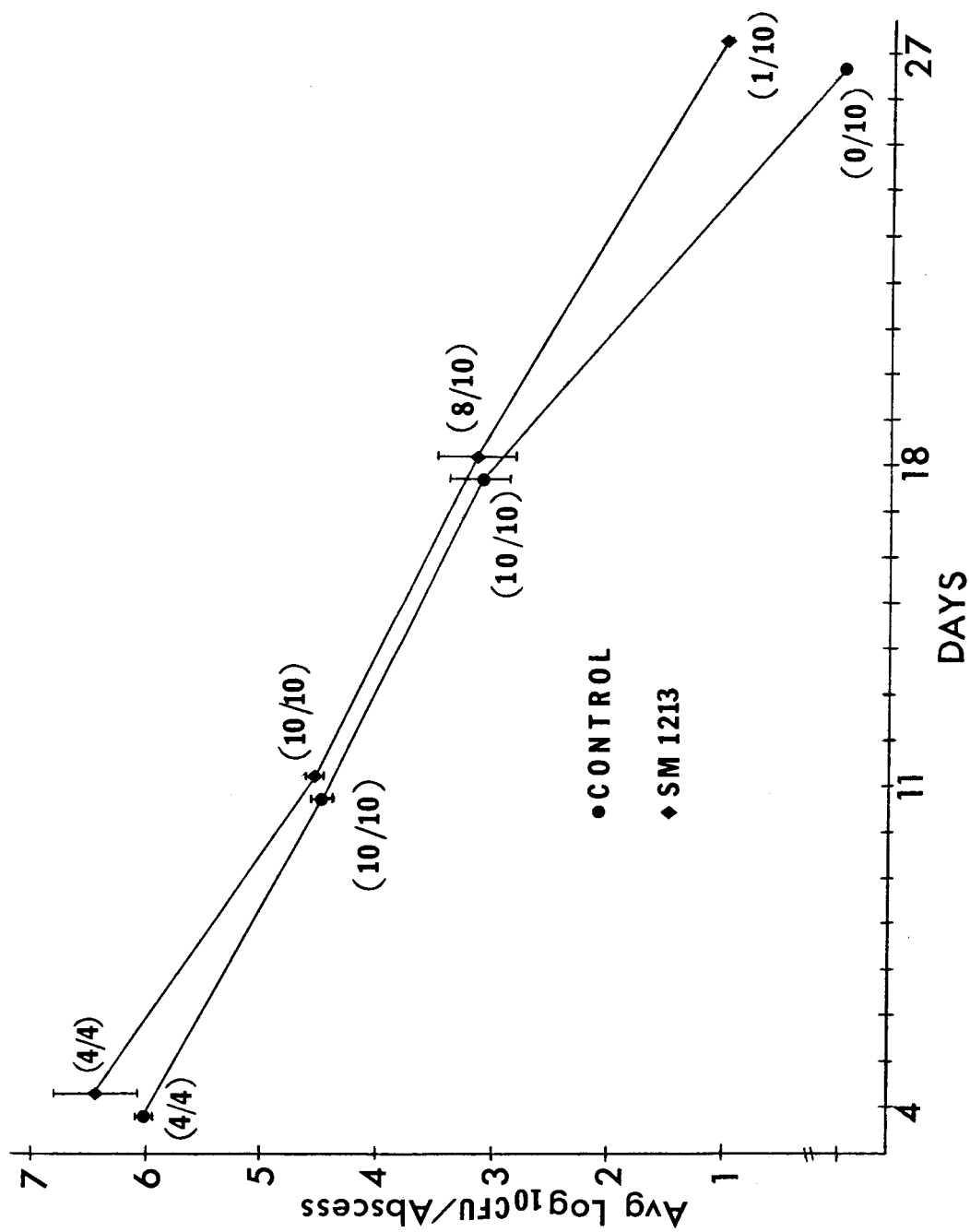


Figure 18. Effect of SM-1213 on the clearance of Candida albicans from the abscesses of CBA/J mice infected intramuscularly with 1.25×10^7 colony forming units.

Mice were sacrificed at day 4, 11, 18, and 27; abscesses were excised, homogenized, and assessed for cfu by plating serial dilutions of the homogenate on SDA. The ratio of the number of mice found to have Candida present in leg lesions to the total number of mice sacrificed is represented in parentheses. Control mice received no drug; drug treated mice received therapy as in Figure 17. The original number of mice in each test group was as follows: (●) Control, N = 40; (◆) SM-1213 treated, N = 40.



Candida had been cleared from the abscesses. Abscesses were homogenized at each sacrifice interval.

The overall average "abscess" size for uninfected control mice was -0.01 ± 0.02 from day 4 to day 27 (N = 10).

Days of sacrifice were pre-determined before the beginning of this experiment based on the previous study. In order to further examine the kidney involvement which occurred in Leg Lesion Study #6, day 11 and day 18 were once again chosen as days of sacrifice. Day 4 (prior to drug treatment) and day 27 were chosen as additional days of sacrifice. One difficulty which arose from such pre-determination was that sacrifices in this experiment happened to occur on days which did not show significant differences in abscess sizes between control and drug treated groups. For the analysis of kidney involvement, such sacrifice days were ideal. However, this interfered with an attempt to examine the relationship between the number of Candida within abscesses and abscess size. It would be of value to determine whether differences in the number of Candida present within abscesses occurred on days of significant abscess size differences between control and drug treated groups. No significant differences in Candida number occurred within abscesses which were not significantly different in size (see Figure 18).

Since sacrifice days did not include such days of significance, control and drug treated group abscess sizes were combined, as were the total average colony forming units per abscess, to determine the correlation of abscess size to number of Candida present within abscesses at the various days of sacrifice; the correlation is strong (Pearson's $r = 0.81$) for those animals sacrificed. The correlation between abscess size and total average colony forming units per abscess is also strong for the entire population of mice (including those not sacrificed) suggesting that, on the days of sacrifice, the animals sacrificed were representative of the entire study (Pearson's $r = 0.87$).

h. Leg Lesion Studies #6 and #7: Combined Data from Leg Lesion Studies of Mice Infected Intramuscularly with 1.25×10^7 Candida albicans Cells

The combined abscess data from Leg Lesion Studies #6 and #7 are shown in Figure 19. Significant differences in abscess sizes are demonstrated early in infection. Measurements of uninfected control mice are also shown at the bottom of the graph.

Table 17 delineates the highly significant combined data obtained on day 18 for the frequency of Candida present in the kidneys of mice in Study #6 and #7. No Candida was detected in uninfected control mice kidneys.

Figure 19. Effect of SM-1213 on the course of leg lesions in CBA/J mice infected intramuscularly with 1.25×10^7 Candida albicans cells.

Leg swelling was measured as in Figure 10. Control mice received no drug; drug treated mice received 80 μ g SM-1213/kg/day for day 4 to 5 post-infection and 400 μ g SM-1213/kg/day for day 7 to 11 post-infection orally in drinking water. The original number of mice in each test group was as follows: (●) Control, N = 60; (◆) SM-1213 treated, N = 60; and (▲) uninfected control (injected with PBS only in the right leg), N = 18. Data shown is compiled from two experiments.

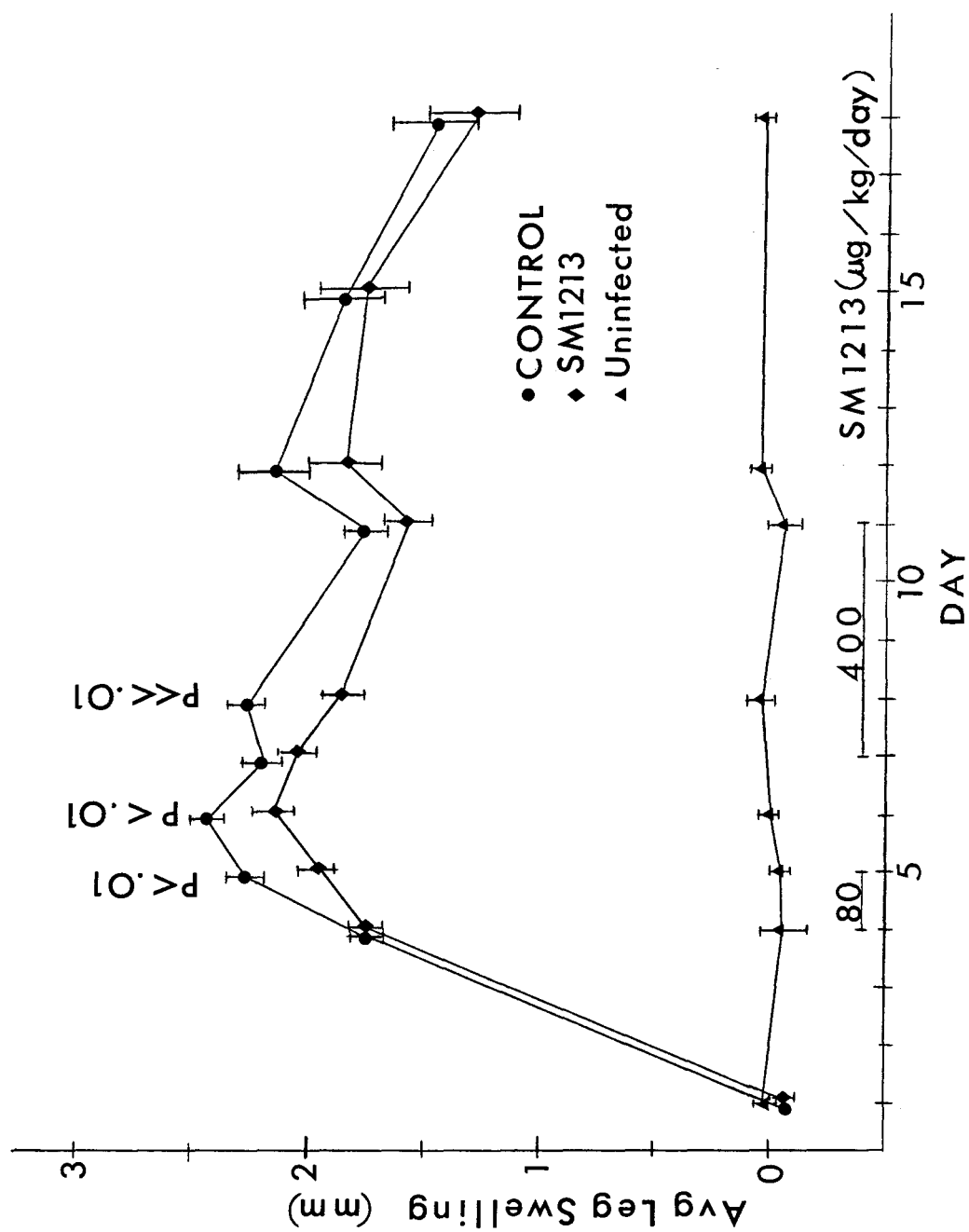


Table 17. Leg lesion studies #6 and #7: Effect of SM-1213 on the frequency of Candida albicans present in kidneys of intramuscularly infected mice^a

Treatment group ^b	Frequency of kidneys containing viable <u>C.albicans</u> on day 11 ^c	Frequency of kidneys containing viable <u>C.albicans</u> on day 18 ^d
Control	6/18	11/19
SM-1213	7/18	0/18
Uninfected control ^e	0/2	0/2

a) Male, CBA/J mice were injected intramuscularly in the calf with 1.25×10^7 C.albicans cells on day 1. On day 11 and day 18 post-infection, mice were sacrificed; kidneys were excised, homogenized, and assessed for cfu on SDA at 37°C.

b) Control mice received no drug; SM-1213 treated mice received therapy as described in Table 15.

c) Control mice versus drug treated mice, $P > 0.05$ by χ^2 analysis on day 11.

d) Control mice versus drug treated mice, $P < 0.001$ by χ^2 analysis on day 18.

e) Uninfected control mice were injected in the calf with PBS only on day 1.

Combined data for the frequency of Candida occurrence in abscesses revealed no significant differences between control and drug treated groups. On day 11, in both control and drug groups, 18 of 18 abscesses contained Candida; on day 18, in the control group 19 of 19 mice had recoverable Candida, while in the drug group 16 of 18 abscesses contained Candida. Uninfected control mice had no recoverable Candida in the kidneys or legs.

The following observations have also been made:

1) Preliminary histological studies conducted on day 11 post-infection in this laboratory indicated that the central area of the leg lesion contained predominantly polymorphonuclear cell types, while the periphery of the abscess consisted primarily of mononuclear cell types. Histological sections were stained with hematoxylin-eosin (H&E) for cell differentiation analysis.

2) Wright-Giemsa stained smears of abscess fluid showed the presence of predominantly polymorphonuclear cells and cell debris. In addition, periodic acid-Schiff-hematoxylin (PASH) stained smears of abscess fluid on day 4 and day 8 of infection showed numerous PAS positive yeast and pseudohyphal forms of Candida present. By day 15, the number of Candida present in the fluid was reduced as compared to day 4 and day 8.

3) Histological sections of the kidneys on day 11 post-infection from mice infected in the leg muscle with

1.25×10^7 Candida cells showed the presence of polymorpho-nuclear cells dispersed within the kidneys and both yeast and pseudohyphal forms of Candida were evident in the kidney pelvis as detected by PASH staining. Such a distribution of Candida is similar to that characterized by Winbald in the chronic type of renal candidiasis (66); that is, similar to mice surviving intravenous infection for over ten days.

CHAPTER IV

DISCUSSION

In Vitro Studies

No direct candidacidal or growth inhibitory effect due to the presence of SM-1213 in the incubation medium was demonstrated against the yeast or hyphal form of Candida albicans (Table 1 and 2). However, SM-1213 was found to be effective in inhibiting intracellular germ tube elongation of Candida (Table 3), as well as in reducing the percentage of viable cells remaining after phagocytosis (Table 6). This data suggests that an indirect candidacidal effect was exerted by SM-1213 mediated by the modulation of phagocyte cell processes.

It has been shown that guinea pig peritoneal exudate cells can suppress the growth of Candida albicans significantly when compared to the unimpeded growth of this organism in cell-free, tissue culture medium (Table 7). The ability of these cells to inhibit candidal germ tube elongation and to kill phagocytized Candida varies, depending upon the type of inducing agent used. Peritoneal exudate cells induced by mineral oil were less efficient than those elicited by glycogen in inhibiting germ tube elongation (Table 9), as well as in reducing the percentage

of viable Candida remaining after phagocytosis (Table 6). If these highly significant differences are due to the cell type produced, this would indicate that the polymorphonuclear cell, which predominates with glycogen induction, is better able to inhibit germ tube elongation and to reduce the viability of intracellular Candida than is the oil induced macrophage. A prediction such as this must be weighed, however, by the fact that the inducing agent itself may interfere with the fungicidal abilities of these cells. This may be particularly true in the case of paraffin oil-sated macrophages. However, other researchers (2,36,60) have also suggested that the PMN provides a better defense against candidal invasion than the macrophage.

In addition, the number of Candida which failed to produce hyphae intracellularly is significantly greater in the glycogen induced cells (Table 8). Apparently there is a mechanism whereby hyphal formation is inhibited within responding leukocytes.

The addition of serum to the test medium caused an inhibition in the average intracellular germ tube length regardless of the inducing agent used (Table 3). However, in the oil induced control group, the germ tube length was significantly decreased upon the addition of serum. Oil induced drug treated groups were not significantly

reduced upon the addition of serum. Since this phenomenon is pronounced in the oil induced studies, it is speculated that SM-1213 treatment may replace an unknown serum factor important to the macrophage in its ability to inhibit intracellular germ tube elongation. Hadden et al (20), working with paraffin oil induced guinea pig macrophages, determined that SM-1213 was capable of activating macrophages and increasing their phagocytosis of Listeria monocytogenes in relation to control samples. In addition, SM-1213 at a concentration of 0.12 µg/ml significantly increased intracellular killing of bacteria without affecting the viability of the macrophage monolayer.

In the glycogen induced studies, the differences in average germ tube length upon the addition of serum were not significantly different in parallel control or drug treated samples (Table 3). However, serum did produce a significant reduction in the number of Candida present which were able to transform into hyphae (Table 8). In other words, a significantly greater number of Candida remained in the yeast form when serum was added. Thus, the presence of serum in the incubation medium has been demonstrated to affect the morphology of the intracellular Candida. The retention of Candida in the yeast form may play an important role in reducing candidal pathogenicity (37,66).

The transformation of Candida from the yeast to the hyphal form has been suggested to be important in the spread of infection, particularly in the kidney (66). Winbald (66) observed that yeast cells evaded the immune system by invading intraluminally into the kidney tubuli in hyphal form. The fungi were protected in this environment and rapidly formed mycelial masses. Leukocyte actions which oppose the conversion of Candida from the yeast to the hyphal form contribute thereby to the prevention of the spread of infection.

Although the fungal hyphae appeared to penetrate the cell membrane of the peritoneal cells, it is possible that a thin cell membrane, invisible by standard microscopy, still surrounded the hyphal germ tubes. This may account for the appearance of a low viability while germ tube lengths were relatively long.

SM-1213 significantly enhanced the ability of both macrophages and neutrophils to inhibit intracellular growth of Candida (Figure 2, 4, 7). In addition, the percentage of viable Candida cells was reduced in those samples treated with the 0.001 $\mu\text{g/ml}$ dose (Table 6). An explanation for the candidacidal effect of SM-1213 treated phagocytes may lie in the activation of such cells by the drug.

Normal phagocytic activity is accompanied by activation of oxidative metabolism in phagocytic cells. A

result of this activation is the generation of oxidative intermediates such as superoxides, hydrogen peroxide, and hydroxyl free radicals (23). The production of such "active oxygens" contributes to the destruction of ingested bacteria, but also results in the escape of cytotoxic agents into the surrounding environment. This can produce a detrimental effect on surrounding healthy tissue or on the phagocyte itself. The result is an increase in inflammation. It has been shown that the anti-inflammatory effect of superoxide dismutase may be due to the protection of nearby tissues from damage by the superoxide radical itself or from its reaction with hydrogen peroxide and the production of secondary radicals (55).

Upon phagocytosis of particles, normal polymorphonuclear leukocytes generate chemiluminescence (light emission) which accompanies the activation of increased oxidative metabolism (23). Such chemiluminescence can be quantitated in a liquid scintillation spectrometer (65).

Studies were conducted by colleagues in this laboratory on glycogen induced guinea pig neutrophils. Through the use of chemiluminescence techniques, a more prolonged generation of microbicidal active oxygens was detected in 0.001 μ g SM-1213/ml treated samples than in controls following phagocytosis of Candida.

However, the activity of SM-1213 appears, in work being conducted at this time, to be biphasic in its effects

depending upon the level of drug administered (personal communication, P. Gordon). Glycogen elicited peritoneal neutrophils were examined by scintillation techniques for chemiluminescent activity representative of superoxide production upon the addition of 5×10^7 heat-killed Candida cells. Cells obtained from mice which had been treated with 16 mg SM-1213/kg in drinking water showed no increase in extracellular chemiluminescence, while controls increased by 12-fold in the presence of luminol amplifier. No differences were detected in intracellular chemiluminescence between control and drug treated animal cells. Thus, it is suggested that the decreased production of harmful extracellular oxidative components associated with decreased extracellular chemiluminescence produces an anti-inflammatory effect akin to that observed for superoxide dismutase (25). At the same time, the continued intracellular production of active oxygens allows for the destruction of micro-organisms. Thus, high doses (16 mg/kg) of SM-1213 treatment in vivo appear to be anti-inflammatory, while low doses in vitro (0.001 $\mu\text{g/ml}$) prolong chemiluminescence.

Also, Gordon et al (19) found that, in guinea pig macrophages elicited with paraffin oil and cultured at 37°C , 5% CO_2 for 24 hours in $\text{HI-WO}_5/\text{BA}_{2000}$ medium, 0.001 μg SM-1213/ml caused enhanced protein secretion (150%),

increased peroxidase excretion relative to controls, and increased acid phosphatase activity in macrophage phagosomes. Colleagues have proposed that SM-1213 may enhance the microbicidal activity of the myelo-peroxidase or related microbicidal systems dependent upon superoxide in these cell types at low levels of drug.

Studies conducted elsewhere have demonstrated the myelo-peroxidase system to be effectively candidacidal (32,35). In addition, in those patients whose immune systems have an impaired peroxidase system such as those receiving chemotherapy for metastatic cancer or those with advanced Hodgkin's disease or acute leukemia (36), as well as those with chronic granulomatous disease (3,33,34), while phagocytosis of Candida appeared to be normal, candidacidal capabilities were significantly reduced. Although the polymorphonuclear cell has been the primary focus of these studies, the mouse peritoneal macrophage has been demonstrated to display a similar anti-microbicidal potential (42).

Thus, while no direct fungicidal or fungistatic effects were observed against the yeast or hyphal form of C. albicans, the effects of SM-1213 in vitro can be explained as an action upon the phagocytic cells present and, as indicated above, are probably mediated through the modulation of intracellular enzymes and their secretion or through other activation products.

In Vivo Studies

In studies concerning mice injected intravenously with Candida, it was determined that the interval of drug administration is highly important to therapeutic effects (Table 13). Over a period of 24 days, cumulative mortality was significantly reduced in mice treated with SM-1213 for day 3 to day 4 post-infection. Earlier drug administration was less effective as the experiments progressed. It will be of importance, therefore, to examine the immune mechanisms which are involved at such time intervals, in that specific enhancement of fungicidal capabilities of SM-1213 may be dependent upon the activation of a particular population of cell types present after the initiation of infection.

A suitable model for studying experimentally induced candidiasis was determined to be the mouse leg lesion model. A consistent, self-limiting lesion, which could be easily measured using a Schnelltaster caliper, was produced by injection of an appropriate inoculum into the mouse calf muscle. The enlargement of the abscess produced served as an indicator of the pathogenesis of the infection. Unlike intravenously infected mice which frequently succumb to infection within two weeks, mice survive intramuscular infection and recover in three to four weeks allowing for the study of immune responses.

Histopathological studies conducted elsewhere (44) have indicated that such leg lesions are composed of a large number of yeasts early in infection with a modest cellular infiltrate during lesion development. As the lesion regresses, the number of fungal cells is reduced and the amount of host cellular infiltrate is increased. Histologically, the lesions have the characteristics of an abscess (44).

The mechanisms of protection from candidiasis have been investigated by Pearsall et al (43), who suggested that neutrophils, along with a contribution from humoral immune forces, may be sufficient to limit the infection in mouse leg lesions. In vitro studies reported in this thesis (Table 9) have suggested that, indeed, neutrophils may be of primary importance. In addition, the presence of normal guinea pig serum enhanced inhibition of candidal germ tube formation (Table 8), suggesting an influence of humoral factors [i.e. Candida albicans is so ubiquitous that even "normal" serum may contain antibodies to this organism (28)].

Pearsall et al (43) also demonstrated that passive transfer of immune serum gave a significant degree of protection against lesion development in murine candidiasis. The transfer of resistance by sensitized lymphoid cells gave negative results, although cutaneous delayed hypersensitivity was transferred by these cells.

There is continued controversy over the role of humoral and cellular immunity in candidal infections.

In studies of experimental systemic candidiasis in mice intravenously injected with Candida albicans, complement intact mice survived significantly longer than complement deficient mice. It was speculated that longer survival was due to the earlier and greater mobilization of phagocytic cells (41). Ray (46) found the formation of neutrophilic microabscesses in C5-intact mice infected intraepidermally with Candida. C5-deficient mice, however, developed no inflammatory response and allowed the proliferation and cutaneous invasion of Candida. In addition, in vitro studies showed Candida-induced neutrophil chemotaxis and complement activation.

Giger et al (14) suggested that the acute inflammatory response due to cutaneous infection of mice with Candida albicans stimulated the production of precipitating antibody and conferred protection against subsequent intravenous challenge due to predominantly humoral response. In addition, Rogers and Balish (51) demonstrated that, in disseminated candidiasis, renal histopathology revealed a prominent role for the polymorphonuclear leukocyte, but not for mononuclear cells in the defense against systemic infection.

The polymorphonuclear cell was also important in patients with chronic granulomatous disease, where

susceptibility to candidal infection resulted from an abnormal function in neutrophils (33).

However, the cellular immune response cannot be overlooked as an important element in the resistance to candidal infection. This was especially evident in those individuals with chronic mucocutaneous candidiasis who were able to produce antibody (34) but displayed abnormalities in the cellular immune system (28,31). Successful treatment of such patients with injections of transfer factor or transfusions of immunocompetent lymphocytes support a role for thymus-dependent cellular immunity in protection against candidiasis (28,29).

Controversy has arisen, however, due to evidence that congenitally athymic mice with deficient thymus-dependent cell mediated immunity may actually have a greater capacity than normal littermates to prevent the growth of Candida in the kidneys and to clear Candida from the liver (52).

Reconciliation between the humoral and cellular defense theories has been suggested in both cutaneous and disseminated candidiasis. Sohnle et al (59) compared the pathology in chronic and acute candidal lesions in guinea pigs infected cutaneously with Candida. Acute lesions (produced by placing occlusive dressings over injection sites) were characterized by intense polymorphonuclear infiltration. In contrast, chronic lesions

(produced without occlusive dressings) generally presented an intense dermal infiltrate of mononuclear cells. However, such mononuclear infiltration was demonstrated in previously immunized guinea pigs, while the acute PMN infiltrate occurred with or without previous exposure to Candida. In addition, a mononuclear response could be acquired by those animals receiving transferred lymphoid cells and clearance of Candida was postulated to be associated with lymphokine release. It would be speculated that, if normal and nude littermates had been previously exposed to Candida (immunized), the accompanying cellular immune protection afforded to the kidney upon antigen challenge would likely be greater in normal than in nude littermates.

In systemic candidiasis, Miyake et al (40), in experiments with intravenously infected normal, nude (athymic), lethally irradiated, or immunized mice, suggested that non-immune phagocytosis was required early in infection, while T-cell mediated immunity was required at late stages in infection in resistance to candidiasis. In this case, Candida was shown to decline in the liver of both nude and normal littermates, but increased in the kidneys of nude mice. It was suggested that, since there are fewer fixed macrophages in the kidney than the liver, an accumulation of macrophages in the kidney

induced by T-lymphocyte dependent cellular immunity may be necessary for an effective defense against Candida in this organ.

In addition, in lethally irradiated normal or nude AKR mice, in which phagocytic functions were decreased, increases in cfu were detected in both the liver and the kidneys from initiation of infection. Macrophages from irradiated mice could not as effectively inhibit the growth of intracellular Candida as could normal macrophages, as demonstrated by the more frequent formation of mycelia.

Lymphoid cell transfer from AKR mice immunized intramuscularly with Candida demonstrated a 20% survival upon intravenous challenge, while few mice receiving non-immune lymphocytes survived by day 20 (40). Prior to day 7, the number of cfu in the kidneys of both groups of mice were similar. By day 7, however, mice receiving immune cells showed a reduction in cfu in the kidney, although both groups demonstrated similar cfu in the liver. Normal or hyperimmune serum was ineffective in reducing mortality or liver or kidney involvement.

It appears, therefore, that, while Pearsall (43) found no lymphoid protection from abscess formation, cellular immune elements as well as phagocytic elements are involved in the protection of the kidney. In

addition, serum transfer does not afford protection in the kidney, while transfer of lymphoid cells does (40).

Thus, both the PMN and the macrophage, as well as other humoral and cellular factors, play important roles in resistance to candidal infection. It would be of interest to examine the effects of immune serum on the in vitro macrophage culture system in place of normal, whole serum previously utilized. Certainly both humoral and cellular elements are important in the defense against candidiasis and warrant further investigation.

Mice injected intramuscularly with C. albicans and subsequently treated with SM-1213 showed a significantly higher weight gain than controls and a weight gain pattern more closely resembling uninfected controls (Figure 11). This data, along with the lower incidence of Candida within the spleens of drug treated mice (Table 14), suggests that SM-1213 treated animals were better able to clear Candida which had spread systemically. Perhaps similar immune mechanisms are involved in the clearance of Candida from visceral organs in intravenous studies, since mice receiving a similar drug regimen and dose of drug as in the original leg lesion study demonstrated the lowest cumulative mortality after intravenous infection (Table 13).

The successive low dose (80 $\mu\text{g/kg}$) followed by high dose (400 $\mu\text{g/kg}$) drug regimen has been proven to be

effective in reducing leg swelling in drug treated mice as compared to control animals (Figure 12, 15, 16) regardless of the inoculum size tested. In an experiment where drug was initially administered by gavage (Figure 13), followed by drinking water administration of SM-1213, significant differences in abscess sizes were also observed between control and drug treated mice. Gavage therapy was discontinued due to the revelation that gavage therapy could cause alterations of immune responses as a result of the additional stress received during this procedure and due to the discovered rapid clearance of SM-1213 in the urine when administration was limited to a single, heavy dosage per day rather than small doses three to four times per day as received in drinking water therapy (personal communication, P. Gordon and J. Majde).

While low doses of drug (80 $\mu\text{g}/\text{kg}$) given early in infection enhanced clearance of Candida from the spleen after leg inoculation (Table 14) and reduced mortality in intravenous studies (Table 13), larger doses (400 $\mu\text{g}/\text{kg}$) effected a reduction in leg swelling relative to controls (Figure 12, 15, 16), while generating neither an increase nor a decrease in the number of recoverable Candida obtained within the abscesses (Figure 18). This suggests that larger doses of SM-1213 are anti-inflammatory, since smaller abscesses apparently contained a similar number of Candida organisms.

This has been borne out subsequently by experiments conducted by colleagues in this laboratory. In studies employing peritoneal exudate cells from SM-1213 treated mice, a biphasic dose response was noted. Twenty-four hours prior to cell harvest, either 10^7 Candida cells or 20 mg of glycogen were injected into the peritoneum. In mice receiving 40 to 80 μ g SM-1213/kg doses for short duration (2 days), a dose dependent increase in total leukocyte count and polymorphonuclear leukotaxis into the peritoneum was revealed. However, in mice receiving 800 μ g SM-1213/kg doses or greater for longer intervals (3 to 60 days), a dose dependent decrease occurred in PMN and leukocyte migration into the peritoneum. However, such a decrease was associated with an increase in microbicidal abilities in the responding cells.

Upon reinoculation of mice in the contralateral leg (Figure 14), significant differences were obtained early after Candida injection between previously drug treated and control CBA/J mice. The appearance of smaller abscesses in the SM-1213 treated mice may be explained as the consequence of an enhanced immunity, since no further drug therapy was administered during the reinoculation interval. According to the work of Pearsall and Lagunoff (43), such protection would be indicative of an enhanced humoral response. However, these authors do not exclude

the possible contribution of immune lymphocytes in the resolution of abscesses based on the available evidence. Further studies conducted by Pearsall et al (45) have demonstrated a direct anti-fungal activity of lymphokine preparations in vitro and suggest that the decrease in fungi in the leg lesions after the second week of the primary infection in vivo may be partly due to the contribution of lymphocytes present in the lesions.

Little or no systemic spreading of Candida occurred in those studies where 7×10^6 Candida cells were injected intramuscularly. For the examination of immune mechanisms in convalescing mice, such a limited, localized lesion should prove ideal. In addition, since this inoculum gives reproducibly significant differences in abscess size between control and drug treated mice (Figure 15), future studies examining the relationship of abscess size to candidal cfu per abscess should utilize this inoculum.

While abscess sizes were reduced in drug treated mice inoculated with 1.25×10^7 C. albicans (Figure 16), no reduction in Candida count was observed on sacrifice days as determined by tissue homogenization procedures. However, no sacrifice days occurred on days of significant abscess size differences. It is unlikely that an erratic clearance of Candida would occur within the abscesses and it is more probable that a steady, progressive clearance

would occur as indicated in Figure 18. The frequency of candidal occurrence, as well as the total number of recoverable Candida, was similar in both the control and drug treated groups on days of sacrifice.

Apparently, on days where no significant difference in abscess sizes was observed, a direct correlation existed between abscess size and the number of Candida cfu per abscess. However, few data points were used to determine this correlation and a greater number of points should be obtained for analysis before this relationship can be stated unequivocally. Also, points occurring where significant differences are present between control and drug treated abscess sizes must be analyzed in the future.

A strikingly significant difference in the frequency of Candida obtained from the kidneys of mice in these studies was obtained, whether culturing or tissue homogenization techniques were employed. Such differences are related to an enhanced clearance of Candida from the kidneys of drug treated mice, since no difference in candidal frequency was observed until day 18 post-infection (Table 16). In addition, the total number of Candida present, as well as the frequency of occurrence, was greater in the kidneys of control mice (Figure 17).

Current studies by colleagues in this laboratory have suggested a role for SM-1213 as a mitogenic agent.

Peritoneal exudate cells from mice treated with SM-1213 have shown a significantly greater uptake of radioactive thymidine relative to controls, especially in the presence of phagocytic activity.

The effect of SM-1213 on the reduction of extracellular active oxygen production suggests an explanation for its ability to promote mitogenicity. The production of active oxygens by PMN leukocytes normally accompanies the process of phagocytosis as a result of an increase in the oxidative metabolism of these cells upon contact with a stimulus such as Candida albicans or zymosan (65). In mouse peritoneal cells which demonstrate chemiluminescence accompanying the phagocytosis of heat-killed Candida, prior in vivo SM-1213 treatment served to protect peritoneal phagocytes and lymphocytes from damage by reducing extracellular active oxygens in vitro.

Damage to lymphocytes and phagocytes by active oxygens has been discussed by other researchers (8,54). Damage to fixed tissue also accompanies the production of active oxygens by stimulated neutrophils (53) and by active oxygens associated with an arthritic response (25).

It may be that SM-1213 protects both fixed tissues and lymphocytes participating in an inflammatory response indirectly by reducing the release of active oxygens from neighboring polymorphonuclear leukocytes. Lymphocyte

mitogenicity could therefore be preserved in these protected cells due to the indirect influence of SM-1213. Thus, SM-1213 treatment reduces lymphoid cell damage at the site of inflammation in vivo, thereby preserving lymphocyte mitogenicity in vitro and therefore presumably in vivo. Such preservation could allow for the enhanced clearance of Candida from the kidney of mice treated with SM-1213, as demonstrated in Figure 17 and Table 17.

In light of the possible mitogenic effect SM-1213 may exert upon lymphocytic cells, as well as its ability to activate macrophages in tissue culture, a cellular role is also indicated in the enhanced clearance of Candida from the kidney in SM-1213 treated mice. Although histological sections of the kidney from mice injected with 1.25×10^7 Candida cells intramuscularly showed predominantly PMN leukocytes present, no sections were made after the second week of infection. Therefore, if a greater accumulation of mononuclear cells were to occur after the second week of infection [as in the abscess model proposed by Pearsall (44)], such an accumulation may not have been detected. However, a small number of mononuclear cells were present in the kidney and at the site of abscess formation as observed by this researcher and by others (44).

Cellular as well as humoral factors have also been suggested for the clearance of systemically spread Candida

from the kidneys after intravenous injection. Corbel and Eades (9) examined the relative susceptibility of CBA and New Zealand Black (NZB) mice, a strain known to spontaneously develop deficiencies in cell-mediated immune function (24) to systemic candidiasis. Six weeks after intravenous injection of Candida, CBA mice developed predominantly mononuclear reactions with lymphocytes and plasma cells readily apparent in the kidneys, while NZB mice developed progressive, disseminated infection with essentially non-specific PMN leukocytes and mononuclear phagocytes but no lymphocytes present in the kidneys. The greater susceptibility of NZB mice to Candida spread was suggested to be due to reduced ability to mount a specific T-lymphocyte dependent response to the fungus.

Rogers and Balish (51) have suggested that humoral factors may be involved in the clearance of Candida from the kidneys of mice. Histological studies conducted by these authors have indicated that, for the first 21 days after infection, the predominant cell type was the PMN leukocyte. Few PMN's were observed at day 7 post-infection; by day 14, however, a dramatic increase in the number of PMN's present occurred with the appearance of a few monocytes and almost no lymphocytes. By day 21, PMN's still predominated, and a small number of monocytes and lymphocytes were present. It was suggested that, if

the PMN leukocyte was responsible for the eventual clearance of Candida from the kidney, the possibility of antibody-mediated protection by antibody plus complement mediated chemotaxis of PMN cells would be consistent with the observed data. However, the possibility of direct lymphokine mediated Candida clearance (45,56), as well as the influence of thymus derived cells upon the function of the PMN itself (7,49), was also suggested. Winbald (66) and Hurley and Winner (26) also suggested a role for the PMN leukocyte early in murine kidney infection (prior to day 6 post-infection).

Finally, in histological studies conducted by Miyake et al (40), irradiated mice, with decreased phagocytic function, exhibited large numbers of mycelial Candida in kidney sections with little cellular infiltrate present two days post-intravenous infection. Normal or nude mice with unimpaired phagocytic function showed little Candida present in the kidneys. Non-immune phagocytosis is therefore important early in infection.

By 20 days post-infection, normal mice exhibited a large cellular infiltrate with an accumulation of macrophages and little or no Candida was observed. In nude mice, however, little cellular infiltrate was present, while large numbers of Candida occurred in the kidney pelvis.

Thus, non-immune phagocytosis confines Candida early in infection, while, at day 20 with the contributions of cellular and humoral elements, Candida can be cleared from the kidney.

SUMMARY

SM-1213, a substituted monosaccharide with immunomodulatory activity, was examined for its effects on pathogenesis and immunity in experimentally induced Candida albicans infections in laboratory animals and for its effects on the intracellular germ tube elongation and viability of Candida in tissue culture.

In vitro studies utilizing non-immune guinea pig peritoneal PMN and macrophages indicated that the glycogen induced PMN leukocyte was better able to suppress the intracellular germ tube elongation of Candida albicans than was the oil induced macrophage. The addition of whole, normal guinea pig serum to the tissue cultures reduced the average germ tube length of the oil induced control sample significantly. The drug treated samples were not significantly affected by the addition of serum. It was speculated that SM-1213 was mimicking a serum factor important to the macrophage for its inhibition of germ tube elongation. In glycogen induced cells, the addition of serum to the tissue culture medium increased the number of Candida remaining in the yeast form. This factor may play an important role in limiting the spread of Candida attributed to the formation of invasive, hyphal

forms. The addition of SM-1213 reduced both the average germ tube length of intracellular Candida and the percentage of viable cells remaining after phagocytosis. This effect was particularly significant at the 0.001 µg/ml dose level.

In order to determine the effects of SM-1213 on parameters of host defense against systemic candidal infection, experiments were conducted on mice infected intravenously with Candida albicans. In systemically infected Ha/ICR mice, SM-1213 could reduce cumulative mortality when administered for specific 24-hour intervals in the drinking water. A dosage of 80 µg/kg/day was highly effective when administered from day 3 to 4 in infection and was less effective when given earlier. This suggests that immune mechanisms may be most sensitive to regulation three days post-infection and that treatment prior to infection is unnecessary, unlike other immunopotentiating drugs.

An injection of 1.25×10^7 Candida albicans cells into the leg muscle of CBA/J mice gave a consistent, self-limiting lesion which could be easily measured and which served as an indicator of the pathogenesis of infection. Histopathology conducted here and by others has indicated that resistance to infection involves the early mobilization of non-immune phagocytes in both the kidney and the

abscess. The predominant cell produced early after infection was the PMN, which has been shown in vitro to be an effective inhibitor of candidal spread.

Under proper drug protocols, consisting of successive doses of low (80 $\mu\text{g/kg/day}$) followed by high dose (400 $\mu\text{g/kg/day}$) treatment, significant differences in abscess sizes between the drug and control groups were observed early after infection (day 5 to 8). If non-immune phagocytes are the primary cell type present early in infection, SM-1213 can reduce inflammation produced by such cells when administered in this manner. The number of Candida recovered from the abscesses of drug and control groups were not significantly different on days of sacrifice. Therefore, while SM-1213 reduces inflammation, it does so without impairing net fungicidal capabilities of the responding leukocytes.

The possible anti-inflammatory effect of SM-1213 may serve to protect proto-immune cells from inflammatory injury so that, when cellular factors are mobilized later in infection, fungal clearance is more rapid and complete. Humoral factors may also play an important role in kidney as well as abscess pathology. Protection of cutaneously immunized mice upon subsequent intravenous challenge has been attributed to a predominantly humoral response. Antibody-mediated transfer of immunity against leg lesion

development has been demonstrated by others and a possible direct candidacidal effect due to lymphokine production has been proposed. The mechanism of Candida clearance from the kidneys of systemically infected mice has been the subject of much controversy; however, early mobilization of non-immune phagocytes followed by subsequent T-cell mediated immunity appear to be important factors.

When larger doses of Candida were used as leg inocula (1.25×10^8), the greatest differences in abscess sizes between drug and control groups occurred late in infection. If clearance of Candida from the abscesses late in infection is due to cellular elements (lymphokine production in particular), SM-1213 could also reduce inflammation caused by this system late in infection. Upon reinoculation of recovered mice, animals which had been treated with SM-1213 during the course of the primary infection produced smaller abscesses than did the control group. Therefore, SM-1213 enhanced the development of immunity to a second infection without additional drug treatment. In addition, the more complete clearance of Candida from the spleen in drug treated mice also suggests an enhancement of immune mechanisms without an enhancement of inflammation. The mitogenicity of SM-1213 would support an opsonin or cellular immune promoted enhanced clearance of Candida from the kidneys.

Finally, when an inoculum of 7×10^6 Candida cells was injected intramuscularly into mice, a more localized, limited lesion occurred with little or no systemic spreading of Candida. This suggested that, if an inoculum was small, non-immune phagocytosis was able to limit the spread of Candida early in infection more efficiently than when larger inocula were used. Abscesses produced as a result of the lower inoculum were smaller and regressed earlier after infection. Highly significant differences were observed in abscess sizes between control and drug treated groups when successive low and high drug doses were administered.

In conclusion, the above studies, along with those of others, have demonstrated that SM-1213 can enhance the host's ability to produce an anti-candidal effect which may be facilitated by an indirect, anti-inflammatory action.

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APPROVAL SHEET

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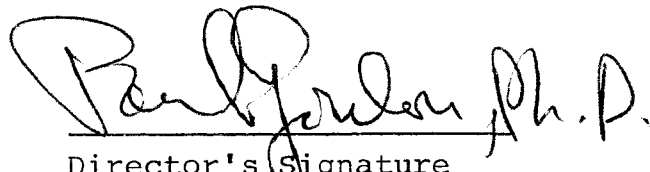
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The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

April 15, 1980

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