Some antifungal activities of guinea pig peritoneal exudate cells against Sporothrix schenckii

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SOME ANTIFUNGAL ACTIVITIES OF
GUINEA PIG PERITONEAL EXUDATE CELLS
AGAINST
SPOROTHRIX SCHENCKII

by

Daryl S Paulson
B.A., University of Montana, 1972
Presented in partial fulfillment of the requirements for the degree of
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Some antifungal activities of guinea pig peritoneal exudate cells against *Sporothrix schenckii*. (46 pp.)

Director: John J. Taylor

Six guinea pig models were treated with *S. schenckii* conidia in various combinations to ascertain the effects of peritoneal exudate cells (PECs) on these conidia, to find whether or not those PEC effects were fungicidal or fungistatic to *S. schenckii* conidia, and to determine the effects hydrocortisone acetate compromise had upon PEC activity. Phagocytic responses were measured using these 6 animal models which included animals immunized with viable conidia, animals immunized with non-viable conidia, untreated controls, animals compromised with cortisone but not immunized, animals both compromised and immunized with viable conidia and finally animals both compromised and immunized with non-viable conidia. Statistical analyses were used to compare the groups. It appears that animals immunized with viable conidia are more efficient in clearing these fungi via phagocytosis, that phagocytic rate and fungicidal properties are positively correlated, and that cortisone compromise, achieved through repeated cortisone injections into these animals, reduces both phagocytic ability and fungicidal properties to PECs.
ACKNOWLEDGEMENTS

This work is dedicated to my two friends and fellow Marines, John Burke and John Osterhaus, who were killed in action at An Hoa Fire Base, South Vietnam, on Operation Taylor Common during the Tet offensive, 1968. Special thanks to Dr. John J. Taylor for his guidance in this work and to the other members of my committee, Drs. Mitsuru J. Nakamura, Clarence A. Speer, and Todd G. Cochran. I also extend my appreciation to Dr. Jon A. Rudbach for his valuable assistance in opening the initial computer account for the Microbiology Department, which allowed me to greatly sophisticate the statistical computations required in the multivariate analyses of this work. Also my thanks to Drs. Rudy Gideon and Don O. Loftsgaarden of the Mathematics Department for their statistical consultations.
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CHAPTER I

INTRODUCTION

A. BASIC REVIEW MATERIAL

Host/Parasite Relationship

For man and other animals, parasitic microbial infection is the invasion of the host's tissue by a multiplying microorganism which consumes the host's nutrients, placing a strain on the host's homeostatic ability. However, through the course of evolution, several effective and efficient antimicrobial defense systems have evolved: the immune system and the reticuloendothelial system which are interfunctional.

Immune System

The immune system (47) composed of primary and secondary lymphoid tissue (spleen, thymus, lymph nodes, bone marrow, lymphocytes and Peyer's patches) can be further divided into two cell type systems: the humoral and the cellular immune systems. The humoral system refers to those lymphoid cells whose primary function is to produce antibodies (glycoproteins) capable of binding non-covalently through electrostatic forces, van der Walls force, hydrogen bonding of hydrophobic/hydrophilic interactions with specific complementary antigens. These humoral cells (B cells) are bone marrow derived and are the precursor cells of immunoglobulin secreting cells or plasma cells. The cellular lymphoid system consists of thymus derived lymphoid cells (T cells), which do not differentiate into immunoglobulin secreting cells, but instead produce lymphokines such as macrophage activation factors, macrophage inhibition factors, lymphocytotoxins, interferon, activation factors for eliciting the humoral immune response and chemotactic factors. They are responsible for hetero-
genous tissue graft rejection, they have the ability to directly lyse and destroy foreign cells including certain tumors, and they are involved in delayed hypersensitivity reactions.

Reticuloendothelial System

The reticuloendothelial system (5,44) is composed of a network of highly phagocytic reticular cells lining organ tissues such as the spleen, the thymus, the liver (Kupffer's cells) and the vascular endothelium of the tunica interna. In addition to these sessile cells, motile phagocytic cells occur in the peripheral pools as neutrophils, basophils, eosinophils, and monocytes. The macrophage, the end cell of the monocyte line and the phagocyte found in various tissue, is free to wander by ameboid movement through tissue. It is especially concentrated within the loose connective tissue, but is not restricted to that tissue. Specialized macrophages are found in the alveoli of the lungs (alveolar macrophages) and in the peritoneal cavity as peritoneal macrophages.

Peritoneal Exudate Cells

The cells of special interest in this thesis are the peritoneal exudate cells (PEC) which include PMNs, monocyte/macrophages, and lymphocytes but particularly the macrophage since it is the most abundant cell normally found in the extravascular area of the peritoneal cavity.

Macrophage: The macrophage, derived from the circulating white blood cell, the monocyte, is a long lived and a very effective phagocytic cell in mammals (13,39,42). Electron microscopy has revealed that a unit membrane exists in the macrophage which has many protuberances (microvilli) and invaginations. The cytoplasm contains smooth and rough endoplasmic reticulum. Two types of vacuoles have been shown to exist: a smaller
pinocytic vesicle and a larger phagocytic vacuole. The macrophage ingests particles smaller than 0.1\,\mu m by pinocytosis and engulfs particles of larger size through phagocytosis. It has a plasma membrane which is capable of recognizing two of four subclasses of the human gamma immunoglobulin class, Ig G\textsubscript{1} and Ig G\textsubscript{3} which differ only in their physicochemical composition in the constant globulin region\textsuperscript{(4)}. It can also recognize the complement fraction C3b. The macrophage has been shown to be capable of both non-immunologically and immunologically controlled phagocytosis/pinocytosis.

The macrophage has also been shown to attach to the Fc portion of the gamma immunoglobulin, which has previously been bound to an antigen. During phagocytosis, a particle is bound to the macrophage's membrane receptors and surrounded by the cell membrane. The particle is then internalized into a phagosome and finally digested after the phagosome containing the particle and a lysosome have been fused\textsuperscript{(45)}.

Metabolism of the macrophage is dependent upon the activation state of the cell. An unactivated macrophage obtains energy primarily through anaerobic glycolysis but the activated macrophage has a significant increase in the hexose monophosphate shunt. A macrophage may become activated by exposure to lymphokines produced by sensitized T cells or by direct antigen contact. An activated macrophage is larger than the unactivated form, an activated macrophage adheres to glass more readily than an unactivated macrophage, and an activated macrophage is more phagocytic than an unactivated type especially in phagocytosing complement-coated C3b, or Ig G\textsubscript{1} and Ig G\textsubscript{3} coated particles. An activated macrophage also exhibits a greater chemotactic ability than the non-activated type.

Mechanisms of intracellular killing are not as well known in macrophages
as in neutrophilic degradation involving myeloperoxidase, an acid pH, a halogen, and hydrogen peroxide. However, lysozyme and hydrogen peroxide are known to be of importance in the killing and degradation ability by the macrophage.

The killing effect of the macrophage is related to the engulfed particle's chemical structure and to the activation of the macrophage. The activated macrophage is especially efficacious in phagocytosing intracellular organisms.

Neutrophil: The neutrophil, a phagocytic leukocyte(8), is also important as a PEC. The neutrophil, a polymorphonuclear leukocyte, accounts for about 60% of the total number of white blood cells contained in the peripheral blood pool, and is of secondary importance to the macrophage in this thesis only because it is not the major phagocytic cell line found in the peritoneal cavity. The neutrophil is a key cell in the phagocytic defense system because of its phagocytic effectiveness and efficiency and its quick response to microbial pathogens.

The neutrophil is 10-20μm in diameter and is distinguished by its 3-to-5 lobed nucleus held together by the nuclear envelope. Its average life span is 4 to 5 days and therefore, it is a short-lived cell. The neutrophil is extremely motile via ameboid movement and may become chemotactic in the presence of certain attracting microbial products, tissue proteases, and C3b and C4b complement components. Opsonized microbial organisms are more readily phagocytosed than non-opsonized ones and neutrophils having surface Fc receptors which recognize opsonins are very effective in phagocytosing opsonized microbial organisms in one of three ways(5,12): (1) by a specific IgG antibody/antigen complex; (2) by a
specific IgG antibody/complement complex; or (3) by a non-specific complement fraction activated by a bacterial or fungal polysaccharide which forms an microbe/complement complex evoking phagocytosis.

Phagocytosis of microbial organisms is also mediated by three additional factors: (1) by the proximity of an antigen and a neutrophil relative to each other since the more packed together they are, the greater the phagocytic activity independent of chemotactic or opsonizing factors; (2) through natural antibodies other than IgG molecules synthesized for specific antigens; and (3) through leukokinin, a type of IgG complex which can coat the neutrophil's surface stimulating neutrophilic phagocytosis. Ostensibly, the biological activity of leukokinin is dependent upon a single peptide chain (Thr-Lys-Pro-Arg) named Tuftsin since it was discovered at Tufts University(25).

The energy requisite for the neutrophil's killing mechanism is provided by the glycolytic pathway. This pathway is significant because neutrophilic phagocytosis often occurs in hypoxic surroundings such as abscessed lesions.

Once a microbe and a neutrophil have made contact, pseudopodia from the neutrophil extend and fuse on the far side of the microbe, engulfing it within a phagosome. The phagosome containing the microbe moves toward the cell's center where it fuses with lysosomes (at a low pH) containing myeloperoxidase, hydrogen peroxide, and a halide. Once fusion of these constituents has been completed, the microbe is killed and degraded. This is an especially effective mechanism in phagocytic removal of intercellular pathogens.
B. SPECIFIC REVIEW MATERIAL

Three Host/Parasite Models

Bacterial Model: The polymorphonuclear leukocyte is usually the first cell to provide defense against pathogenic bacteria(4). Upon entry into a host, specific bacterial end products are chemotactic for neutrophils. Once a neutrophil has come in contact with a bacterial cell, the bacterium is phagocytosed. The phagocytic efficiency is enhanced if the bacterial cell has been previously opsonized, for example, by IgG antibody.

If the bacterial pathogen is a long-lived and/or an intracellular organism, the activated macrophage is more effective in its removal than the neutrophil. Mackaness(34) studied extensively the macrophage's role in intracellular bacterial infection. He introduced, intravenously, a sub-lethal dose of virulent Listeria monocytogenes into a mouse model and ascertained that it was initially removed by neutrophils and sessile cells of the reticuloendothelial system. However, L. monocytogenes was found to continue multiplying in these cells for 3 to 4 days after being phagocytosed. But after the 4th day, the bacterial population dropped off rapidly until L. monocytogenes was eliminated from the infected mice. The mice proved to be highly resistant to a rechallenge of these bacteria and suffered no pathological effects with a rechallenge of up to a LD$_{50}$ dose of L. monocytogenes.

The reason for the high level of resistance was that the bacterial antigens stimulated the cellular immune system, elicited by the T lymphocytes, between the 1st and 2nd days of the challenge. The sensitized T cells released lymphokines such as macrophage activation factors and macrophage inhibition factors into the area where the T lymphocytes and bacterial cells interacted. The lymphokines activated the macrophages.
and they readily phagocytosed the bacteria. Resistance to *L. monocytogenes* was long-lived in these mice due to the T memory cells which were capable of reactivating the macrophages upon a rechallenge and subsequent recognition of *L. monocytogenes*.

A different bacterial model, *Mycobacterium tuberculosis*, a long-lived, acid fast bacterium and the causative agent of tuberculosis, has provided similar results. With low level doses of *M. tuberculosis* in healthy, well nourished mammals, the disease is usually averted through neutrophilic phagocytosis. However, when *M. tuberculosis* is not completely destroyed by neutrophils, the activated macrophages become a preeminent factor in the phagocytosis of these bacteria.

In 1965, Lurie and Dannenburgh(32), using inbred rabbits as hosts, studied macrophage/*M. tuberculosis* interaction. They concluded that resistance or susceptibility to tuberculosis in the rabbits was dependent on the macrophage's phagocytosing ability. In addition, when they injected cortisone into these rabbits, they found that the cortisone (which they thought prevented the fusion of the macrophage's phagosome and lysosome bodies) severely reduced the macrophage's ability to eliminate *M. tuberculosis*. However, Hart and Armstrong(18) reported in 1974 that *M. tuberculosis* survived degradation in their study, even after being phagocytosed and both the phagosomal and lysosomal bodies had in fact fused.

Moreover, it had previously been shown in several studies that the cellular immune system accounted for a major portion of the phagocytic elimination of *M. tuberculosis*. For example, in 1960, Hsu and Kapral(25) compared guinea pig immune peritoneal macrophages with non-immune peritoneal macrophages. They concluded that the immune and therefore activated macrophages were more effective in phagocytosing *M. tuberculosis* than non-immune macrophages.
Youmans and Youmans in 1969(48) reported that *M. tuberculosis* was not only eliminated faster by immunized (activated) macrophages than by non-activated macrophages, but the activated macrophages, immunized specifically for *M. tuberculosis* were as expeditious in removing non-related organisms such as *L. monocytogenes* as those macrophages sensitized with *L. monocytogenes*.

In a bacterial model using *Salmonella typhimurium*, a gram negative bacterium the causative agent of salmonella gastroenteritis, several studies (3,35) concluded that macrophages activated by T lymphocytes were more efficient in killing *S. typhimurium* than non-activated macrophages. Also, it was reported that humoral components (B cells) were not a significant factor in the activation of the macrophages against *S. typhimurium*.

**Viral Model:** Since viral infections are intracellular, macrophages may have an antiviral function in this type of infection (2,37). T lymphocytes, upon recognition of viral antigen, release lymphokines which in turn activate macrophages. These activated macrophages then pinocytose the free virus particles. It has been demonstrated in vitro that activated macrophages do restrict intracellular replication of some viruses through phagocytoses. It has also been shown that activated macrophages protect new born mice from herpesvirus infection(19). There is evidence that macrophage-produced interferon offers resistance to general viral infections; however, no conclusive evidence has been shown to date.

**Fungal Model:** Studies pertaining to subcutaneous and systemic fungal infections in mammalian hosts have been neglected, for the most part, relative to the extensive and intensive studies conducted upon bacterial and viral models. One reason for this is the low invasiveness many fungi have upon healthy and well nourished mammals. However, with the advent of
broad-spectrum antibiotics, synthetic anti-inflammatory drugs such as the corticosteroids, and present oncological treatment methods (e.g. chemotherapy), the occurrence of fungal diseases such as candidiasis, mucormycosis, cryptococcosis, and aspergillosis has been increasing significantly (29,45).

Natural resistance appears to be a major factor in protecting the host against pathogenic subcutaneous and systemic fungal infections. Neutrophils rapidly provide initial protection against these organisms until the macrophages become activated. Once activated, macrophages can be effective antifungal agents especially in, for example, fungal infections like Cryptococcus neoformans.

Early attempts (28) in 1925 by Shapiro and Neal and Hoff in 1942, and Macus and Rambo (36) in 1955 failed to find an immunization treatment for or an immune mechanism to explain cryptococcosis relative to the humoral immune system.

Gadebush (14) in 1958, provided an interpretation of natural immunity against C. neoformans in rabbits which was accepted as valid. He introduced C. neoformans into rabbits through aerosols. Once C. neoformans was situated in the alveolar septa and the alveoli of the lungs, the majority was removed within 24 hours by neutrophils. However, the fungi that were not phagocytosed by neutrophils proliferated freely. The phagocytic obligation soon shifted to the monocyte. It is thought that during this shift the fungi spread into areas outside the pulmonary tract (15). After several days, the monocytes which initially confronted C. neoformans matured into macrophages. They and other macrophages were activated by delayed hypersensitivity reactions as both an effect of the cellular immune response and through previous contact with the fungi as monocytes,
i.e., direct contact activation. The existing *C. neoformans* were then swiftly phagocytosed and destroyed. Complete recovery from this infection was common if the infective *C. neoformans* dose was not so massive as to overwhelm the host's system.

Abrahams (1) in 1966 demonstrated that natural resistance to cryptococcosis in mice was dependent on the cellular immune system and not the humoral system directly. However, it was demonstrated that opsonization of these fungi by IgG greatly enhanced phagocytosis. Abraham's findings were supported by Goren (17) in 1967.

**Rabbit Macrophage:** In previous study in our laboratory (11) it was found that alveolar macrophages may be activated by either viable or non-viable *S. schenckii* conidia regardless of the route of administration. It was discovered that fungicidal activity was 2-to-3 fold greater in those macrophages which had been recovered from animals sensitized by aerosols than from those sensitized by intramuscular, intravenous, or intraperitoneal injections of equivalent numbers of conidia.

**S. schenckii Neutrophil Model:** In 1973, Howard (23), using guinea pig neutrophil cell cultures, demonstrated that neutrophils were highly effective in killing extracellular *Histoplasma capsulatum* fungi cells. Other studies (24) have also shown that neutrophils are highly effective against certain extracellular fungi, such as *Candida albicans, krusei, parapsilosis, stellatordia, pseudotropicalis, Saccharomyces cerevisiate, Geotrichium canidium, Aspergillus niger, fumigatus, and neoformans.*

**Macrophage Model:** Howard (20), in 1959 maintained normal (nonimmune) mouse peritoneal exudate macrophage cultures inoculated with yeast phase *H. capsulatum*. He found that as few as 30 fungus cells per macrophage overwhelmed the macrophages.
In 1965, Howard(21) expanded his previous studies dealing with nonimmune mouse macrophages to other models. He used five different isolates of _H. capsulatum_; he used both guinea pig and mouse normal macrophages, and he used guinea pig and mouse macrophages in the presence of specific hyperimmune serum and macrophage cells from specifically immunized guinea pigs and mice. He concluded that the generation time of _H. capsulatum_ in mammalian macrophages was very constant, that the rate of growth for the five _H. capsulatum_ isolates was very similar in macrophage cells; that the generation time of fungus within guinea pig macrophages was nearly the same as in mouse macrophages, and that the exposure of yeast phase _H. capsulatum_ to antibody and complement did not affect intracellular fungal proliferation in the macrophages. He reported no significant difference in fungus proliferation within macrophage cells from immunized compared with those from nonimmunized guinea pigs and mice.

In 1973 Howard(22) used freshly harvested macrophages from immunized mice instead of those grown on tissue cultures. He found that _H. capsulatum_ was inhibited by these immune activated macrophage cells but was not killed.

In 1976 Howard and Otto(24) reported that the effectiveness of inhibition of _H. capsulatum_ was a product of the cellular immune system and not the humoral system.

Kashkin _et al._(27) in 1976, using the pathogenic fungi _Coccidioides immittis_, the causative agent of the pulmonary infection coccidioidomycosis, reported a similar finding that fungicidal and fungistatic responses are dependent upon the cellular immune system which activated the peritoneal macrophages. This is important to this author's research in that the fungicidal and fungistatic effects of the cellular immune system against
S. schenckii shall be studied.

Statement of Purpose

This author proposes a slightly different approach in continuing pathogenic fungal research. The previously cited fungal models used fungi that are not normally encountered by peritoneal exudate cells, particularly peritoneal macrophages. H. capsulatum, C. neoformans, and C. immitis are basically pathogenic to the pulmonary system. To date no study concerning a subcutaneous fungus/PEC model has been reported. Hence, it is felt that a guinea pig model using S. schenckii would be of significance since peritoneal exudate cells do normally encounter subcutaneous pathogenic fungi.

Therefore, the purpose of this thesis is to determine some antifungal properties of guinea pig peritoneal exudate cells when challenged by the pathogenic fungus, S. schenckii, the causative agent of sporotrichosis, a lymphocutaneous infection.

The first question to be evaluated is: what phagocytic effects, if any, do guinea pig peritoneal exudate cells have upon S. schenckii?

The second question to be evaluated is: given phagocytosis does occur, is it fungistatic or is it fungicidal?

The third questions to be evaluated is: what are the effects of cortisone compromise on PEC activity?

Plan of Development

In this study, S. schenckii conidia will challenge thioglycolate-induced guinea pig peritoneal exudate cells in three specific modes: normal, immune, and immune suppressed.

1. Normal condition: Defined as a guinea pig having had no previous exposure to S. schenckii. This is also the control condition.
2. **Immunized condition:** Defined as a guinea pig having been immunized for both a primary and a secondary response against *S. schenckii* antigens and therefore presumably capable of antigenic memory and macrophage activation.

3. **Immune suppressed condition:** Defined as the state in which the capacity to elicit the immune response has been greatly reduced by the use of corticosteroids.
CHAPTER II

MATERIALS AND METHODS

Animal Models

Guinea pigs, the animals models used in this research, were obtained from Rocky Mountain Laboratories, Hamilton, Montana, were housed in the animal room at the Department of Microbiology, were approximately the same age, were immune competent, and were fed a nutritionally complete commercial guinea pig ration.

Fungal Cultures

Cultures of S. schenckii, strain 425, were obtained from stock cultures stored at the Department of Microbiology, University of Montana. Subcultures of S. schenckii were inoculated onto Mycosel agar (Baltimore Biological Laboratory) slants; were incubated in screw cap tubes at 25°C, and stored at minus 20°C. Before any S. schenckii culture was employed, the culture was examined for contamination through microscopy and gross observation. Broth cultures used for harvesting conidia contained 150 ml Sabouraud dextrose broth (Difco Laboratories) combined with 0.1% yeast extract (Difco Laboratories). For conidia harvesting, a subculture of S. schenckii was streaked on a Mycosel agar plate, which was incubated at 25°C for 96 hours. After incubation, a 0.5 X 0.5 mm section of agar containing S. schenckii was removed and inoculated into a 500 ml Erlenmeyer flask containing 150 ml of the conidia broth described above. Each inoculated flask was then incubated at room temperature on a horizontal shaker for 10 to 12 days. At this time, the conidia were harvested by transferring about 75 ml of each broth culture into a 250 ml sterile plastic centrifuge bottle. The bottles were centrifuged at 75 x G, at 4°C, for 10 minutes and the
supernatant was discarded, leaving in each centrifuge bottle a pellet mass of conidia. To each of the plastic centrifuge bottles, 100 ml of sterile physiological saline (0.85%) were added. The bottles were hand agitated 25 times to insure an adequate washing of the conidia. The conidia were then recentrifuged as previously described and after centrifugation, a second conidia washing was performed. These washed conidia were stored in the plastic centrifuge bottles at minus 20°C for future use as either cellular antigens or for use in establishing the effectiveness of PEC phagocytosis in vitro.

**Tissue Culturing Media**

Eagles Minimum Essential Media (MEM) with Earles' balanced salt solution (Microbiological Associates) was the tissue culture medium employed. Two grams crystalline MEM were reconstituted in 1,000 ml triple distilled water, buffered with 2.2 grams NaHCO₃, and the hydrogen ion content was adjusted to pH 7.2 using 1 N NaOH or 1 N HCl depending upon the acid or base concentration. This completed medium was filter sterilized using a Seitz filter, pore size 0.22μm and stored at 4°C.

For the tissue culturing process, 100 ml MEM was enriched with 2% heat inactivated fetal calf serum (Microbiological Associates), 1 ml tricine (Sigma Chemical Co.) as a buffering agent, 10,000 units penicillin G (Sigma Chemical Co.) and 10,000 μg streptomycin sulfate (Sigma Chemical Co.) each to inhibit gram positive and gram negative bacterial growth respectively, 1 ml heparin (Sigma Chemical Co.) to prevent clumping of red blood cells and 1 ml L-glutamine (Sigma Chemical Co.) to ensure adequate levels of this amino acid. In this thesis, this solution shall be known as complete MEM.

Phosphate Buffered Saline (PBS) was utilized as a physiological buffer-
ing agent and in the tissue culture washing process to be described later. Stock solutions of PBS were prepared as described by Dulbecco and Vozt(9).

Glassware

All glass items utilized in this work were first rinsed in cool water to avoid protein coagulation, then washed in warm soapy water, rinsed 5 times in distilled water, and finally acid rinsed for a minimum of 4 hours in a 0.1 N HCl immersion bath. In cases where direct contact between PECs and glassware occurred, those glasswares were siliconized with Silaclamp (Biological Associates) with the exception of the 15 mm-diameter cover slips positioned in the bottom of each culture well in order to augment PEC adherence.

Antigen Preparations

Two types of cellular antigens were used in this work; viable and non-viable S. schenckii. (1) Viable S. schenckii: As required, samples of S. schenckii conidia were transferred from the 250-ml plastic centrifuge bottles, plated on Mycosel agar, incubated at 25°C, and inspected for growth on the 5th day of incubation in order to ensure viability. Preparations of viable conidia were produced by adding conidia to sterile physiological saline until a concentration of $10^7$ conidia per ml saline was achieved. These viable conidia preparations were stored at 4°C in screw cap bottles. (2) Non-viable S. schenckii: S. schenckii viable conidia were added to a 1:10,000 concentration of merthiolate, incubated at 35°C for 24 hours and washed in PBS to remove the merthiolate. This was accomplished by centrifuging the merthiolate aliquot for 15 minutes at 4°C, at 75 x G. Once completed, the merthiolate supernatant was decanted followed by the addition of 50 ml PBS. This mixture was hand agitated 25 times to ensure adequate washing, followed by recentrifugation as previously
described. At this time, a sample of the conidia from this washed aliquot was plated on Mycosel agar, incubated at 25°C, and inspected for growth after 72 hours. Non-viability was validated by the absence of any conidia growth when plated on Mycosel agar. Non-viable conidia were also stored at a $10^7$ conidia per ml physiological saline concentration at 4°C in screw cap bottles.

**Experimental Models**

**Group I:** This, the control group, received neither an immunization of fungal conidial antigen or cortisone.

**Group II:** This group was immunized with merthiolate-treated, non-viable *S. schenckii* conidia. The guinea pigs in this group were initially immunized intraperitoneally at day 1 with a $10^7$ conidia dose followed by a second $10^7$ conidia dose at day 11, and the PECs were extraced at day 22.

**Group III:** The animals in this group were immunized with *S. schenckii* viable conidia. Each animal received two $10^7$ doses of viable conidia, the first at day 1 and the second at day 11, administered intraperitoneally. PECs were collected at day 22.

**Group IV:** The guinea pigs in this group were administered hydrocortisone acetate (Sigma Chemical Co.) but no fungal antigen. Each animal received 0.05 mg hydrocortisone acetate (cortisone) per gram body weight intraperitoneally at 5-day intervals until day 22. At this time the PECs were removed.

**Group V:** This group was treated with cortisone and immunized with non-viable *S. schenckii* conidia. Two days prior to the first $10^7$ non-viable conidia immunization, 0.05 mg cortisone per gram body weight was injected intraperitoneally into each guinea pig; the injection was repeated at 5-day intervals until day 22. Non-viable conidia in a $10^7$ dose were
injected into each animal at days 1 and 11, followed at day 22 with the collection of PECs.

Group VI: This group was injected with cortisone and immunized with viable *S. Schenckii* conidia. Guinea pigs received 0.05 mg dose of cortisone per gram body weight intraperitoneally 2 days prior to the first immunization of $10^7$ viable conidia. Cortisone was readministered at 5-day intervals thereafter until day 22. At days 1 and 11, non-viable conidia were injected into each guinea pig intraperitoneally and at day 22 the PECs were collected.

It should be mentioned that in each of these 6 models, 72 hours prior to removal of the PECs (or on day 19), each guinea pig was induced with an injection of 4 ml of thioglycolate fluid medium (Difco Laboratories) intraperitoneally.

**In Vitro Methodology**

In order to study the in vitro effects of PECs when exposed to *S. schenckii* conidia, PECs were introduced to viable conidia through a tissue culturing procedure.

**Procedure**

1. At day 22, 20 ml complete MEM was injected intraperitoneally via an 18 gauge needle into each guinea pig. This was followed by a gentle massage of the abdominal area for approximately 1 minute to increase the number of PECs collected. At this time the needle was removed from its syringe and replaced, bevel up, into the initial puncture wound at an acute angle of 20-40° from the plane of the guinea pig's abdomen until it was in the peritoneal cavity. A prechilled 40-ml sterile siliconized centrifuge tube was held under the needle's butt to collect the returning PEC suspension. To ensure the maximum recovery of the PEC suspension, a gentle downward abdomen milking was provided each animal.
2. After the PEC/MEM aspirates were collected from each of the six guinea pig models, they were centrifuged 5 minutes, at 4°C, at 75 x G, in the 40-ml centrifuge tubes.

3. Once centrifugation was completed, the supernatant was aspirated, leaving a pellet of PECs at the bottom of each of the tubes. At this point 1 ml 9.17 M Tris-(hydroxymethyl)-aminomethane (Sigma Chemical Co.) and 9 ml 0.83% NH₄Cl were added to each centrifuge tube containing the PECs. This suspension was allowed to stand at room temperature for 15 minutes to ensure lysis of any red blood cells collected.

4. The cells were then re-centrifuged as in step 2 followed by aspiration of the supernatant fluid. Once completed, 10 ml PBS were added to each centrifuge tube to wash the PECs, followed by centrifugation as in step 2.

5. At this point, the supernatant was aspirated and the PECs were resuspended in 5 ml of complete MEM per each centrifuge tube. A small volume of PEC/MEM suspension from each tube was collected and placed on a hemocytometer. The PECs were then counted by standard methods(4) and these counts estimated the number of PECs per ml within the tubes. Complete MEM was added to each of the 40-ml centrifuge tubes until a $10^5$ PEC count per ml was confirmed via counting.

6. After a uniform cell count of $10^5$ cells per ml was achieved for each of the 6 models, 1 ml PEC aliquots were added to each of the 24, 1.7 X 1.6 cm wells contained in each flat bottom tissue culture plate (Flow Laboratories). Each well in tissue culture plates contained a round 15 mm flat non-siliconized glass cover slip for PEC adherence.

7. These tissue culture plates were incubated at 37°C in 0.4% CO₂ for 45 minutes. At this time any non-adherent PECs within each culture
well were removed by dispensing 5 ml PBS into each well simultaneously removing the PBS/MEM solution through a Pasteur pipette via negative pressure. To prevent the PECs from osmotic shock, approximately a 2 mm layer of PBS covering the adherent cells on the coverslips was not removed. Quickly, 1 ml of MEM was reintroduced into each of the tissue culture wells. Once completed, the PEC tissue culture plates were re-incubated. Refeeding was performed every 4 hours in an identical manner until the PECs had been cultured for a total of 12 hours. It was found that 12 hours incubation was necessary for an even monolayer of PECs to form on each of the cover slips.

8. At hour 12, each of the tissue culture wells was washed with 5 ml PBC as stated in step 2, followed by the introduction of $10^4$ viable *S. schenckii* conidia suspended in 1 ml of complete MEM.

9. At times 0, $\frac{1}{4}$, 1, 2, 4, 8, and 12 hours, a cover slip containing adherent PECs, representing each of the 6 models, was removed from the appropriate tissue culture well, washed in physiological (0.85%) saline, and stained with a 1:10,000 dilution of acridine orange in a modified procedure described by Newcomer(40). Following the acridine orange staining method, conidia which are viable appear bright green while conidia which are non-viable appear orange or red when examined with fluorescence microscopy.

10. Two methods of conidia counting were employed. In the first method, 20 PECs, selected at random, were observed microscopically. The number of conidia contained within each of these 20 phagocytes was tabulated and averaged. The second counting method used the same data as the first except any of the PECs not actually demonstrating phagocytoses via the acridine staining method were dropped from the tally and an adjusted
average number of conidia phagocytosed per PEC was calculated. This was performed in order to allow for the possibility that the PECs which did not demonstrate intracellular conidia may have been non-viable PECs instead of viable, non-phagocytosing PECs.

**Quantitative Analysis**

The Statistical Package for Social Science (SPSS) computer program format was employed on the University of Montana's DEC-20 computer system when possible. When hypothesis testing was encountered, the 0.05 level of significance (95% confidence) for type I error was used. When appropriate, the descriptive level of significance or P value is given. This is defined as the probability of realizing an observed value or a more extreme value, given that the null hypothesis of equivalence amount models is true.
CHAPTER III

RESULTS

Plotted on figure 1 are the average numbers of phagocytosed conidia per PEC, for each of the 6 models based on microscopic observations of 20 randomly selected PECs per model, plotted vs time in hours. Note that the time intervals from time zero, when viable conidia were first introduced into each tissue culture plate wells, to the first observed cytotoxic effects of these PECs on the conidia are also provided. Table 1 provides the mean data of these observations from which graph 1 was plotted.

Figure 2 provides the plot of the average number of phagocytosed conidia for each guinea pig model relative to time; however, this graph portrays only those PECs having actually demonstrated phagocytosis of conidia. Those PECs having not phagocytosed any conidia as demonstrated microscopically by means of the acridine staining procedure, were dropped from this statistical design. Table 2 provides the mean data from which Figure 2 was plotted.

A standard 1-factor, 2-way analysis of variance(46), Table 3, was computed using the mean data contained in Tables 1 and 2 and other required parameters, for example, the errors of the means, sums of squares, F statistics and variances of these sample data necessary in this Anova design were computed from the raw experimental data. Computational analysis was performed through an ANOVA SPSS computer program(41), followed by both the computation of 4 multiple contrasts and hypotheses testing by this author, using a hand-held programmable HP-41C calculator. Since the same relative statistical results were achieved when using the data from either Table 1 or 2, (as confirmed through a non-parametric statistical process involving
FIGURE 1. AVERAGE NUMBER OF CONIDIA PHAGOCYTOSED PER PEC BASED ON 20 RANDOMLY SELECTED PECs.
Figure 2. Average number of conidia phagocytozed per PEC based on PECs containing conidia only.
TABLE 1

Average number of conidia phagocytosed per PEC based on 20 randomly selected PECs.

Animal Treatment Schedule

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Viable conidia</th>
<th>Non-viable conidia</th>
<th>Normal control</th>
<th>Cortisone comp., plus viable conidia</th>
<th>Cortisone comp., plus non-viable conidia</th>
<th>Cortisone control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1/4</td>
<td>1</td>
<td>0.5</td>
<td>0.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1/2</td>
<td>1</td>
<td>1</td>
<td>0.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>1.5</td>
<td>1</td>
<td>1</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>1.5</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>16</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>20</td>
<td>4</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>20</td>
<td>5</td>
<td>4</td>
<td>4.5</td>
<td>2.5</td>
<td>1.5</td>
</tr>
<tr>
<td>12</td>
<td>20</td>
<td>6</td>
<td>5</td>
<td>5</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>
TABLE 2

Average number of conidia phagocytosed per PEC based on only those PECs containing conidia.

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Viable conidia</th>
<th>Non-viable conidia</th>
<th>Normal control</th>
<th>Cortisone comp., plus viable conidia</th>
<th>Cortisone comp., plus non-viable conidia</th>
<th>Cortisone control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1/4</td>
<td>1</td>
<td>0.5</td>
<td>0.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1/2</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>18</td>
<td>6</td>
<td>5</td>
<td>5</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>20</td>
<td>6</td>
<td>5</td>
<td>5</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>8</td>
<td>20</td>
<td>7</td>
<td>6</td>
<td>5</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>12</td>
<td>20</td>
<td>8</td>
<td>7</td>
<td>7</td>
<td>4</td>
<td>3</td>
</tr>
</tbody>
</table>
ANOVA summary table of the average number of conidia phagocytosed per 20 randomly selected PECs.

<table>
<thead>
<tr>
<th>Variation Source</th>
<th>Degrees Freedom</th>
<th>Sum of Squares</th>
<th>Mean Squares</th>
<th>F Computed</th>
<th>F Tabled</th>
</tr>
</thead>
<tbody>
<tr>
<td>Due treatments</td>
<td>5</td>
<td>696.3</td>
<td>139.26</td>
<td>3.29</td>
<td>2.13</td>
</tr>
<tr>
<td>Due time</td>
<td>7</td>
<td>296.3</td>
<td>42.33</td>
<td>5.21</td>
<td>1.57</td>
</tr>
<tr>
<td>Residual (unex-</td>
<td>12</td>
<td>97.5</td>
<td>8.12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>plained by ANOVA)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ANOVA summary table of conidia phagocytosed per PEC based only on those PECs containing conidia

<table>
<thead>
<tr>
<th>Variation Source</th>
<th>Degrees Freedom</th>
<th>Sum of Squares</th>
<th>Mean Squares</th>
<th>F Computed</th>
<th>F Tabled</th>
</tr>
</thead>
<tbody>
<tr>
<td>Due treatments</td>
<td>5</td>
<td>693.8</td>
<td>138.76</td>
<td>3.32</td>
<td>2.13</td>
</tr>
<tr>
<td>Due time</td>
<td>7</td>
<td>292.5</td>
<td>41.79</td>
<td>5.16</td>
<td>1.57</td>
</tr>
<tr>
<td>Residual (unex-</td>
<td>12</td>
<td>97.2</td>
<td>8.10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>plained by ANOVA)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Variation Due to Treatments = \[ SS_a = \sum_{i=1}^{a} \sum_{j=1}^{b} (\bar{y}_{ij} - \bar{y})^2 \]

Variation Due to Time = \[ SS_b = \sum_{j=1}^{b} (\bar{y}_{j} - \bar{y})^2 \]

Residual or Unexplained Variation = \[ SS_e = \sum_{i=1}^{a} \sum_{j=1}^{b} (y_{ij} - \bar{y}_{ij} + \bar{y})^2 \]
Summary table of the nested ANOVA design based on phagocytic efficiency on time

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>Sum of Squares</th>
<th>Degrees Freedom</th>
<th>Mean Square</th>
<th>F computed</th>
<th>F tabled</th>
</tr>
</thead>
<tbody>
<tr>
<td>Due treatments</td>
<td>1583.51</td>
<td>5</td>
<td>316.701</td>
<td>11.54</td>
<td>4.26</td>
</tr>
<tr>
<td>Within treatment among PECs on time</td>
<td>576.11</td>
<td>21</td>
<td>27.434</td>
<td>12.75</td>
<td>2.04</td>
</tr>
<tr>
<td>Residual (random error)</td>
<td>1032.95</td>
<td>480</td>
<td>2.152</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Variation Due Treatments = $b n \sum \frac{1}{2} (\bar{y}_i - \bar{y})^2$

Variation Within Treatments
Contrasts among PECs, with time in the model
$= b n \sum \frac{1}{2} \sum (y_{ij} - \bar{y}_i)^2$

Residual (unexplained error)
$= \sum \frac{1}{2} \sum (y_{ik} - \bar{y}_i)^2$
### TABLE 5

ANOVA Summary table for average numbers of conidia phagocytosed for each model regressed on (X₁) treatment, and contrasted with the addition (X₂) Age, and/or (X₃) Sex.

<table>
<thead>
<tr>
<th>Variation Source</th>
<th>Degrees Freedom</th>
<th>Sum of Square Variation</th>
<th>F computed</th>
<th>Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>X₁ due treatment</td>
<td>1</td>
<td>582.95</td>
<td>19.67</td>
<td>( \hat{Y} = \beta_0 + \beta_1 X_1 + \epsilon; \epsilon \sim N(0, \sigma^2) )</td>
</tr>
<tr>
<td>X₂/X₁ due age with treatment in the model, but controlled</td>
<td>1</td>
<td>0.24</td>
<td>0.08</td>
<td>( \hat{Y} = \beta_0 + \beta_2 X_2 + [\beta_1 X_1] + \epsilon; \epsilon \sim N(0, \sigma^2) )</td>
</tr>
<tr>
<td>X₃/X₁ due sex with treatment in the model, but controlled</td>
<td>1</td>
<td>0.03</td>
<td>0.001</td>
<td>( \hat{Y} = \beta_0 + \beta_3 X_3 + [\beta_1 X_1] + \epsilon; \epsilon \sim N(0, \sigma^2) )</td>
</tr>
<tr>
<td>X₁/X₂/X₃ due treatment with age and sex in the model, but controlled</td>
<td>1</td>
<td>0.29</td>
<td>0.11</td>
<td>( \hat{Y} = \beta_0 + \beta_1 X_1 + [\beta_3 X_3 + \beta_2 X_2] + \epsilon; \epsilon \sim N(0, \sigma^2) )</td>
</tr>
<tr>
<td>Unexplained Variation</td>
<td>8</td>
<td>195.19</td>
<td>22.69</td>
<td></td>
</tr>
</tbody>
</table>

\[
R^2 = \frac{\sum (y_i - \bar{y})^2 - \sum (\hat{y}_i - \bar{y})^2}{\sum (y_i - \bar{y})^2}
\]

\[
F \text{ computed } = \frac{R^2}{1 - R^2} \frac{n-k-1}{k}
\]

\( Y = \) number of conidia phagocytosed

* computed via an SPSS Algorithm
a modified Chi Square procedure (38) measuring a goodness of fit which provided a P value of 0.005, which is the probability of a significant difference between the average data contained in Tables 1 and 2, which is insignificant), only those data based solely on those PECs containing intracellular conidia (Table 2) will be used throughout the remainder of this work.

Based on the 2-factor 1-way ANOVA using the data on Figure 2, two separate groups of models could be isolated: the first group, the model immunized with viable conidia; and the second group, which contained the other 5 models, which as a unit, were less efficient in antifungal properties. It can be seen that the PECs extracted from guinea pigs immunized with viable conidia were the most effective of those models tested, in both their phagocytosing and their fungicidal activity against S. schenckii. The probability of this group not being the most efficient and active phagocytic group studied is less than 0.001. This group phagocytosed an average of more than 20 conidia per PEC within the 8 hour time frame and that intracellular counting of PECs had to be terminated at hour 8, because the numbers of intracellular conidia could no longer be accurately counted, since the PECs were literally "stuffed" with intracellular conidia. The phagocytic rate between hours 1 and 4 was about 7 conidia per hour, which was more than double the average rate of any of the other 5 groups. Intracellular conidia lost viability less than 2 hours after initial exposure to PECs in this group as demonstrated by the acridine orange staining procedure.

The non-cortisone compromised animals immunized with non-viable conidia and the control group were statistically comparable in both fungicidal effects and phagocytic effects, phagocytosing an average of 8 - 7 conidia per PEC respectively within 12 hours. The first fungicidal effects against the conidia appeared about 4 hours after initial exposure to the PECs.
Neither phagocytic nor fungicidal activity of PECs from any of the cortisone compromised animals sensitized with viable conidia differed significantly from the normal untreated, unsensitized animals. The phagocytic efficiency of PECs from the compromised animals challenged with non-viable conidia was minimal and did not differ significantly from the unchallenged animals. The first fungicidal effects against the conidia appeared 8 hours after initial exposure to the PECs for each of the 3 compromised groups.

Using only one animal from each model for each experimental run attributed to large internal statistical variations, causing loss in quantitative precision via the 1-way, 2-factor ANOVA procedure. To achieve a greater degree of quantitative resolution through statistical analysis, a nested ANOVA design(10) was programmed into the DEC-20 computer system, using the SPSS format. Unlike the 1-way, 2-factor ANOVA design, which contrasted the variation of the 6 guinea pig models to each other based on time, the nested ANOVA design provided a 3-dimensional test by comparing the variation among the 6 guinea pig models based on time with the additional subsample contrast, that of phagocytic variation among PECs, both within each of the 6 models (internal variation) and between each of the 6 guinea pig models. Since a statistical sampling procedure had been violated using the nested ANOVA design in that for each model, several guinea pigs within that model should have their extracted PECs pooled, but did not, the P value is significantly high (the probability of this nested ANOVA design being untrue is P=0.30), which provided a higher probability of being incorrect than the 2 factor 1 way ANOVA of P 0.001 does.
Via the nested design, 3 distinct animal groups, based on phagocytic efficiency, could be realized using multiple contrast analyses based on the data contained in the Nested ANOVA Summary table, Table 4. The most obvious difference between the 3 groups appeared in the animal model immunized with viable conidia. Within the other 2 models, separated via the nested design, was the second most efficient phagocytic group consisting of 3 models: the normal animal model immunized with non-viable conidia, the normal control model, and the cortisone compromised model immunized with viable conidia. These three models were statistically identical at the 95% confidence level based on their similar phagocytic efficiency.

The third group isolated and the least efficient of the 3 models included the two remaining cortisone compromised models, those animals immunized with non-viable conidia and the cortisone compromised control animals. The probability of this third group being statistically identical to the second class in phagocytic efficiency is 0.30.

Of concern in this work was the possible bias effects that both differences in sex and age might have in this study. Prior to actually initiating any of the phagocytic and fungicidal assays as described in this thesis, a multiple/partial and a partial regression analysis were performed using the SPSS and BASIC programming formats. Four experimental contrasts were devised using a 2 X 2 factorial format similar to those discussed by Cochran and Cox(6) so as to ensure that each animal model was both age and sex adjusted. That is, each model was randomly assigned 1 of 4 variables: an older guinea pig, a younger guinea pig, a female guinea pig, and a male guinea pig. Each of these variables was then used one time within each model during the 4 experimental runs used in completing this study. Internal variation within each model was then measured and a
Partial F Test ANOVA procedure (28) was employed (Table 5), measuring first the variation explained solely by phagocytic variation due to the 6 treatments which was high (F computed = 19.67). Next the variable, sex, was measured to find how much additional phagocytic variation could be explained through this influence. As can be seen on Table 5, the additional phagocytic variation explained by sex is negligible (F computed = 0.08). Next the sex variable was removed and the guinea pig factor tested. The amount of phagocytic variation explained solely by age was also negligible (F computed = 0.03). Finally, both variables, age and sex, were simultaneously measured to find their joint contribution to explaining phagocytic variation. Again, the contribution in explaining additional phagocytic variation was negligible (F computed = 0.11).

Hence, it can be concluded that sex and the age differences in this work did not bias the phagocytic results at a probability level of confidence of 0.999.
DISCUSSION AND CONCLUSIONS

The immunological mechanisms of resistance to the pathogenic fungus *S. schenckii* are not fully understood. According to the literature, the PEC/fungus relationship is similar to the PEC/bacterium relationship, in that when conidia or bacteria are encountered in a wound such as a traumatic implantation within cutaneous tissues, neutrophils play a paramount antimicrobial role since they are physically present at the wound site from the onset of the inflammatory process. Since neutrophils require no activation to initiate efficient phagocytosis, this phagocytic response occurs whether or not the animal has been previously immunized against that foreign body. In both human mycoses and bacterial infections, neutrophils soon become of lesser phagocytic importance against those organisms, relative to the activated macrophage, once T-cell secretion of lymphokines such as MAF and MIF has occurred mainly due to the lower numbers of neutrophils but higher number of activated macrophages found in cutaneous tissue. Also, the physically smaller neutrophil simply is not capable of phagocytosing the large number of microbial organisms that the macrophage can.

However, a significant post-phagocytic difference between the bacterial and fungal models may exist. Recall that once a bacterial or fungal cell comes in physical contact with a neutrophil, pseudopodia from this white blood cell extend and surround the organism, encasing it within a portion of inverted plasma membrane, the phagosome. Once the organism is encased in the phagosome, the entire body, assisted by microtubules, moves toward lysosomes. Once the phagosome and lysosomes
come in physical contact, they fuse, exposing the organism to an environment of low pH, myeloperoxidase, hydrogen peroxide, lactoferrin, glycosidases, proteases, lipases, granular cationic proteins, and a halide, usually iodide. For the majority of bacteria, the fate is degradation but this may not be the case with fungi. Fungi have cell wall structures significantly different biochemically from either gram negative or positive bacteria. The fungal cell wall consists mainly of polysaccharide polymers of cellulose, of chitin, of beta-glucans, and of mannans, either in combination with each other or appearing as single repeating polymers. Mammalian PECs are known to lack enzymes capable of degrading these unique polysaccharide configurations, even after intracellular killing of these fungi has been demonstrated(7). Therefore, the retention of fungal bodies may persist intracellularly. This may play a significant role in human resistance to fungal infections since most of the non-degradable fungal cells are partially degraded by phagocytes. This partial degradation, however, is at best, slow, hence it may provide an immunological "boosting" effect by intensifying the immune response.

Some disagreement exists as to whether non-viable fungal antigens are as immunogenic to a mammalian host as viable fungal antigens. Howard (19) reported that immune peritoneal macrophages in both mice and guinea pigs were activated to a greater extent when immunized with viable \textit{H. capsulatum} than with non-viable \textit{H. capsulatum} conidia. Others (13,14,47) have reported similar findings. However, using rabbit models, Ferguson (11) reported that activation of alveolar macrophages was relatively constant regardless of whether viable or non-viable \textit{S. schenckii} were administered as antigens. Using bacterial models, Mackaness(34) reported that viable \textit{L. monocytogenes} were more antigenic than non-viable \textit{L. monocytogenes},
as did others (2,36,37) using various other bacterial models. It appears then that both viable fungi and viable bacteria are generally more antigenic than non-viable types. From the results of this present work, it has been demonstrated that viable conidia are more highly antigenic than non-viable S. schenckii conidia when administered into guinea pigs. Both the phagocytic rate and the numbers of conidia phagocytosed by PECs of normal animals immunized with viable conidia were at least twice that of the PECs from animals immunized with non-viable conidia. Also, from these results, it can be seen that both those animals immunized with non-viable conidia as well as the non-immunized controls were equivalent in phagocytic ability, which probably means that immunization with non-viable conidia has no detectable effect on the immune system in terms of PEC activation. In the present study, the results obtained through the 2-factor, 1-way ANOVA statistic, indicate that these 6 guinea pig models could be separated into 2 groups based on their phagocytic effectiveness. The most efficient animals in phagocytotic ability were those immunized with viable conidia. The other 5 models were statistically indistinguishable from each other. The conclusions reached using this statistical analysis are probably not complete in describing the phagocytic variation among the 5 models contained in group 2, since it may consist of 2 separate subclasses. With a more sensitive nested ANOVA design, it was determined that 3 separate classes may exist within the 6 models. The most effective phagocytic group consisted of those animals immunized with viable conidia. The second most effective group consisted of 3 animal models: control animals immunized with non-viable conidia; animals compromised with cortisone and immunized with viable conidia; and animals immunized with non-viable conidia. These models were equivalent in phagocytic efficiency. The third
and least effective group isolated by this statistical process was composed of the two cortisone-compromised animal models, those immunized with non-viable conidia and the non-immunized controls.

Unfortunately this nested design is not above reproach as portrayed by the relatively low (0.70) probability of being the correct model of representation, because the requirement for pooling guinea pig PEC samples within each of the 6 models was not met: too large a number of guinea pigs would be required for such a test. However, the 3 phagocytic group models appear logical and may be quite valid.

Note that the normal controls and the cortisone-compromised models, both those immunized with viable conidia and those non-immunized, are equivalent in phagocytotic efficiency. Cortisone is known to prevent the fusion of phagosomes and lysosomes(16) which leads to the inhibition of enzymatic degradation and alters the plasmalemma inhibiting phagocytosis. It is also known that cortisone is lymphotoxic and may lead to reduction in the number of T lymphocytes which are required in PEC activation, as well as to increased release of neutrophils from the bone marrow into peripheral blood pools.

It is possible that the cortisone-compromised animals which were immunized with viable conidia demonstrated PEC activity equivalent to the non-immunized control group because immunization with highly antigenic viable conidia may have compensated for the immune-compromising effect of cortisone treatments.

Examining the last group, those models cortisone-compromised and immunized with non-viable conidia and the non-immunized cortisone-compromised group, it can be argued that there was a more dramatic repression of the immune response, because this group, based on the
nested ANOVA design, was least efficient in phagocytosis than the other model groups.

Using lower levels of cortisone and/or different injection intervals might have produced other results. For example, it was discovered that a dose of 0.005 mg cortisone per gram animal weight had little repressive response on the guinea pig immune system, nor did a 0.05 mg per gram body weight cortisone injection, administered in 3 divided doses at ten-day intervals. Conversely, it was found that a 0.05 mg cortisone per gram body weight injection administered in 7 divided doses at 2-day intervals killed all guinea pigs receiving that treatment, perhaps because the animals lost all immunological resistance or suffered toxic effects of the drug. Hence the 0.05 mg cortisone per gram body weight administered in 5 divided doses was found most suitable for this work.

A significant point in this research was the association between PEC activation states and cytotoxic activity. One can see (Fig. 1) that fungicidal effects occurred with those conidia phagocytosed by the non-cortisone compromised animals and immunized with viable conidia within half the time required for fungicidal effects to be first noticed in the non-cortisone compromised animals immunized with non-viable conidia and the normal controls. This observation has not previously been reported. One would, of course, expect the fungicidal effects of the cortisone-treated animals to be reduced for reasons stated above.

Controversy exists as to the role of the humoral immune system in relationship to mycotic disease. Recall that antibodies are produced in most mycotic infections. Their production of course depends in part on actual physical contact between fungi and/or fungal metabolites and/or B lymphocyte recognition of those antigens.
In the case of acute respiratory infection as in histoplasmosis, (caused by \textit{H. capsulatum}) detectable IgG, IgM, and IgA immunoglobulins corresponding to \textit{H. capsulatum} protein and polysaccharide antigens can be demonstrated from 10-14 days after the initial exposure to the fungus. Medically, to some extent, the detection of serum antibodies is useful in determining the status and progress of deep fungal infections, including human sporotrichosis. But evidence for direct antibody killing of fungi is scarce. Ferguson (11) however, reported that \textit{S. schenckii} conidial growth was inhibited by incubating conidia in the presence of antiserum. Lurie and others (31,33) have demonstrated that \textit{S. schenckii} conidia and/or yeast-phase cells are inhibited within the "asteroid body" , an eosinophilic immune complex precipitate occurring in histological sections from sporotrichic lesions, and may be converted into "chlamydospires" or (sclerotic bodies), i.e., thick-walled, non-proliferating cells, in the presence of increasing immunoglobulin titers. Also, it has been reported that \textit{C. neoformans} has been killed in the presence of anti-cryptococcal antibody. Also that the C-3 component of complement when activated by \textit{C. albicans}, via the alternate pathway, has been responsible for direct killing of this fungus. Others state that beneficial effects of antibody relative to the host against fungi are inconclusive mainly due to lack of serious study.

Aside from any fungicidal effects immunoglobulins may have upon fungi, the ability of PECs to phagocytose fungi may be enhanced when immunoglobulins coat fungal cells opsonizing them for PECs, augmenting phagocytosis.

It is commonly observed in clinical settings that only low numbers of fungal elements are normally seen in exudates, tissues, and aspirates from humans infected with \textit{S. schenckii}. Given that human lymphatic phago-
cytes and interstitial cutaneous histiocytes are approximately equal to the guinea pig PECs in phagocytic effectiveness, the result of the present work may offer insight into this phenomenon. Since S. schenckii antigens are probably ubiquitous in nature, especially on wood products, it is likely that humans are normally exposed to at least low numbers of these antigens. That exposure can often be confirmed via positive skin tests of persons never having had demonstrable sporotrichosis. It stands to reason, then, that immunological memory is present to elicit the immune response when S. schenckii is encountered in a wound. The PECs, of course activated in part by T memory cells, would be highly efficient in fungal cell killing. In fact, the observation of low numbers of fungal elements seen in fixed cutaneous and other localized atypical sporotrichic infections may be indicative of even more effective activation of human interstitial histiocytes. The results of Ferguson(11) indicate that this concept may reach maximum significance in the alveolar macrophage which are generally so efficient in conidial killing that pulmonary disease rarely occurs in the normal individual.

In the presence of demonstrable antifungal phagocytic activity as portrayed in this work (or equivalently in humans, the interstitial and paralymphatic phagocytes) the chronicity of sporotrichosis in the presence of highly efficient phagocytes may be based on the low numbers of normal or activated phagocytes present in tissues to remove S. schenckii completely, and/or the failure of some phagocytes to be activated, possibly because of reduced immune response to S. schenckii antigens actually present in the infection process due to fungal elements being walled off in granulomatous lesions and the formation of non-phagocytosing giant cells by macrophage fusion.
In sporotrichosis, as caused by traumatic implantation of \textit{S. schenckii} into cutaneous and lymphocutaneous tissues, after about 3 weeks from the initial time of fungal implantation, a small, hard movable, nontender nodule appears. Histological sections reveal this to be a granulomatous lesion containing fungal debris, walled off by phagocytes, elastic fibers, collagenous fibers, and fibrocytes. This wailing off of fungal cells may prevent B cells from recognizing fungal surface antigens and T cells from receiving processed internal, partially degraded, fungal antigens from giant cells, thus impairing the immune response. In chronic sporotrichic infections where the fungal elements multiply faster than they can be phagocytosed, however, they disseminate through lymphatic vessels, which are normally sparse in phagocyte numbers, so fungal proliferation may not be inhibited. Once these fungi have spread to a lymph node, the majority of fungi suspended in the lymph are removed by filtration and phagocytosed by both granulocytes and monocytes. Those fungal elements escaping phagocytosis are free to proliferate into the subcutaneous tissue and renew this process over and over until a regional lymph node is encountered. Rarely will \textit{S. schenckii} escape complete phagocytosis once contained in one of these nodes.
CHAPTER V

SUMMARY

Six guinea pig models were treated with \textit{S. schenckii} conidia in various combinations to ascertain the effects of peritoneal exudate cells (PECs) on these conidia, to find whether or not those PEC effects were fungicidal or fungistatic to \textit{S. schenckii} conidia, and to determine the effects cortisone compromise had upon PEC activity. Phagocytic responses were measured using these 6 animal models which included animals immunized with viable conidia, animals immunized with non-viable conidia, untreated controls, animals compromised with hydro-cortisone acetate but not immunized, animals both compromised and immunized with viable conidia and finally animals both compromised and immunized with non-viable conidia. Statistical analyses were used to compare the groups. It appears that animals immunized with viable conidia are more efficient in clearing these fungi via phagocytosis, that phagocytic rate and fungicidal properties are positively correlated, and that cortisone compromise, achieved through repeated cortisone injections into these animals, reduces both phagocytic ability and fungicidal properties of PECs.
REFERENCES CITED


