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IMMUNE RESPONSES IN RABBITS TO SPOROTHRIX SCHENCKII

by

Robert B. Ferguson

B.A., Olivet College, 1968

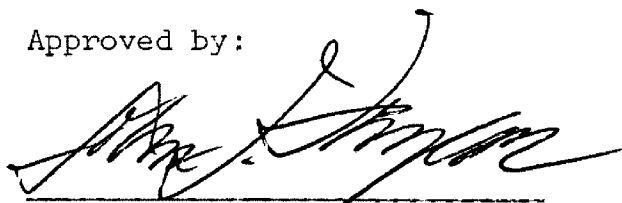
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Microbiology

Immune Response in Rabbits to Sporothrix schenckii (56 pp.)

Director: John J. Taylor 

The nature of the immune response to Sporothrix schenckii and its role in resistance to pulmonary sporotrichosis are equivocal. Although case reports indicate a relatively low incidence of pulmonary disease when contrasted with the typical lymphocutaneous infection, skin test surveys suggest that inapparent pulmonary infections may be quite common in areas endemic for the more characteristic lymphatic syndrome. Evidence for a more effective immune response to S. schenckii in pulmonary than in lymphocutaneous tissues is lacking.

The present study was undertaken to determine whether immunoglobulins contribute to resistance through opsonizing and/or fungistatic (fungicidal ?) activity, and to correlate the phagocytic and antifungal activities of alveolar macrophages with pulmonary resistance.

Rabbits sensitized with S. schenckii introduced by several routes were examined for their immune response. Humoral activity was assayed by agglutination, precipitation, and fungal inhibition. Cellular responses were examined using skin tests, macrophage migration inhibition analysis, phagocytic efficiency and fungistasis assays. Comparisons were made between sensitized and unsensitized animals.

Sera from both sensitized and unsensitized animals were fungicidal when mixed with S. schenckii conidia, but there was no well-defined difference evident in the majority of the dilutions tested. However, when compared with sera from unsensitized (normal) rabbits, sera from sensitized (immune) animals significantly enhanced both phagocytic and fungicidal activities of alveolar macrophages. Macrophages recovered from animals sensitized by inhalation possessed greater phagocytic and fungicidal activities than those from animals sensitized by other routes, and sensitization with viable cells produced greater macrophage activity than sensitization with killed cells.

The results suggest that inhalation of viable S. schenckii conidia leads to a dramatic sensitization of pulmonary macrophages, and a decreased potential for expression of a pulmonary syndrome. Immunoglobulins appear to contribute to phagocytic and perhaps antifungal resistance. Artificial immunization with living S. schenckii conidia confers a more efficient resistance in rabbits than immunization with killed cells.

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Chapter 1

INTRODUCTION

General Introductory Statements

Sporotrichosis is generally manifested as a chronic lymphocutaneous disease of man and other animals. It is acquired by cutaneous inoculation, or occasionally by inhalation of conidia produced by the polymorphic fungus, Sporothrix schenckii. Characteristics of the common form are frequently ulcerating and draining nodules of subcutaneous and cutaneous tissues and adjacent lymphatics. Spread to other tissues, such as the central nervous system, muscle, bone, and genitourinary tract may occur.

Schenck (46) at Baltimore's John Hopkins University in 1898, recorded the first clinical picture of sporotrichosis. Two years later, Hektoen and Perkins (20) reported the second case in the United States, described a sporotrichic skin lesion on the finger of a small boy, and named the fungus, Sporothrix schenckii. In the early 20th century, deBeurmann, Raymond, Matruchot, and Gougetot (46) reported numerous cases in France, and Schenck's organism was renamed, Sporotrichum schenckii. From the early 1900s until the 1920s, the incidence of sporotrichosis in Europe was high, but has decreased measurably since then.

In 1907, in Brazil, Lutz and Splendore (46) identified natural

sporotrichosis in rats and later Carougeau (46) described the infection in horses. Sporotrichosis has also been recorded in dogs, cats, insects, boars, mice, hamsters, mules, foxes, camels, and parrots (16,38, 46, 50, 58).

In 1962, Carmichael (46) suggested that there were indeed differences between Sporotrichum and Sporothrix based on sporulation. Despite these differences, there is a tendency among medical personnel to use the two names synonymously. Recent revisions in the taxonomy and systematics have resulted in the two genera being mutually exclusive. The generic name of Sporothrix has been adopted for the etiologic agent of sporotrichosis. Sporotrichum aureum, the lectotype of the genus Sporotrichum, is characterized on the basis of a new isolate as an imperfect basidiomycete (1, 9, 57). Sporothrix, unlike Sporotrichum, is classified solely as a hyphomycete; no clamp connections have been observed, thus negating any affinity with the basidiomycetes. Barron (3) related that in Sporothrix the conidia are sympodulospores and in Sporotrichum they are aleuriospores.

Taylor (53) suggested that S. schenckii is a heterogeneous form-species complex consisting of aniscigerous states of several Ophiostoma (=Ceratokystis) species. His conjecture has been supported by recent antigenic (43), rhamnomanan (56), and whole cell analyses (55) of S. schenckii isolates.

Ajello and Kaplan (1) described a new variant of S. schenckii, S. schenckii var. luriei which differs in the tissue phase from the typical yeast-like phase, in that the former had larger cells, produced sclerotica, and frequently multiplied by fission rather than by budding

alone.

In 1968, Mariat (36) hypothesized that physiological properties of Ophiostoma and S. schenckii are remarkably similar. Taylor (53) recognized similarities between S. schenckii and several species of Ophiostoma based on culture, morphologic, serologic characteristics, and mouse virulence. Andrieu (2) has recently determined that there was sufficient antigenic relationship among several strains of S. schenckii tested to assume that they were a homogeneous group, and that Ophiostoma sp. represented the perfect state of S. schenckii. Similar observations have been made by DeHoog (12).

The relatively slender hyphal dimensions of S. schenckii are of diagnostic significance (although not pathognomonic) since hyphae of most other pathogenic fungi are considerably broader. Spherical or pyriform asexual spores (2.0-5.0 μ m in length) develop laterally or sympodially from the conidiophores. Initially one may assume that the conidia are separated from the bearing structure by a short, but constant distance; but after careful oil immersion observation, fine hair-like stalks (sterigmata) of attachment are seen. By modern definition, Sporotrichum does not possess sterigmata; conidia are borne as aleurio-spores directly from the lateral hyphae, and thereby serve as another basis for differentiation.

Some of the conidia may produce one or more buds at their tips after separating from their conidiophores. Taylor (54) described the presence of secondary conidia and their mechanism of production. Primary sympodulospores may germinate to produce one or more sympodulae, which in turn bear secondary sympodulospores. It is likely that the

latter represent the yeast-like tissue phase of typical forms of S. schenckii.

At 37°C, or in host tissues, the mycelial phase converts to a so-called yeast-like phase. Strains isolated from nature vary in their ability to grow at 37°C. Tissue phase variation at the species level substantiates claims that S. schenckii should be referred to as polymorphic rather than a dimorphic organism (1). Phase transition is dependent on thermal state, but generally appears to be independent of the nutritional status of the supporting medium (30).

S. schenckii is distributed as a soil saprobe in temperate and tropical climates. Through skin testing, sporotrichosis is found to be highly endemic in humid areas such as Brazil, Uruguay, and South Africa. In Mexico, where the climate is, in contrast, considerably more arid, sporotrichosis poses as the most common subcutaneous mycosis. In temperate countries such as the United States, Canada, and France, infection is commonly encountered through gardening accidents.

The distribution of cases within a given population appears to be associated with occupation, rather than with genetic, age, or racial influences. Those individuals who are in the most frequent contact with the soil generally present the majority of infections.

According to Rippon (46), deBeurmann and Gougerot considered sporotrichosis to be an opportunistic disease, infecting only the pre-disposed. Other investigators assert that predisposition is not a requirement, citing many subclinical cases from which the organism has been cultured. Perhaps one may postulate that underlying disease, malnutrition, or a faulty immunological system may be necessary for the

dissemination of the disease, but not required for the common cutaneous and subcutaneous form of infection.

Early reports stated that numerous cigar-shaped bodies of the yeast-like phase were observed from sporotrichic lesions. But today, pathologists rarely find tissue-phase organisms by direct examination of lesion aspirates. Lurie (33) observed in tissue sections stained with hematoxylin and eosin (H&E) what appeared to be a capsular halo around the organisms, but later attributed this phenomenon to shrinkage of the fungus during the fixation process.

The asteroid body, which is a common histopathological observation in South American and South African cases, is rarely seen in the United States. It consists of a compact central mass, about which radiates a homogeneous eosinophilic material. The diameter of the central mass ranges from 5.0-10.0 μm and stains poorly with H&E. Serial sections are necessary to detect asteroid bodies, and they are more frequently observed from secondary than from primary lesions (46). There have been various theories as to the nature of their formation. Lurie (33) postulated that the homogeneous eosinophilic projections composing the stellate arms of the asteroid represent a host humoral response to antigenic stimulation. Although this host response theory seems to be the most recent and accepted view, other investigators have based its appearance on associated growth of particular bacteria, or the result of an actual product of the invading fungus. Lurie (34) stated that the asteroid body is formed by the precipitate of a soluble antigen-antibody complex which is deposited on the surface of the tissue-phase propagule.

Immunological Aspects

Natural resistance to sporotrichosis in unsensitized individuals is quite high. Although subclinical infections may occur in many healthy individuals, for some reason(s), they are frequently able to abort clinical disease. Using two groups of children (clinically asymptomatic for sporotrichosis), Pereira (45) skin tested them with sporotrichin. One group of children, from a low socioeconomic class, presented a 10.8% positive skin reactivity, whereas the other group of children, which was isolated in a sporotrichosis-free area, yielded a 6% reactivity. The resulting immunity to infection and/or disease is perhaps due largely to a humoral and/or a cell-mediated response mounted by the host. The characterization of the response leading to resistance to sporotrichosis is basically unknown, hence the relevance of the research conducted in this paper.

Once the etiologic agent successfully penetrates the host's primary line of defense, body constituents (humoral and/or cellular) must act if the host is to resist clinical disease. There is much speculation as to the immunological mechanism(s) involved in the suppression of clinical sporotrichosis.

The vast majority of research involving antifungal effects of cell-free serum has dealt with dermatophytic infections. Blank and co-workers (5) determined that cell-free serum was fungistatic to pellet inocula of Trichophyton mentagrophytes and T. rubrum. After prolonged incubation, the fungi began to grow out. Heating the serum to 56°C for 30 minutes reduced the inhibitory activity, but did not destroy it completely. Lorincz, Priestley, and Jacobs (32) reported dializable,

heat labile, antidermatophytic substances in human and mouse serum and tissue fluids.

Baum and Artis (4) were able to detect inhibitory influence of human cell-free serum on the growth of Cryptococcus neoformans, Histoplasma capsulatum, and S. schenckii. They postulated that the fungistasis was unrelated to past clinical infections and appeared to be a nonspecific resistance inherent in the serum. Of these three fungi tested, inhibition of S. schenckii was least dramatic, but still observable.

Roth and co-workers (47) suggested that the inhibition of a yeast (Candida albicans) by cell-free serum may be based on a different mechanism than that active against a dermatophyte. The antidermatophyte capacity of serum is present from birth and is in a similar concentration to that found in maternal blood. This antidermatophytic titer can be reduced by dialysis and heating the serum to 56°C for 30 minutes. The serum of "normal" human adults has inhibitory activity against C. albicans, but unlike the activity against dermatophytes, its level is much reduced until the third month of life. Heating the serum does not reduce the anti-Candida activity.

It appears that with certain fungi, complement may be required for fungistasis, either by itself, or in a helper role. Depending on the fungus, an acquired specific antibody response may be protective (as in C. albicans), whereas the serum factors directed against the dermatophytes may be totally nonspecific. Dr. Gerald Baum (personal communication) hopes that newer techniques of protein separation may help elucidate the fungistatic serum protein fractions.

Complement-fixing (CF) antibodies present in sporotrichosis, as in coccidiomycosis and histoplasmosis, do not seem to be protective (6, 17, 44, 48, 59). In fact, elevated CF antibody titers appear to parallel the activity of infection or degree of tissue involvement, rather than the degree of host resistance mounted. Jones and co-workers (25) followed the CF antibody titer during therapy of two patients with extracutaneous sporotrichosis. A drop in titer was manifested by clinical improvement. Norden (44) broadly stated that a rising CF titer in systemic mycoses is usually an unfavorable sign, indicating dissemination of the fungus.

Karlin and Nielsen (26) related that patients with articular or pulmonary sporotrichosis have higher agglutination titers than patients with the cutaneous form of the disease. Blumer (8) concluded from her comparative evaluation of 5 serological techniques that the slide latex agglutination had greater specificity and sensitivity than immunodiffusion, complement fixation, or indirect fluorescent antibody antibody in the diagnosis of sporotrichosis. Jones (25) concluded that either yeast or broth-coated latex antigens serve as a better reagent for detecting antibodies to S. schenckii than gel precipitation or complement fixation.

Karlin and Nielsen (26) postulated that individuals with humoral antibodies to S. schenckii probably also exhibit delayed hypersensitivity to the fungus. Skin testing is an extremely useful diagnostic tool in detecting individuals who are subclinically infected with S. schenckii (45). Norden (44) was unable to demonstrate a delayed dermal hypersensitivity or agglutination reaction in rabbits which received

a subcutaneous injection of viable mycelial-phase S. schenckii.

Rabbits in which Norden (44) administered a single intravenous injection of viable yeast-phase S. schenckii, demonstrated positive agglutination and precipitin reactions 10-15 days following infection, but the rabbits showed no visible signs of disease. Rabbits which received repeated daily intravenous and intraperitoneal injections in increasing doses, showed a slight appetite and weight loss. Norden (44) stated that pigmentation or phase variation had no effect on the agglutination response. Agglutination reactions were fairly equal regardless whether antigenic stimulation was provided by pigmented strains (yeast- and mycelial-phase) or nonpigmented strains (yeast- and mycelial-phase). There was no difference in stimulatory response of agglutinins or precipitins when he altered his antigen by subjecting it to heat or formalin treatment.

In vitro complement fixation and agglutination titers appear to discount any protective value of these antibodies against sporotrichosis. However, Lurie's (33) studies indicate that in sporotrichosis, the presence of asteroid bodies probably reflects an in vivo humoral response that effectively localizes the antigen and prevents dissemination. If opsonins also constitute, in part, the in vivo humoral response of the asteroid body (14, 37, 41), then it would appear that antibodies may be active in resistance to S. schenckii by aiding phagocytosis.

Howard (21) working with mouse peritoneal macrophages, determined that the generation time of H. capsulatum in specifically sensitized macrophages was significantly increased over the generation time within nonsensitized macrophages. Sensitized macrophages that were washed prior to sensitization, had a decreased inhibitory effect, but fungi-

static activity was restored when sensitized lymphocytes were added. This would imply the importance of lymphocytes as mediators of fungistasis. Although lymphocytes are most likely the mediators of this reaction, they have no direct effect themselves (29).

Howard (22, 23) emphasized the observation that yeast-phase H. capsulatum still retained viability following a 24-hour residence within sensitized macrophages.

Epidemiologic studies indicate a relatively low incidence of pulmonary sporotrichosis (primary and secondary) compared to the typical lymphocutaneous form. Despite this, Baum (5) urged caution in dismissing the possibility of clinical pulmonary sporotrichosis. Feeling that the disease has frequently been overlooked in the past, he attributed the lack of reports to the following: pulmonary sporotrichosis markedly resembles other granulomatous diseases based on x-rays and clinical patterns, lack of familiarity with mycotic techniques, difficulty in finding the organism histologically, and the mere lack of appreciation that S. schenckii can cause pulmonary disease. Skin test surveys suggest that inapparent pulmonary sporotrichosis may be common in endemic areas, but supporting data and evidence for enhanced pulmonary response to conidia contact are lacking.

The nature of the immune response and its relationship to pulmonary sporotrichosis is equivocal. The conducted research was intended to determine whether immunoglobulins can contribute to host resistance through opsonizing or fungistatic (fungicidal ?) activity. Additionally, attempts were made to correlate resistance with phagocytic and fungistatic (fungicidal ?) action of alveolar macrophages.

Immune responses of rabbits to S. schenckii will be determined fol-

lowing (1) intramuscular injection of viable conidia and hyphal fragments, (2) immunization with merthiolate-killed conidia and hyphal fragments injected intravenously, and (3) pulmonary sensitization with aerosolized viable conidia.

Agglutination and/or precipitation titers will be determined on sera collected at various time intervals from rabbits sensitized by each of the above mentioned routes.

Onset of delayed skin reactivity will be determined in each of the experimental models.

Cell-free serum studies will be conducted to determine whether sera possess fungistatic activities.

Alveolar macrophages will be procured from both sensitized and unsensitized rabbits. Using these cells, in vitro correlates of delayed hypersensitivity will be conducted by migration inhibition factor (MIF) assays and phagocytic and fungistatic (fungicidal ?) indices will be determined. All cellular studies will be performed with "immune" and "normal" serum to determine if there are humoral components which affect phagocytosis and fungistasis.

Chapter 2

MATERIALS AND METHODS

Fungal Cultures

Cultures of S. schenckii 425 isolated from a case of sporotrichosis were initially obtained from the Center for Disease Control, Atlanta, Georgia. From stock cultures, subcultures were inoculated onto slants of potato dextrose agar (PDA) (Difco Laboratories, Detroit, Michigan), Sabouraud dextrose agar (Difco Laboratories), and Mycosel agar (Baltimore Biological Laboratory, Cockeysville, Maryland), and incubated in screw-cap tubes at 25°C in a humidity-controlled incubator.

Broth flasks were prepared by inoculating 500 ml Erlenmeyer flasks containing 100 ml Sabouraud dextrose broth (Difco Laboratories) plus 0.1% yeast extract (Difco Laboratories) with S. schenckii 425. These flasks were incubated at room temperature on an Eberbach horizontal shaker for a period from 10 days to 2 weeks.

Tissue Culture Media and Buffer Solutions

Minimal essential medium (MEM) with Earle's balanced salt solution (Grand Island Biological Company, Grand Island, New York) was used as the tissue culture medium. Crystalline MEM was reconstituted with triple distilled water. The medium was buffered by addition of 2.2 g/l NaHCO₃. Penicillin and streptomycin were incorporated to a final con-

centration of 100 units and 100 µg respectively, to retard bacterial growth. The solution was adjusted to pH 6.8 with 1N HCl or 1N NaOH. Following filter sterilization, the pH of the MEM was 7.0 +/- 0.1. The medium was then tightly sealed and stored at 4°C.

Phosphate-buffered saline (PBS) was utilized as a physiological buffering medium. Stock buffer solutions of Na₂HPO₄ and KH₂PO₄ (0.15M) were prepared in deionized distilled water and added to an equal volume of 0.85% NaCl. The PBS (pH 7.2) was autoclaved and stored in tightly capped prescription bottles at room temperature.

Antigen Preparation (unless stated otherwise, all cellular antigens had a concentration of 10⁷ colony forming units/ml (cfu))

1. Merthiolate-killed S. schenckii 425: Merthiolate (final concentration of 1:10,000) was added to aliquots of hyphae and conidia. After several hours incubation at 37°C, platings were made on nutrient agar (Difco Laboratories) and Mycosel agar to check for contamination and loss of viability. The antigen suspensions were stored in PBS at 4°C.

2. Viable S. schenckii 425: Viable cultures were grown in Sabouraud dextrose broth containing 0.1% yeast extract in flasks incubated at room temperature on an Eberbach horizontal shaker. Conidia were isolated from the hyphal mat by passing the culture material through an absorbent pad (47 mm diameter; 0.85 mm thick) (Millipore Filter Corporation, Bedford, Massachusetts). The conidial suspension was washed twice in PBS, placed in 3.0 ml aliquots, and stored at 4°C.

3. S. schenckii 425 protoplasm: Crude protoplasm was obtained by grinding the fungus in a mortar and pestle with type 305 alumina (Sigma Chemical Company, St. Louis, Missouri). Following 1 hour of pulverization at 4°C, the mixture was centrifuged and the supernatant fluid was concentrated by pervaporation at 4°C. The protein concentration of the protoplasm suspension was estimated by ultraviolet absorption at 260 nm and 280 nm (31), and sterilized by passing it through a 0.22 µm filter (Millipore Filter Corporation). Aliquots consisting of 0.5 ml were prepared and stored in the frozen state.

Immunization of Test Animals

1. Inoculation of viable S. schenckii 425 by intramuscular injection: Rabbits were initially sensitized with 3.0 ml viable S. schenckii 425 inoculated intraperitoneally (ip) and 1.0 ml intramuscularly (im). Monthly im booster injections of viable fungus were given and the animals were sacrificed approximately 10 days following the last booster and after becoming skin test positive.

2. Inoculation of merthiolate-killed S. schenckii 425 by intravenous injection: Rabbits were initially sensitized with 1.0 ml merthiolate-killed S. schenckii 425 given intravenously (iv). Booster iv injections of the same dosage were given every 3-4 days until the animals were skin test positive. The rabbits were sacrificed 3 days following the last booster injection.

3. Inoculation by aerosolization of viable S. schenckii 425: Test and control rabbits were blindfolded and placed in the Middle-

brook chamber. A suspension of viable S. schenckii conidia (6.0 ml suspension) was nebulized into the compartment. Immediately following aerosolization, duplicate control rabbits were removed from the chamber and sacrificed with a nembutal overdose. The lungs from these animals were removed, weighed, homogenized in a Waring blender, and plated onto Mycosel agar to determine the number of conidia that reached the lungs.

To establish what effect the homogenization procedure would have on the viability of conidia, lungs of a rabbit were removed, weighed, and placed in a Waring blender jar containing a known number of viable conidia. Plate counts were performed following homogenization.

Ten days after becoming skin test positive, duplicate test animals were sacrificed. Lungs were lavaged and alveolar macrophages were obtained for cellular studies described below.

Another set of duplicate test animals received a second inoculum of conidia delivered by aerosol 10 days after becoming skin test positive. Two control rabbits simultaneously exposed were sacrificed immediately and plate counts were made of lung homogenates. Five days later, these 2 test rabbits were sacrificed and alveolar macrophages obtained for upcoming cellular studies

In Vivo Testing

1. Skin testing: Test animals (before and after sensitization) and control animals were skin tested by intradermal injection of 0.1 ml crude protoplasm (0.27 mg protein/0.1 ml) and washed merthiolate-killed S. schenckii 425. Intradermal injections consisting of 0.1 ml

PBS and 0.1 ml distilled water were used as skin test controls. Arthus and delayed skin reactions were recorded qualitatively (without the use of calipers).

In Vitro Testing

1. Agglutination testing: Test animals were bled prior to, and at various intervals following sensitization. Aliquots of whole sera and serum dilutions (to 1:2048) were combined with an equal volume of merthiolate-killed S. schenckii 425. The tubes were mixed, sealed, and placed in a 37°C incubator for 2 hours, followed by overnight storage at 4°C. The agglutination titers were observed microscopically and were compared to agglutination titers of similar dilutions of sera from unsensitized rabbits. The comparison was utilized as the baseline value.

Agglutination titers of sera from 8 unsensitized rabbits were determined using saline controls (PBS plus conidia); these titers were also used as a baseline.

"Immune" sera were so designated by their ability to agglutinate merthiolate-killed antigen at titers in excess of "normal" sera. Increased fungistasis of cell-free serum (see below) was also used as a criterion to define "immune" serum.

2. Precipitin testing: Sera from test animals (excluding those exposed to aerosols) were used to determine precipitin titers. Serum (showing no precipitin bands) from unsensitized rabbits was used as a baseline control. Serial dilutions (to 1:512) of the test and control

sera were prepared. Tubes 6 mm in diameter were partially filled with undiluted crude protoplasm (0.27 mg protein/0.1 ml) and overlaid with whole serum or the diluted serum, forming a definite interface between the antigen and antiserum. These tubes were sealed and left at room temperature in the vertical position. Periodic observations for the appearance of precipitin bands over a 24-hour period were made.

3. Cell-free serum assays: Five dilutions were made from serum of unsensitized and sensitized rabbits and combined with suspensions of known viable S. schenckii 425 populations. Using MEM as a tissue culture support medium, the suspensions (serum plus conidia) were maintained over a period of 12 hours. Duplicate platings on Mycosel agar were made at 5 different time intervals. Differences in colony counts between test and control rabbits were plotted.

4. Cellular studies:

- a. Harvesting alveolar macrophages: The technique of Myrvik, Leake, and Fariss (39) was used with the following exceptions: the lungs were not removed from the rabbits, but a 45-ml volume of cold, heparinized MEM was injected into the trachea of the sacrificed rabbit; the thorax was gently massaged, the trachea severed, and the macrophages collected by gravity into cold siliconized glassware. The macrophage yield was determined by direct cell count.
- b. Migration inhibition factor (MIF): The technique of George and Vaughan (18) was used with the following exceptions:

the capillary tubes, 2/3 filled with cellular suspension, were sealed by heat; the coverslip was held in place, not with paraffin, but with a rubber gasket and a securable screw type washer. Following incubation, the migration pattern of the macrophages was enlarged with the aid of a Simon Omega model D-2 enlarging rapture. The patterns were then traced on bond paper and weighed on a Mettler H2OT balance. The index of migration was calculated by comparison with control migration patterns (28).

All MIF tests were carried out incorporating 10% serum from either unsensitized or sensitized rabbits. The state of sensitization was previously determined by agglutination testing. Delayed hypersensitivity of the cellular constituents was ascertained, to this point, by skin testing.

- c. Phagocytic and fungistatic assays: With the remaining macrophages (those not used in the MIF), phagocytic and fungistatic tests were performed. Alveolar macrophages at the concentration of 10^5 /ml (10^6 /total volume) were combined with 10^6 conidia/ml in MEM with 10% "immune" serum or 10% "normal" serum. At 8 different time intervals over a 48-hour period, smears were made of the macrophage/conidia suspension, and stained with periodic acid schiff (PAS) as described by Humison (24). Upon examination of these slides, 50 macrophages were counted. The actual

number of intracellular conidia based on these 50 macrophages was plotted versus time. The maximum number of intracellular conidia/macrophage was also recorded at each time interval.

Following preparation of the smear for the phagocytic index, aliquots of the macrophage/conidia suspension were taken at each time interval (8 intervals over the same 48-hour period) and passed through a distilled water dilution followed by a series of additional 10-fold dilutions in PBS. The first dilution (10^{-1}) in distilled water was performed in order to create a hypotonic environment to lyse the macrophages and expose the phagocytized, but still viable, conidia for plate counts. The subsequent 10-fold dilutions in PBS were performed to re-create physiological conditions, so as not to lyse conidia, and also to establish a dilution of viable conidia which could be counted (approximately 100 colonies/plate). A conidia control (conidia in MEM without macrophages) was performed to establish a baseline viability curve and a basis to compare the effect of macrophages on conidia. The platings were made in order to determine what, if any, loss of conidial viability had occurred in the presence of macrophages over the 48-hour period. Also the effects of "immune" and "normal" sera on the cellular clearance of conidia were noted.

Chapter 3

RESULTS

All the test animals were skin test negative when checked prior to sensitization. All control sites (PBS and distilled water) were also nonreactive.

A rabbit sensitized with viable S. schenckii 425 im reacted to intradermal injections of merthiolate-killed S. schenckii 425 conidia and hyphae, as well as to crude protoplasm (0.267 mg protein/0.1 ml). Arthus and delayed reactions were negative 2 days after sensitization, but both were positive at 5 days. In another rabbit similarly sensitized, protoplasm (3.0 mg protein/0.1 ml) yielded a positive Arthus reaction, but only a borderline delayed reaction on the fifth day following injection.

Two and 5 days following initial injection, the rabbit which received merthiolate-killed S. schenckii 425 iv was nonreactive to intradermal injections of both killed cells and protoplasm (0.267 mg protein/0.1 ml). However, positive Arthus and delayed reactions appeared 1 week post-injection. Also a similarly sensitized rabbit yielded a positive Arthus reaction, but a negative delayed reaction at the 1 week interval.

Two days following aerosol immunization, 4 rabbits were nonreactive to intradermal injections of killed cells and protoplasm (0.267 mg protein/0.1 ml). On the third day following initial exposure, the entire

group of rabbits was Arthus positive, as well as being skin test (delayed reaction) positive to both antigens.

Two rabbits sensitized with viable S. schenckii 425 im showed the first qualitative agglutinin reaction at 1 week. These agglutination tests (figure 1) indicated the 2 rabbits had a maximum titer of 1:512 and 1:1024 respectively.

A rabbit sensitized with repeated iv injections of merthiolate-killed S. schenckii 425 did not show a positive agglutinin reaction until 2 weeks. The maximum agglutinin titer mounted by this rabbit was 1:512 at 158 days post-sensitization.

Two rabbits exposed to a single and double aerosol inoculation of viable S. schenckii 425 conidia (4 rabbits altogether) showed initial positive agglutinin reactions at 3 and 5 days respectively. Two rabbits similarly exposed to a single aerosol of viable conidia yielded a 1:64 agglutinin titer at 13 days following aerosolization. Two other rabbits, both of which were re-exposed to a second aerosol of viable conidia had a 1:64 titer at 16 and 19 days respectively post-aerosolization.

One of the 2 rabbits sensitized with viable S. schenckii 425 im first showed a positive precipitin reaction at approximately 3 weeks, whereas the other rabbit similarly sensitized did not show a positive reaction until 48 days. Precipitin testing (figure 3) showed these 2 rabbits maximum titers of 1:4 at 85 and 176 days respectively.

The rabbit sensitized with repeated iv injections of merthiolate-killed S. schenckii 425 yielded the first observable precipitin titer 171 days following initial inoculation. The first reaction (and maxi-

mum titer) was 1:2.

Fungistatic activities of cell-free serum from all test animals and control animals are fully outlined in table 1.

Migration inhibition factor (MIF) assays are presented in table 2. The data have been tabulated in terms of % migration (migration index) when compared to controls lacking antigen.

A large composite outline of fungicidal activity of rabbit alveolar macrophages in 10% "immune" serum or 10% "normal" serum is summarized in table 3. The tabulated values indicate the viable population of conidia remaining at each time interval.

Phagocytic activities of macrophages from the lungs of both sensitized and unsensitized rabbits are indicated in figures 4-8.

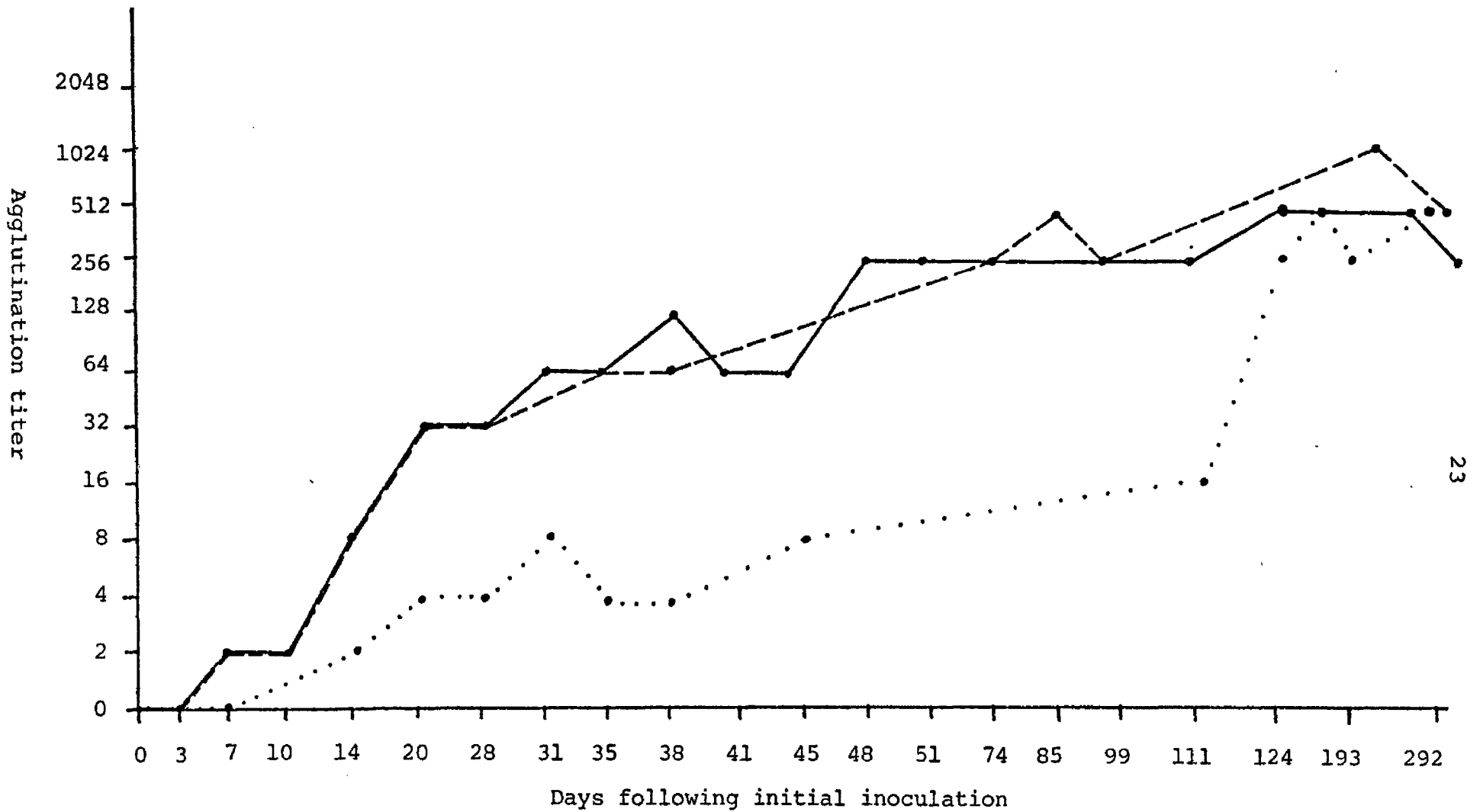


Figure 1 Agglutination titers of sera collected from 2 rabbits sensitized with viable *S. schenckii* 425 im (solid line and broken line) and 1 rabbit sensitized with merthiolate-killed *S. schenckii* 425 iv (dotted line)

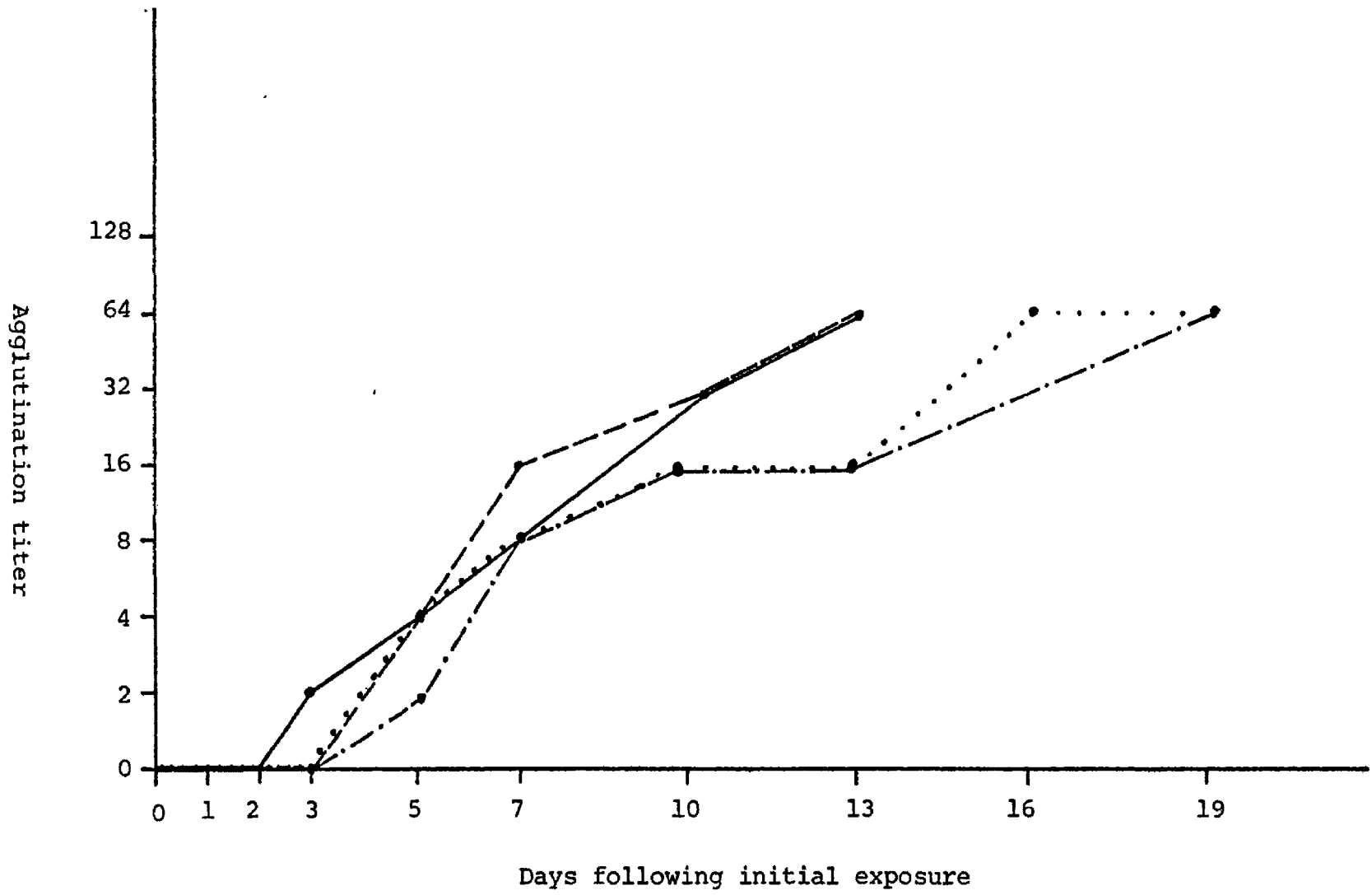


Figure 2 Agglutination titers of sera collected from 2 rabbits which received 1 aerosol exposure (solid line and broken line) of viable S. schenckii 425 conidia and 2 rabbits which received 2 aerosol exposures (dotted line and dash-dot line) of viable S. schenckii 425 conidia

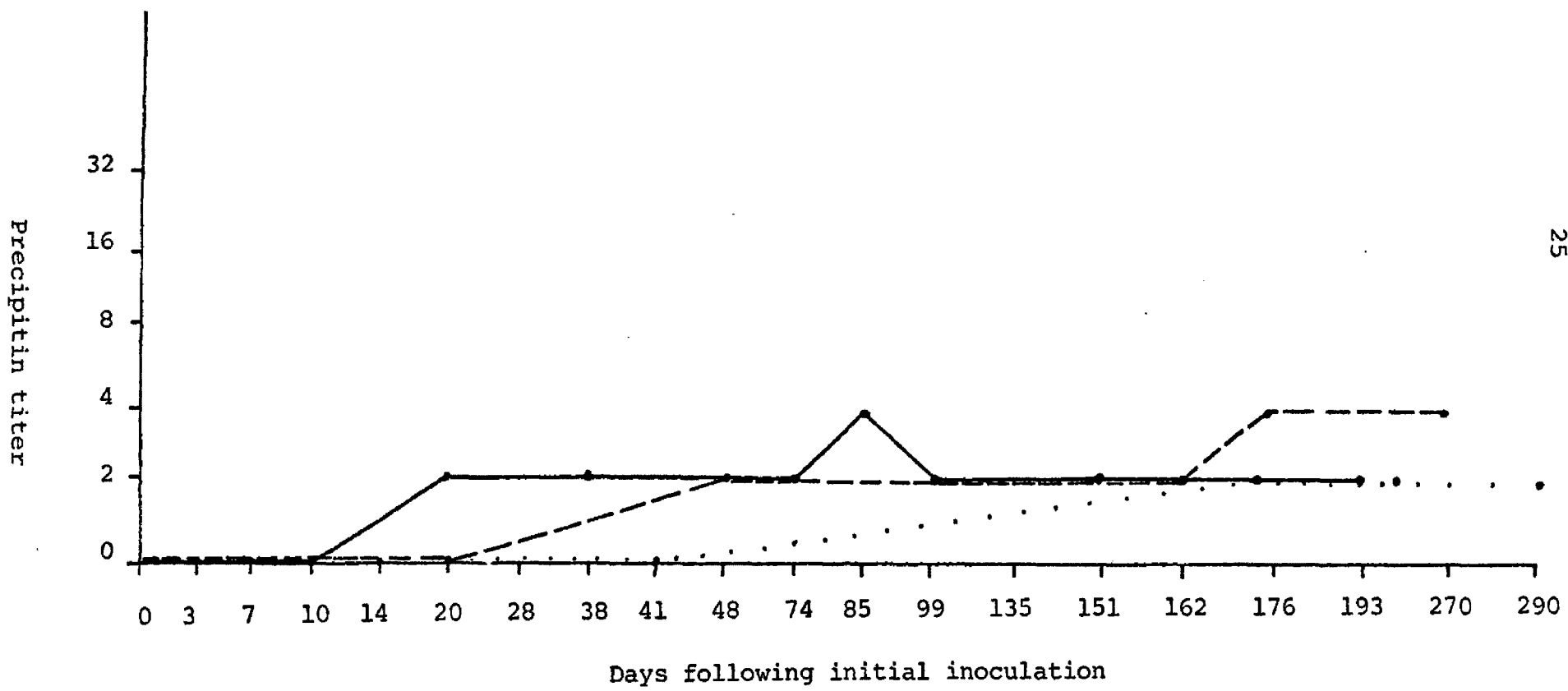


Figure 3 Precipitin titers of sera collected from 2 rabbits sensitized with viable S. schenckii 425 im (solid line and broken line) and 1 rabbit sensitized with merthiolate-killed S. schenckii 425 iv (dotted line)

Table 1 Fungistatic Activities of Cell-Free Serum From Sensitized and Unsensitized Rabbits

<u>Sensitized Rabbits (Test)</u>		<u>Unsensitized Rabbits (Control)</u>	
<u>Mode of Sensitization</u>			
Rabbit sensitized with viable <u>S. schenckii</u> 425 i.m.			
<u>Serum dilutions:</u>	<u>Difference in colony number between 0 hrs. and 12 hrs.</u>	<u>Serum dilutions:</u>	<u>Difference in colony number between 0 hrs. and 12 hrs.</u>
1:10	-8	1:10	-35
1:50	+10	1:50	+20
1:100	-50	1:100	-31
1:200	+2	1:200	+1
1:500	+6	1:500	+30
			<u>Difference in the changes between the test and control rabbits</u>
			+27
			-10
			-19
			+1
			-24
Rabbit sensitized with viable <u>S. schenckii</u> 425 i.m.			
<u>Serum dilutions:</u>	<u>Difference in colony number between 0 hrs. and 12 hrs.</u>	<u>Serum dilutions:</u>	<u>Difference in colony number between 0 hrs. and 12 hrs.</u>
1:10	+2	1:10	-7
1:50	-54	1:50	+1
1:100	-20	1:100	+57
1:200	-27	1:200	+30
1:500	-3	1:500	+37
			<u>Difference in the changes between the test and control rabbits</u>
			+9
			-55
			-77
			-57
			-40
Rabbit sensitized with merthiolate killed <u>S. schenckii</u> 425 i.v.			
<u>Serum dilutions:</u>	<u>Difference in colony number between 0 hrs. and 12 hrs.</u>	<u>Serum dilutions:</u>	<u>Difference in colony number between 0 hrs. and 12 hrs.</u>
1:20	-81	1:20	-145
1:100	-109	1:100	-8
1:200	-109	1:200	+83
1:400	-98	1:400	+89
1:1000	-69	1:1000	+24
			<u>Difference in the changes between the test and control rabbits</u>
			+64
			-101
			-192
			-187
			-93
Rabbit which received one aerosolization with viable <u>S. schenckii</u> 425 conidia			
<u>Serum dilutions:</u>	<u>Difference in colony number between 0 hrs. and 12 hrs.</u>	<u>Serum dilutions:</u>	<u>Difference in colony number between 0 hrs. and 12 hrs.</u>
1:10	-53	1:10	-27
1:50	-29	1:50	-25
1:100	-8	1:100	+21
1:200	-13	1:200	+3
1:500	0	1:500	+13
			<u>Difference in the changes between the test and control rabbits</u>
			-26
			-4
			-29
			-16
			-13
Rabbit which received one aerosolization with viable <u>S. schenckii</u> 425 conidia			
<u>Serum dilutions:</u>	<u>Difference in colony number between 0 hrs. and 12 hrs.</u>	<u>Serum dilutions:</u>	<u>Difference in colony number between 0 hrs. and 12 hrs.</u>
1:10	-34	1:10	-27
1:50	-23	1:50	-25
1:100	+8	1:100	+21
1:200	+9	1:200	+3
1:500	-1	1:500	+13
			<u>Difference in the changes between the test and control rabbits</u>
			-7
			-2
			-13
			+6
			-14
Rabbit which received two aerosolizations with viable <u>S. schenckii</u> 425 conidia			
<u>Serum dilutions:</u>	<u>Difference in colony number between 0 hrs. and 12 hrs.</u>	<u>Serum dilutions:</u>	<u>Difference in colony number between 0 hrs. and 12 hrs.</u>
1:10	-31	1:10	-27
1:50	-32	1:50	-25
1:100	+28	1:100	+21
1:200	-17	1:200	+3
1:500	-26	1:500	+13
			<u>Difference in the changes between the test and control rabbits</u>
			-4
			-7
			+7
			-20
			-39
Rabbit which received two aerosolizations with viable <u>S. schenckii</u> 425 conidia			
<u>Serum dilutions:</u>	<u>Difference in colony number between 0 hrs. and 12 hrs.</u>	<u>Serum dilutions:</u>	<u>Difference in colony number between 0 hrs. and 12 hrs.</u>
1:10	-46	1:10	-27
1:50	-48	1:50	-25
1:100	+23	1:100	+21
1:200	+20	1:200	+3
1:500	0	1:500	+13
			<u>Difference in the changes between the test and control rabbits</u>
			-19
			-23
			+2
			+17
			+13

Alveolar Macrophage Source

Migration Index

	No Antigen Incorporated	<u>S. schenckii</u> 425 Protoplasm (0.27 mg protein/0.1 ml) Incorporated	Merthiolate-killed <u>S. schenckii</u> 425 (10 ⁶ cfu/0.1 ml) Incorporated
Unsensitized Rabbits			
*a	100%	94.3% *** (94.6%, 94.0%)	96.2% *** (95.2%, 97.2%)
b	100%	93.6% * (92.7%, 94.5%)	97.8% *** (97.0%, 98.6%)
Rabbits Sensitized to Merthiolate-killed <u>S. schenckii</u> 425 iv			
*a	100%	68.2% *** (65.1%, 71.3%)	66.7% *** (62.8%, 70.6%)
b	100%	74.1% * (73.7%, 74.5%)	65.5% *** (63.8%, 67.2%)
Rabbits Sensitized to Viable <u>S. schenckii</u> 425 im			
*a	100%	77.6%	67.3%
**b	100%	78.6%	73.2%
Rabbits Sensitized to 1 Aerosol of Viable <u>S. schenckii</u> 425			
*a	100%	77.0% *** (75.4%, 78.6%)	67.6% *** (67.2%, 67.9%)
b	100%	77.9% * (80.3%, 75.5%)	72.3% *** (73.2%, 74.1%)
Rabbits Sensitized to 2 Aerosols of Viable <u>S. schenckii</u> 425			
*a	100%	81.6% *** (76.4%, 86.7%)	65.4% *** (62.7%, 68.0%)
b	100%	82.7% * (81.0%, 84.3%)	71.7% *** (77.6%, 65.7%)

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Table 2 Migration Inhibition Factor

*a= system contained 10% "immune" serum
 **b= system contained 10% "normal" serum
 ***() = results from 2 separate rabbits representing average

Table 3. FUNGICIDAL ACTIVITIES OF RABBIT ALVEOLAR MACROPHAGES IN "IMMUNE" AND "NORMAL" SERA

	TIME (Hrs.)										Overall Change			
	0	3	5	9	12	15	24	48						
Conidia control (no macrophages)														
"immune serum"	5.3 X 10 ⁵ /ml ±(±0.3) ** (10.0%)	4.9 X 10 ⁵ /ml ±(±0.0) ** (-7.5%)	4.4 X 10 ⁵ /ml ±(±0.3) ** (-11.0%)	3.9 X 10 ⁵ /ml ±(±0.1) ** (-18.0%)	3.7 X 10 ⁵ /ml ±(±0.1) ** (-50.0%)	4.4 X 10 ⁵ /ml ±(±0.2) ** (+16.5%)	4.2 X 10 ⁵ /ml ±(±0.3) ** (-55.5%)	4.4 X 10 ⁵ /ml ±(±0.6) ** (+2.6%)					-17.5%	
"normal serum"	6.5 X 10 ⁵ /ml ±(±0.4) ** (10.0%)	5.6 X 10 ⁵ /ml ±(±0.2) ** (-11.5%)	5.2 X 10 ⁵ /ml ±(±0.0) ** (-7.5%)	5.1 X 10 ⁵ /ml ±(±0.3) ** (-2.0%)	4.5 X 10 ⁵ /ml ±(±0.2) ** (-12.0%)	5.4 X 10 ⁵ /ml ±(±0.3) ** (+17.0%)	5.0 X 10 ⁵ /ml ±(±0.2) ** (+2.0%)	6.0 X 10 ⁵ /ml ±(±0.5) ** (+30.0%)						-5.0%
Unsensitized rabbit														
"immune serum"	6.3 X 10 ⁵ /ml ±(±0.1) ** (10.0%)	4.3 X 10 ⁵ /ml ±(±0.6) ** (-32.5%)	3.5 X 10 ⁵ /ml ±(±0.5) ** (-19.0%)	2.5 X 10 ⁵ /ml ±(±0.3) ** (-28.5%)	3.2 X 10 ⁵ /ml ±(±0.5) ** (-20.5%)	3.1 X 10 ⁵ /ml ±(±0.6) ** (-24.0%)	1.9 X 10 ⁵ /ml ±(±0.3) ** (-38.5%)	2.1 X 10 ⁵ /ml ±(±0.3) ** (-18.5%)						-67.0%
"normal serum"	8.2 X 10 ⁵ /ml ±(±0.5) ** (10.0%)	7.4 X 10 ⁵ /ml ±(±0.3) ** (-10.0%)	7.0 X 10 ⁵ /ml ±(±0.2) ** (-6.0%)	6.3 X 10 ⁵ /ml ±(±0.5) ** (-18.5%)	6.7 X 10 ⁵ /ml ±(±0.2) ** (-6.5%)	6.9 X 10 ⁵ /ml ±(±0.9) ** (+8.0%)	6.4 X 10 ⁵ /ml ±(±0.4) ** (-4.5%)	6.9 X 10 ⁵ /ml ±(±0.1) ** (+7.5%)						-16.0%
Rabbit sensitized with merthiolate killed <i>S. schenckii</i> 425 1.v.														
"immune serum"	5.3 X 10 ⁵ /ml ±(±0.6) ** (10.0%)	2.5 X 10 ⁵ /ml ±(±0.2) ** (-38.0%)	2.9 X 10 ⁵ /ml ±(±0.5) ** (+13.0%)	2.1 X 10 ⁵ /ml ±(±0.4) ** (-28.5%)	2.3 X 10 ⁵ /ml ±(±0.2) ** (-18.0%)	2.0 X 10 ⁵ /ml ±(±0.1) ** (-13.0%)	9.8 X 10 ⁴ /ml ±(±0.3) ** (-51.0%)	1.3 X 10 ⁵ /ml ±(±0.1) ** (-22.5%)						-75.0%
"normal serum"	4.8 X 10 ⁵ /ml ±(±0.2) ** (10.0%)	3.2 X 10 ⁵ /ml ±(±0.7) ** (-34.0%)	2.9 X 10 ⁵ /ml ±(±0.1) ** (-18.0%)	3.1 X 10 ⁵ /ml ±(±0.6) ** (+8.0%)	2.7 X 10 ⁵ /ml ±(±0.1) ** (-21.0%)	2.6 X 10 ⁵ /ml ±(±0.1) ** (-4.0%)	1.7 X 10 ⁵ /ml ±(±0.1) ** (-35.0%)	2.6 X 10 ⁵ /ml ±(±0.2) ** (+37.0%)						-46.0%
Rabbit sensitized with viable <i>S. schenckii</i> 425 1.v.														
"immune serum"	4.6 X 10 ⁵ /ml ±(±0.1) ** (10.0%)	3.0 X 10 ⁵ /ml ±(±0.2) ** (-35.5%)	2.3 X 10 ⁵ /ml ±(±0.1) ** (-23.5%)	1.8 X 10 ⁵ /ml ±(±0.2) ** (-22.5%)	1.9 X 10 ⁵ /ml ±(±0.3) ** (+5.0%)	1.6 X 10 ⁵ /ml ±(±0.1) ** (-15.0%)	9.6 X 10 ⁴ /ml ±(±0.1) ** (-40.0%)	1.0 X 10 ⁵ /ml ±(±0.3) ** (-44.5%)						-76.0%
"normal serum"	6.1 X 10 ⁵ /ml ±(±0.1) ** (10.0%)	4.1 X 10 ⁵ /ml ±(±0.3) ** (-33.5%)	4.3 X 10 ⁵ /ml ±(±0.1) ** (+5.0%)	3.8 X 10 ⁵ /ml ±(±0.7) ** (-18.0%)	3.6 X 10 ⁵ /ml ±(±0.5) ** (-9.5%)	3.2 X 10 ⁵ /ml ±(±0.4) ** (-11.5%)	3.0 X 10 ⁵ /ml ±(±0.0) ** (-5.0%)	3.1 X 10 ⁵ /ml ±(±0.4) ** (+2.5%)						-49.5%
***Rabbit which received one aerosolization of viable <i>S. schenckii</i> 425 conidia														
"immune serum"	5.1 X 10 ⁵ /ml ±(±0.6) ** (10.0%)	3.1 X 10 ⁵ /ml ±(±0.4) ** (-39.5%)	3.0 X 10 ⁵ /ml ±(±0.6) ** (-9.0%)	1.9 X 10 ⁵ /ml ±(±0.3) ** (-36.0%)	1.5 X 10 ⁵ /ml ±(±0.2) ** (-28.5%)	1.3 X 10 ⁵ /ml ±(±0.3) ** (-14.5%)	5.0 X 10 ⁴ /ml ±(±0.2) ** (-60.0%)	5.5 X 10 ⁴ /ml ±(±0.2) ** (-69.5%)						-89.5%
"normal serum"	5.3 X 10 ⁵ /ml ±(±0.5) ** (10.0%)	4.5 X 10 ⁵ /ml ±(±0.5) ** (-15.5%)	4.3 X 10 ⁵ /ml ±(±0.8) ** (-5.5%)	4.2 X 10 ⁵ /ml ±(±0.8) ** (-2.5%)	3.4 X 10 ⁵ /ml ±(±0.9) ** (-20.5%)	2.8 X 10 ⁵ /ml ±(±0.4) ** (-15.0%)	1.9 X 10 ⁵ /ml ±(±0.3) ** (-33.0%)	2.2 X 10 ⁵ /ml ±(±0.4) ** (+14.0%)						-54.0%
***Rabbit which received two aerosolizations of viable <i>S. schenckii</i> 425 conidia														
"immune serum"	5.5 X 10 ⁵ /ml ±(±0.4) ** (10.0%)	4.4 X 10 ⁵ /ml ±(±0.9) ** (-20.5%)	3.6 X 10 ⁵ /ml ±(±0.1) ** (-15.0%)	3.1 X 10 ⁵ /ml ±(±0.3) ** (-38.5%)	1.9 X 10 ⁵ /ml ±(±0.3) ** (-38.5%)	1.2 X 10 ⁵ /ml ±(±0.2) ** (-37.0%)	8.5 X 10 ⁴ /ml ±(±0.2) ** (-36.5%)	8.0 X 10 ⁴ /ml ±(±0.1) ** (-6.0%)						-85.5%
"normal serum"	5.1 X 10 ⁵ /ml ±(±0.7) ** (10.0%)	4.4 X 10 ⁵ /ml ±(±0.0) ** (-12.0%)	3.8 X 10 ⁵ /ml ±(±0.4) ** (-14.0%)	3.4 X 10 ⁵ /ml ±(±0.7) ** (-11.5%)	2.7 X 10 ⁵ /ml ±(±0.3) ** (-19.0%)	2.4 X 10 ⁵ /ml ±(±0.5) ** (-12.0%)	1.5 X 10 ⁵ /ml ±(±0.2) ** (-36.5%)	1.5 X 10 ⁵ /ml ±(±0.4) ** (+2.0%)						-71.0%

* () = variation X 10⁵/ml or 10⁴/ml (whichever applicable)

** () = percentage increase or decrease in colony count compared to immediately preceding value

*** = average values from two rabbits

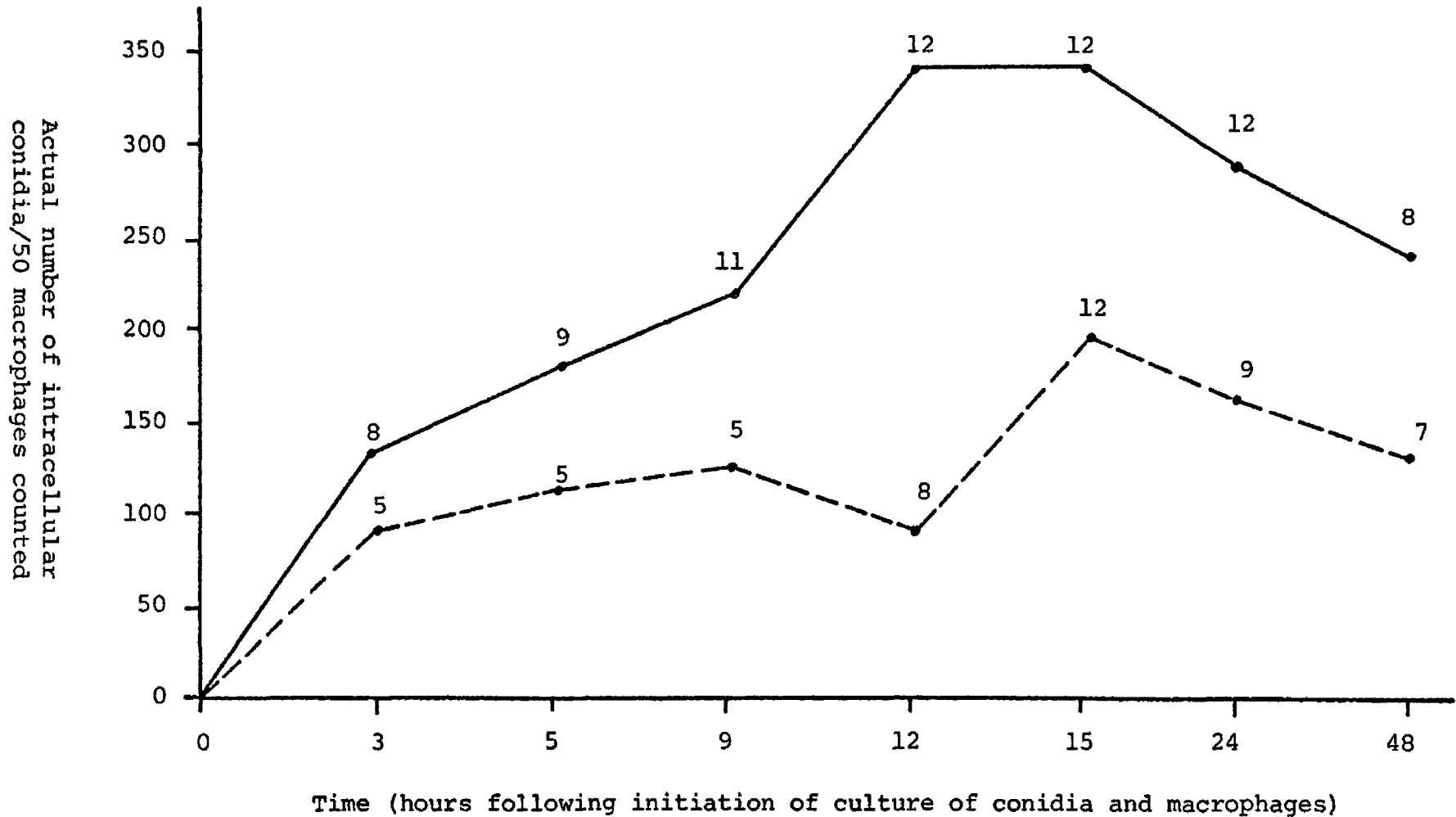


Figure 4 Phagocytic activity of alveolar macrophages from an unsensitized rabbit
solid lines= culture contains 10% "immune" serum
broken lines= culture contains 10% "normal" serum
values above plots= maximum number of conidia within a single macrophage

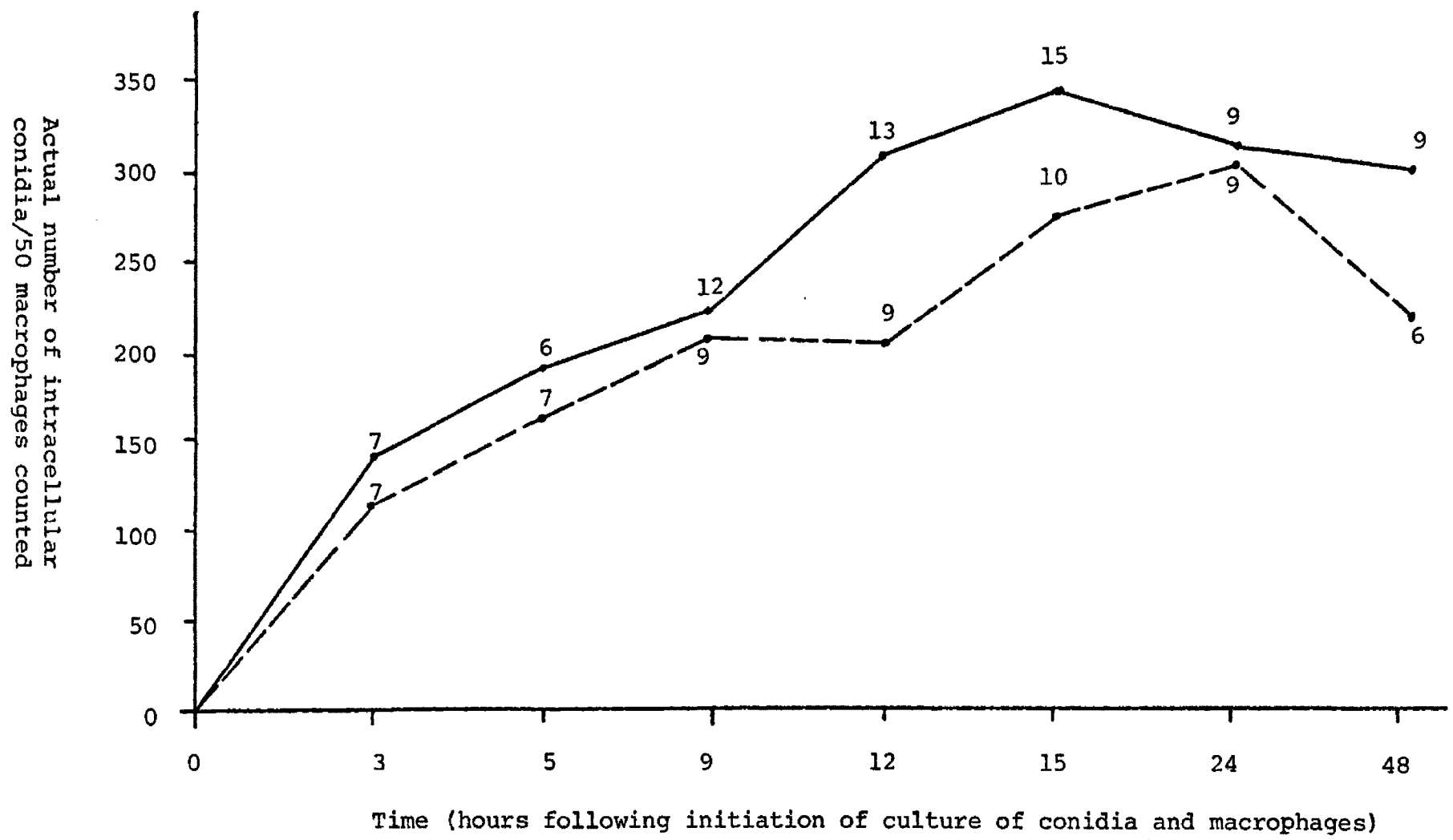


Figure 5 Phagocytic activity of alveolar macrophages from a rabbit sensitized with merthiolate-killed S. schenckii 425 iv
solid lines= culture contains 10% "immune" serum
broken lines= culture contains 10% "normal" serum
values above plots= maximum number of conidia within a single macrophage

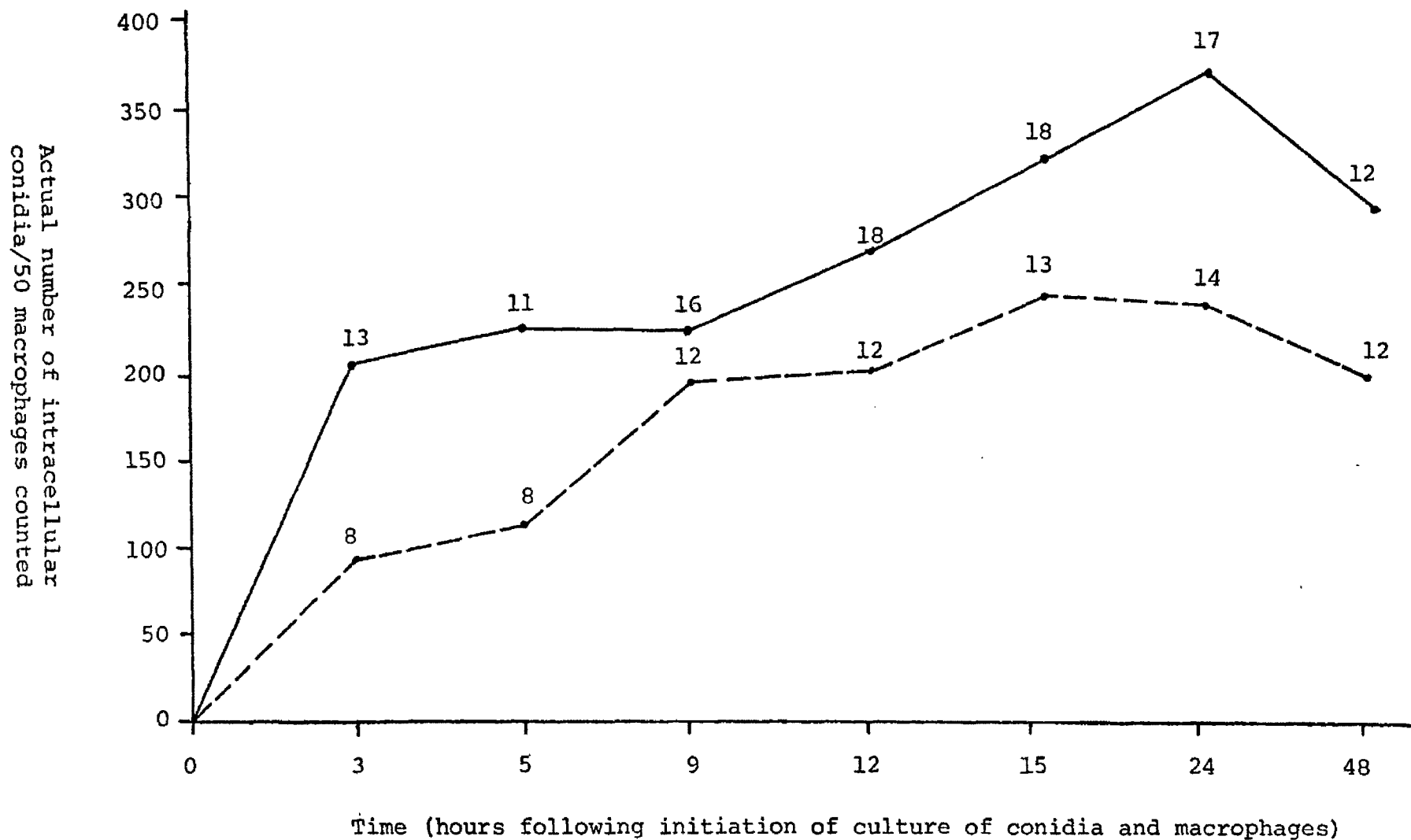


Figure 6 Phagocytic activity of alveolar macrophages from a rabbit sensitized with viable S. schenckii 425 iv

solid lines= culture contains 10% "immune" serum

broken lines= culture contains 10% "normal" serum

values above plots= maximum number of conidia within a single macrophage

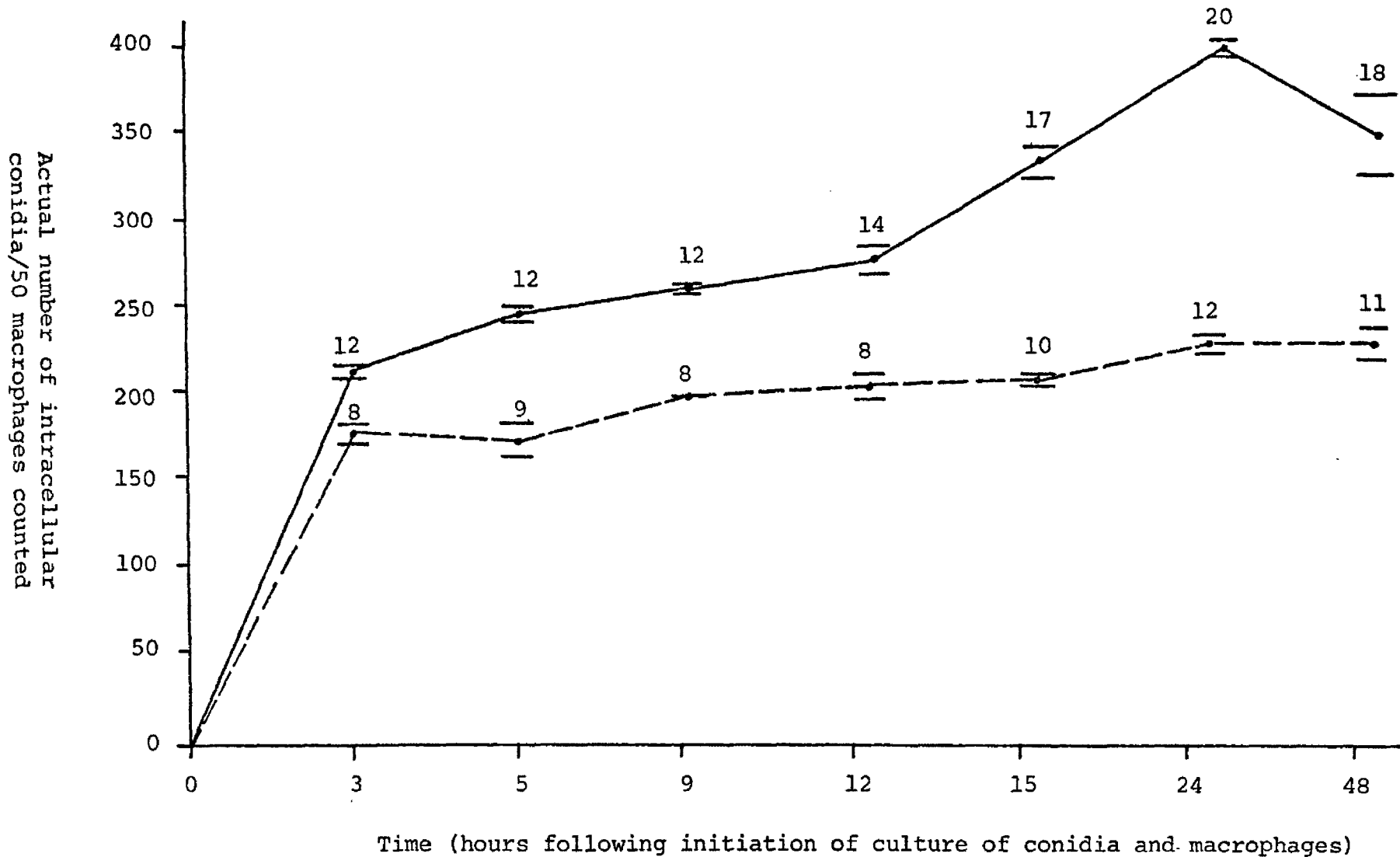


Figure 7 Phagocytic activity of alveolar macrophages from 2 rabbits which received 1 aerosol exposure to S. schenckii 425 conidia
 solid lines= culture contains 10% "immune" serum
 broken lines= culture contains 10% "normal" serum
 values above plots= maximum number of conidia within a single macrophage
 horizontal slash marks above and below plots= actual value of individual rabbits

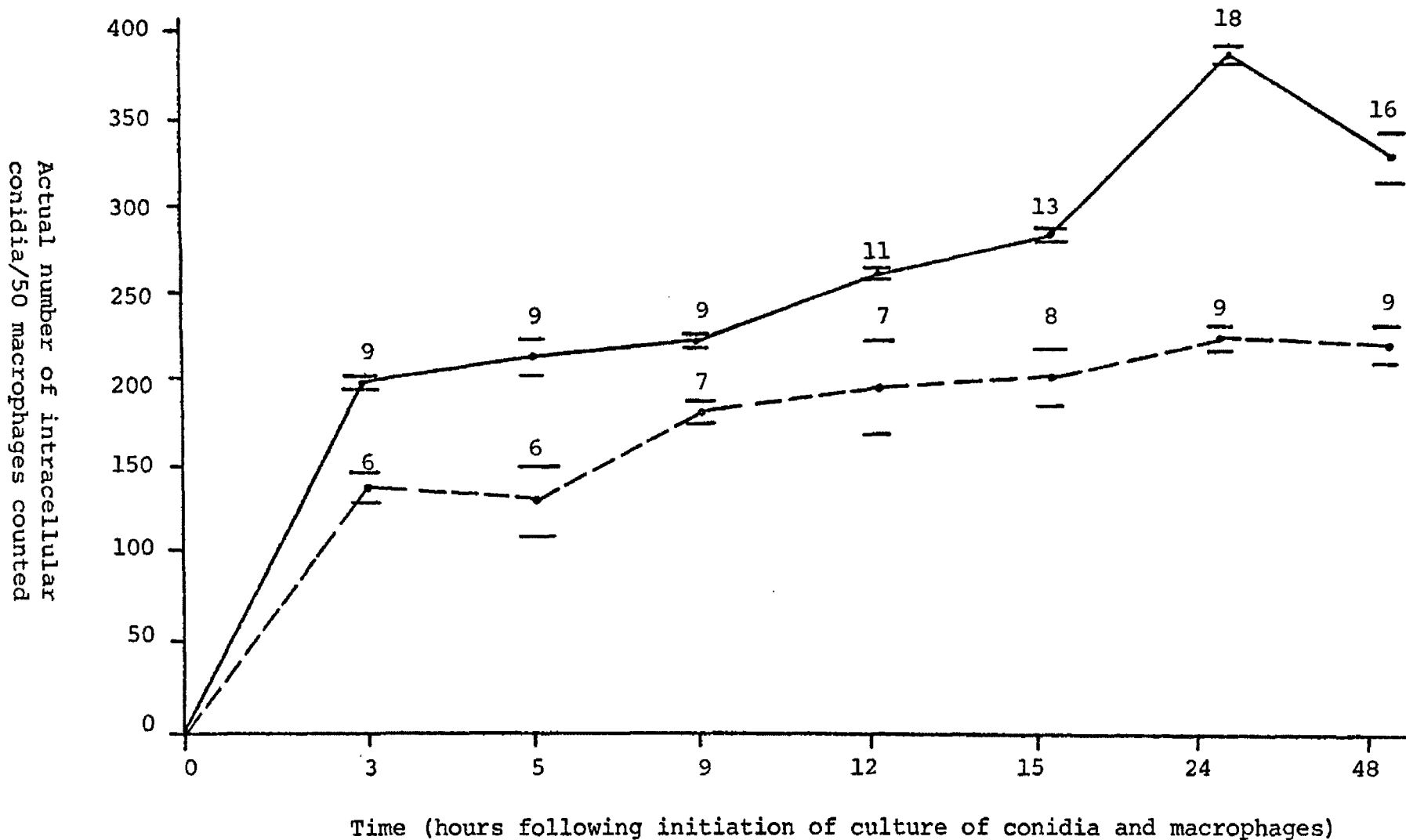


Figure 8 Phagocytic activity of alveolar macrophages from 2 rabbits which received 2 aerosol exposures to S. schenckii 425 conidia

solid lines= culture contains 10% "immune" serum

broken lines= culture contains 10% "normal" serum

values above plots= maximum number of conidia within a single macrophage

horizontal slash marks above and below plots= actual value of individual rabbits

Chapter 4

DISCUSSION

General Statements

Mechanisms of resistance to sporotrichosis are still obscure. Various reports (8, 26, 44) indicate that standard serological procedures are of some diagnostic value in extrapulmonary and chronic lymphocutaneous cases, but the actual role of the humoral response in the recovery from or protection against the disease is equivocal. Kite (29) stated that passive transfer of immune sera from infected animals failed to protect recipients against subsequent homologous challenge to Coccidioides immitis, Cryptococcus neoformans, Histoplasma capsulatum, and Candida albicans. However, antibody titers sufficiently high to be protective may not have been present. If one assumes that sufficient specifically immune globulins were being transferred, then one may postulate that antibodies, by themselves, are not protective against these diseases. A recent conversation with Leo Kaufman (27) at the Center for Disease Control prompted him to say that to his knowledge, he was unaware of any type of antibody response that appeared to be protective against sporotrichosis.

Working with C. neoformans, Diamond (13) found evidence for an antibody-dependent cell-mediated cytotoxicity. Hyperimmune human

serum, specific against C. neoformans, cultured with human blood mononuclear cells significantly reduced the original population of C. neoformans.

Delayed hypersensitivity as determined by skin reactivity to sporotrichin or some other suitable antigen, has proved valuable in the detection of sporotrichosis (past and present infection). Experimental human infection with S. schenckii results in positive skin tests as early as the fifth day after lesions appear (29). Skin tests are of particular value since these cellular reactions are frequently detectable before the humoral response (29, 52). Specificity of the skin test varies accordingly to the test antigen, dilution, and techniques employed, but the yeast-phase antigens of S. schenckii appear to be highly specific.

Skin Testing

In the present study, a rabbit injected intramuscularly with viable S. schenckii 425 reacted to both merthiolate-killed and protoplasmic skin test antigens 5 days following initial inoculation. Agglutinins first appeared at about the same time (1 week post-inoculation), but precipitins did not appear until 28 days following inoculation (see sections later in discussion). These results are consistent with other observations that cell-mediated responses frequently appear before humoral responses. The concentration of protoplasmic test antigen was crucial: 0.27 mg protein/injection was insufficient to elicit a delayed reaction, while 3.0 mg protein/injection did so. A minimum effective dosage of protein was not performed.

A rabbit sensitized with repeated iv injections of merthiolate-killed S. schenckii showed delayed dermal sensitivity to both skin test antigens at 1 week post-inoculation, but not at 5 days. In this case, animals sensitized with non-viable inoculum took a longer period of time to mount a response than did animals sensitized intramuscularly with viable inoculum.

All 4 rabbits receiving aerosolized conidia developed delayed skin reactivity in 3 days. Sensitization by inhalation evidently must evoke a delayed immune response with an equal or greater efficiency than parenterally administered viable or non-viable conidia.

Sethi (49) demonstrated that the majority of mice receiving aerosolized conidia had positive lung cultures for S. schenckii. The same was shown to occur in rabbits in this study. Perhaps the antigen in the alveoli of the rabbit remained in contact with the tissues for a relatively longer period of time (compared to the antigen administered parenterally) and was cleared less rapidly, thereby serving as a more persistent source of sensitization. Inoculum sizes and the time required for contact of conidia with the immune system are 2 other variables which must also be considered. Although the parenterally (im and iv) sensitized rabbits received repeated inoculation, the inoculum involving the aerosolization study was 6 times as great (6.0 ml of 5.5×10^7 conidia/ml). It also seemed logical that conidia entrapped within alveoli of lung tissue would be less susceptible to non-specific clearance by PMNs than would be conidia in the circulation or muscle tissue. If this is true, then perhaps the time required for the conidia to sensitize the T-lymphocytes, and in turn activate monocytes and macrophages

may be lessened compared to the time necessary to activate mononuclear cellular constituents by parenterally administered antigen.

Two major conclusions can be deduced from the skin testing procedures conducted on these groups of rabbits. First, viable immunogens appear to confer a more rapid development of skin hypersensitivity to test antigens. This is in agreement with the literature (15). Secondly, pulmonary exposure to aerosolized conidia represents a very effective and efficient method of reducing the time required for mounting delayed skin hypersensitivity.

Wilson (60) reported that an experimentally infected (with S. schenckii) human reacted to an intradermal skin test antigen as early as the fifth day. This is consistent with the skin responses of the test rabbits which received aerosolized conidia in this study.

Despite small variations in time of onset of skin sensitivity, all rabbits did develop delayed hypersensitivity. It is interesting to note all 3 groups of rabbits sensitized by different routes presented no overt evidence of clinical sporotrichosis. No nodular lesions were noted on the limbs of the rabbits which received im injections of viable S. schenckii. Their immune status, demonstrated by in vivo and in vitro reactions, may therefore have aborted overt infection.

Agglutination Testing

Agglutination testing is a commonly used serological procedure for the rapid diagnosis of sporotrichosis. More than 2 decades ago, Norden (44) noted agglutinins appeared in rabbits approximately 2 weeks following a single iv injection of viable mycelial-phase S. schenckii. Following a single im injection of the same inoculum, he was unable to

detect a response. Blumer (8) related that the tube agglutination test was both very specific and sensitive in a comparative study she made of various serological techniques using human sera (from culturally proven cases of cutaneous, subcutaneous, and extracutaneous forms of sporotrichosis) and merthiolate-killed S. schenckii antigen.

In this study, 2 rabbits injected with viable S. schenckii im yielded agglutinin titers which first appeared 1 week following initial injection. Maximum titers (1:512-1:1024) appeared approximately 6½ months post-injection, and remained elevated for at least 1 year.

A rabbit which received repeated iv injections of merthiolate-killed S. schenckii mounted a peak agglutinin titer (1:512) which approximated the titers mounted by the 2 rabbits injected with viable S. schenckii im. However, these 2 im inoculated first mounted demonstrable agglutinins 1 week sooner than the rabbit sensitized with merthiolate-killed S. schenckii 425 iv.

Two rabbits which were exposed once to aerosolized conidia first exhibited agglutinin titers at 3 and 5 days respectively. Maximum levels of 1:64 were attained at the 13th day following aerosolization, and appeared even before a titer was demonstrable in the rabbit sensitized with iv injections of killed S. schenckii. By the 13th day following inoculation, the parenterally (im and iv) sensitized rabbits mounted an agglutinin titer of 1:2, significantly lower than the 1:64 titer mounted by the rabbits which received aerosolized conidia. Although antibody formation is most frequently obtained (clinically as well as experimentally) by parenteral administration of immunogen, aerosoli-

zation appeared to be a more efficient method of eliciting a humoral response. Serological studies of those rabbits receiving aerosolized conidia were abbreviated because they had to be sacrificed for cellular studies. However, I would expect the agglutinin levels of these rabbits to equal, or even surpass the levels attained by the rabbits sensitized by im or iv injection. This seems plausible since 1:64 agglutinin activity in the im and iv sensitized rabbits took 18 days and more than 3 months longer respectively to occur than in the rabbits which received a single aerosol.

Two rabbits which were exposed twice to aerosolized conidia mounted an agglutinin titer (following the second exposure) identical to the titer mounted by the 2 rabbits which received only a single exposure. Agglutinins appeared in the doubly exposed rabbits at approximately the same time as did the agglutinins in the singly exposed rabbits. I am not convinced that the enhanced response was significant. However, since only 5 days elapsed between the second exposure and animal sacrifice, perhaps the time interval was too short to note significant amplification of the response.

With reference to all the test animals used in the agglutination studies, those rabbits which received viable immunogen responded sooner than the rabbit which received killed inoculum.

The 8 uninoculated rabbits tested for agglutinins either exhibited a maximum titer of 1:2 or no titer at all. This marginal reactivity could be attributed to either subclinical infection, cross reaction, or the value could be discounted as insignificant. Since microscopic analysis was necessary to determine the endpoints, a 1-tube variation in either direction could be considered experimental error.

Karlin and Nielsen (26) recognized some minor cross reactivity of hyperimmune antiserum against S. schenckii with antigens of other fungi. However, when compared to other standard serological procedures, the tube agglutination was quite specific and sensitive.

Precipitation Testing

Precipitin reactions are not as sensitive as agglutination reactions. Only 20 µg of antibody can be detected in the former (15). Probably for this reason, low precipitin titers are of more significance than corresponding agglutinin titers. The rate of precipitin appearance is also considerably slower than that of agglutinin reactions, making the former less desirable as a routine diagnostic procedure.

In this study, 2 rabbits injected with viable S. schenckii im produced only slightly elevated precipitin titers. It took 1 of these animals 3 weeks to mount a 1:2 precipitin titer, and another 9 weeks for the titer to reach its maximum at 1:4. The other rabbit similarly inoculated took 7 weeks for an initial precipitin titer of 1:2 to appear, and 18 weeks later, the maximum titer of 1:4 was reached.

A rabbit sensitized with repeated iv injections of merthiolate-killed S. schenckii took 24 weeks to mount an initial (and maximum) precipitin titer of 1:2.

It is noteworthy that the 2 rabbits which received viable S. schenckii im mounted demonstrable precipitin titers within 28 days of each other. The rabbit sensitized with iv injections of killed inoculum took an additional 4½ months to mount a demonstrable titer. I attribute little significance to the quantitative differences in the

precipitin titers between these 2 groups of rabbits; however, the fact that any titer was achieved at all is important. I also contend that the substantial time lag apparently necessary for the iv sensitized animal to react to non-viable immunogen is significant.

Eisen (15) and Mackaness and Blanden (35) stated that living cells are required to stimulate the maximum delayed-type immune response. It certainly appears that viable cells may also function better than killed cells to stimulate precipitin reactions. The difference in time required for initial demonstration of antibody is not as obvious in the agglutination reactions, although the same trend exists. This observation may be real or may be attributed to increased agglutination testing (compared to precipitin testing) in the initial stages of sensitization. If the former is true, the greater sensitivity inherent in agglutination testing (compared to precipitin testing) could account for this phenomenon.

Cell-free Serum Studies

Cell-free serum fungistasis has been documented by a number of researchers (4, 7, 32, 47). Baum and Artis (4) indicated that although human serum inhibited the growth of S. schenckii, the effect was not so pronounced as noted for other fungi tested.

As evidenced by my work, in most instances cell-free serum from all sensitized rabbits was fungicidal at the lower dilutions. Sera collected from unsensitized rabbits were similarly fungicidal, but the fungicidal activity of these sera appeared to be even more restricted to the lower dilutions (compared to the sera of the sensitized rabbits).

Unequivocal comparison of the fungicidal activity of sera procured from the 3 different groups of rabbits is not possible. However, when compared to the control sera, fungicidal activity was present in the majority of serum dilutions. From these tests alone, participation of antibodies in cell-free fungistasis seems plausible. The increased fungicidal activity of serum from test animals (when compared to respective controls) yields support to immune globulins being the active fraction involved in fungistasis. Since lability studies were not conducted in this research, participation of complement cannot be ruled out as a possible mechanism.

Migration Inhibition Factor (MIF)

The in vitro macrophage migration inhibition factor test is commonly correlated with delayed type hypersensitivity to various microorganisms. Neta and Salvin (42) noted reduced migration patterns of guinea pig peritoneal macrophages when incubated with the same antigen to which they were originally sensitized (C. albicans). It was also noted that these same guinea pigs were resistant to experimental candidiasis.

The presence of a population of sensitized lymphocytes is necessary for migration inhibition. It is these cells that become sensitized and produce the soluble mediators, or lymphokines (of which MIF is one) which inhibit the migration of macrophages (29). The inhibition of macrophage migration is possible upon the addition of specific antigen, even when as little as 1% of the cell suspension consists of sensitized lymphocytes (15).

As expected, the migration of alveolar macrophages from unsensitized

rabbits was not inhibited appreciably with the addition of soluble and cellular antigens of S. schenckii. In contrast, migration patterns of alveolar macrophages from rabbits sensitized to S. schenckii parenterally (im and iv) and by inhaled conidia were substantially reduced. Kirkpatrick (28) stated that a 20% reduction of migration, when compared to the controls without antigen, constituted a positive MIF test. According to this criteria, macrophages taken from the rabbits sensitized with duplicate aerosolization, yielded borderline MIFs when incubated with protoplasm of S. schenckii. When incubated with merthiolate-killed cells, the macrophages from these animals in this study yielded unquestionably positive MIF patterns.

It is interesting to note in all but 1 of the systems involving sensitized alveolar macrophages, serum from sensitized rabbit enhanced migration inhibition over that seen with non-immune serum. The difference in the MIF patterns was only 1.2% in the exceptional situation. This observation is difficult to explain since MIF is mediated through sensitized T-lymphocytes and not cell-free serum. Although greater MIF activity was noted in the systems which incorporated serum from sensitized rabbit, the systems which utilized serum from nonsensitized rabbit also yielded positive MIF tests. This is to be expected since the macrophages were also sensitized, however this observation would substantiate claims that MIF is qualitatively dependent upon sensitized cells as opposed to immune serum. Quantitatively, immune serum may very well enhance the degree of migration inhibition already expressed by sensitized alveolar macrophages as observed in this study.

Another generality that seems evident is that the killed cellular

antigen (protein content not determined) prompted a better MIF response than to the protoplasmic antigen (0.27 mg protein/0.1 ml). The most obvious explanation for this is that the cells probably presented a greater concentration of antigen than the protoplasm. Since the cellular antigen was washed prior to use in the MIF tests, it is doubtful that residual merthiolate toxicity would be present to inhibit migration inhibition. Perhaps the macrophages and lymphocytes elaborated proteinases which degraded reactive protein. The chitin content in the filamentous-phase of S. schenckii is quite high (11), and although rabbit alveolar macrophages are richly endowed with hydrolytic enzymes (10), chitinase activity in mammalian cells is marginal (19). If chitin in the cell walls of S. schenckii in any way protects cell wall peptides from structural degradation by the action of macrophage peptidases, then these peptides would retain their immunogenicity. Immunological studies performed by Shimonaka (51) indicated that peptides are generally responsible for stimulation of delayed-type hypersensitivity reactions. In the present study, the observation that cellular antigen provided greater stimulation in the MIF tests than protoplasmic antigen could be because of greater exposure to wall antigen than to protoplasmic antigen during sensitization.

On the basis of the MIF test alone, it is difficult to substantiate claims that living cells are the most effective immunogens in the sensitization of delayed hypersensitivity. In fact, the MIF patterns of macrophages taken from a rabbit sensitized with killed S. schenckii showed greater inhibition, in certain instances, than the patterns involving macrophages from animals sensitized with viable cells of the fungus.

Quantitatively, however, the difference in migration patterns is one of questionable significance.

Cellular Fungicidal Activity and Phagocytosis

The rabbit alveolar macrophage has the capacity to reduce the viability of S. schenckii conidia in vitro, as evidenced by the cellular fungicidal assays conducted in this study. This activity was observed with alveolar macrophages taken from both sensitized and nonsensitized rabbits. The macrophages taken from rabbits sensitized by parenteral (im and iv) injection and by aerosolization showed significantly elevated fungicidal activity compared to the macrophages taken from non-sensitized animals. The difference in fungicidal activity between macrophages taken from animals inoculated with killed S. schenckii iv and those sensitized with viable inoculum im is probably insignificant. However, rabbits sensitized with aerosolized conidia yielded macrophages that demonstrated much greater fungicidal activity than macrophages taken from any of the other animals.

An obvious and somewhat expected trend that must be mentioned is that in all in vitro tests for cellular fungicidal activity, the macrophages showed greater activity when serum from sensitized rabbit was incorporated. This generality suggests the presence of an active humoral substance, perhaps an opsonin, that aids the macrophage in phagocytosis

Diamond (13) recently observed an antibody-dependent cell-mediated cytotoxicity with C. neoformans. Prevention of phagocytosis by mononuclear cells was drug induced. However, a much greater proportion of original C. neoformans inoculum was eliminated when both leukocytes and antibody were present, than when either or both of these components were

absent. Although not tested in this study, cytotoxic humoral factors may have also been present and contributed to fungal death.

Rabbits sensitized with viable cells yielded macrophages with greater phagocytic activity (i.e. conidia/macrophage) than found in macrophages from animals sensitized with killed cells. This difference was especially obvious with animals exposed to aerosolized inoculum. If the latter observation is reproducible, then the alveolar macrophages possibly provide significant protection against inhaled conidia.

Publications dealing with cell-mediated immunity and delayed hypersensitivity in pulmonary sporotrichosis are not abundant. H. capsulatum probably represents the most discussed and studied model of a pulmonary mycosis. Conclusions and hypotheses may be applicable to pulmonary sporotrichosis. Wilson (60) philosophized by saying, "As Nature does not bother to invent numerous basic processes where one will serve adequately, it is extremely unlikely that she has furnished the human body with a different mechanism for resisting each infectious disease. Instead of emphasizing the differences in immunologic response, we should seek diligently for similarities..."

For some time H. capsulatum has been thought to be an intracellular parasite, able to reside and multiply within reticuloendothelial cells. Howard's (22) work confirmed this after a 24-hour residence within sensitized macrophages. In the present study, following a 24-hour residence within sensitized rabbit alveolar macrophages, S. schenckii was still found to retain viability, although the original inoculum had been reduced drastically.

In another study conducted by Howard (21), the intracellular sur-

vival of H. capsulatum in sensitized mouse peritoneal macrophages was less than within unsensitized macrophages. He also attached much significance to lymphocytes as mediators to this enhanced fungicidal activity. Despite the fact Howard's work dealt with a different organism, a different host, and a different source of macrophages (peritoneal versus alveolar), I was also able to show evidence of loss of viability of conidia in macrophages.

The viability of S. schenckii declined decisively during the first 24 hours when cultured in vitro with alveolar macrophages. A longer incubation would be required (in excess of 48 hours) to determine the eventual plight of the S. schenckii inoculum, but based over 24 hours, it is obvious that the action of the macrophages on the conidia is fungicidal. Perhaps in the in vivo situation, viable organisms may be inactivated by nonspecific factors that are not inherent in the in vitro macrophage assays; nonspecific killing by fungicidal globulins, PMNs, or macrophages which retain their fungicidal activity over a longer period of time. Rabbit alveolar macrophages, in addition to being antifungal, are also able to phagocytize the conidia efficiently. This was obvious with macrophages taken from both sensitized and unsensitized rabbits.

It is my contention that the parallel between phagocytosis and fungicidal activity is not coincidental. The overall reduction of S. schenckii conidia viability, when incubated with alveolar macrophages, generally corresponds to increased phagocytosis. Immune sera in each of these models in table 3 affords a greater overall fungicidal activity compared to the systems incorporating normal sera. This

trend is also evident in the phagocytosis experiments.

Cellular studies in the present research show that the route of antigen administration is important in determining the fungicidal and possibly phagocytic responses of rabbit alveolar macrophages. Rabbits sensitized by aerosolization yielded cells which were significantly more fungicidal and perhaps more phagocytic than cells from the rabbits sensitized parenterally. The difference between fungicidal activity and phagocytic activity among the im and iv sensitized rabbits should be discounted as insignificant.

Rabbits have the advantage over mice and other small animals, in that greater yields of alveolar macrophages are possible, therefore eliminating any need to pool cell populations. In order to better evaluate the cellular response to pulmonary sporotrichosis, alveolar macrophages were used in preference to peritoneal macrophages. In addition to alveolar macrophage suspensions being quite pure (generally less than 1% PMN contamination), these cells permit a more direct interpretation of data obtained from studies of respiratory diseases and eliminate the extrapolation that would be necessary if peritoneal macrophages were used (39).

I was hesitant to attempt to increase the yield of alveolar macrophages through the use of BCG as described by Myrvik (40). Although a larger cell yield would have been mechanically helpful, working with tuberculin sensitive rabbits would have introduced another variable, making interpretation more difficult.

General Comments

Since only 2-3 weeks elapsed from the time of initial aerosol exposure to the time of sacrifice, the rabbits most closely represented examples of acute sporotrichosis. Although stained smears of alveolar macrophage preparations in culture with conidia of S. schenckii revealed mononuclear cells, PMNs may have been the primary cell responding to earlier inflammation in vivo.

A much longer time period elapsed between initial exposure of antigen and sacrifice of the im sensitized rabbits. Therefore, based solely on this protracted time period, these rabbits more nearly simulated chronic lymphocutaneous sporotrichosis (although actual infection was not confirmed in these animals). Compared to the aerosol exposed animals, longer periods of time were required for agglutinins to appear. Although unequivocal comparisons regarding cell-free serum fungistasis are not possible, cellular fungicidal activity of the parenterally sensitized rabbits did not attain the magnitude of that achieved by cellular preparations from rabbits sensitized by aerosol.

Pulmonary sporotrichosis is a very real, and perhaps underestimated problem in urban hospitals (46). Just how the human alveolar macrophage and humoral substances act against the fungus in vivo remains to be seen. From the data presented, it is obvious that when compared with the unsensitized rabbit, the sensitized rabbit has alveolar macrophages with an increased capacity to respond to S. schenckii in vitro. Isolated tests indicate that the cells are highly phagocytic and fungicidal toward S. schenckii conidia in tissue culture. The question that must be answered is: Is this what really happens in the infected animal? Do these alveolar macrophages act as killer cells,

or is it a situation of antibody-dependent cell-mediated cytotoxicity as Diamond (13) suggested in cryptococcosis? It could be both these activities working in conjunction with one another. Obviously experiments precluding phagocytosis are in order to determine whether a cell-mediated (non-phagocytic) antibody-mediated cytotoxicity is active in sporotrichosis. Finally, as a follow-up to the cell-free serum studies performed, additional work should be conducted involving heat-inactivated serum to determine whether complement and other heat-labile serum factors are anti-fungal. Examination of serum proteins by separation may isolate specific anti-fungal fractions.

Chapter 5

SUMMARY AND IMPLICATIONS

Obviously, both humoral and cellular constituents (alveolar macrophages) participate in anti-Sporothrix activity in rabbits, thereby contributing to host resistance. Although cell-free serum was fungicidal at the majority of the dilutions tested, a well defined trend was not evident. However, immune serum did significantly enhance the phagocytic and fungicidal activity of alveolar macrophages compared to the systems incorporating normal serum. This observation, I believe, is attributable to the presence of specific opsonins in the immune serum. The concomitant reduction of S. schenckii in culture with alveolar macrophages coupled with increased phagocytic activity provides evidence for a phagocytic mechanism in host resistance to sporotrichosis.

Rabbits sensitized with viable S. schenckii, especially by the aerosol route, appeared to demonstrate greater phagocytic and fungicidal activity compared to rabbits which received killed inoculum. This would imply that immunization with viable S. schenckii confers a more efficient host resistance of rabbits against Sporothrix than killed cells.

Chapter 6

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