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ASPECTS OF AEROSOL SENSITIZATION AND

INFECTION OF GUINEA PIGS WITH

HISTOPLASMA CAPSULATUM

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

ASPECTS OF AEROSOL SENSITIZATION AND INFECTION OF GUINEA PIGS WITH *HISTOPLASMA CAPSULATUM*

CHAPTER I

INTRODUCTION

Human histoplasmosis is presumably contracted by inhalation of viable particles of aerosolized mycelial phase of *Histoplasma capsulatum*. Areas of high incidence of histoplasmin sensitivity have been mapped in the valleys of the Ohio and Mississippi rivers (Christie and Peterson, 1945; Palmer, 1945). The relation of this skin test hypersensitivity to subsequent reinfection has not been studied under laboratory conditions. However, in laboratories where repeated exposure to the organism is frequent, very few of the previously skin test positive personnel develop clinical symptoms of the disease (Furcolow, 1963).

Little experimental work has been reported which is concerned with the infection, hypersensitivity, and immune response initiated by respiratory exposure of laboratory animals to the organism. Farrell, et al. (1953) produced acute, progressive, fatal histoplasmosis in dogs using the

intratracheal route when the yeast phase was employed. Although the mycelial phase failed to produce the disease, dogs subsequently challenged with the yeast phase were found to be resistant. Cortisone treated dogs were susceptible to both phases. Using the Henderson Aerosol Apparatus, Larsh (1960) reported that guinea pigs became histoplasmin sensitive after exposure to airborne mycelium and that the sensitivity developed more rapidly in those animals given viable particles than in those receiving killed ones. Also noted in this work was the low viability and sporadic aerosol characteristics of mycelial fragments of H. capsulatum isolates. The yeast phase of the organism is more reliably aerosolized as was shown by Larsh and Cozad (1965) when they reported that as few as five yeast cells per animal would infect approximately 10% of their white Swiss mice. With 5 to 24 thousand viable cells per animal, 90 to 100% infections could be expected.

Another systemic mycotic agent, Coccidioides immitis, has been more frequently studied by airborne methods. Vogel, et al. (1954) reported that intramuscular injection of heat killed spherules, which produced positive coccidioidin skin tests in guinea pigs, prevented lymphatic dissemination of the organism and reduced the severity of lung pathology following an airborne challenge of viable arthrospores. Pappagianis, et al. (1960) showed that monkeys subcutaneously vaccinated with viable arthrospores

were partially protected in that three of the seven monkeys were healthy 73 days after a challenge with viable aerosolized arthrospores. Although the other four were ill or moribund, the roentgenograms of all vaccinated animals were clear and histopathology showed only immature spherules in widely scattered lesions of the lungs. Converse, et al. (1963) concurred with these results but found in addition that monkeys immunized with formalin killed arthrospores were not protected from the disease although its progression was appreciably delayed. The protected animals were healthy in appearance and had negative chest films, minor histopathology, and 80% negative lung cultures at autopsy. The complement-fixing and precipitating antibody titers were found to be lower in the vaccinated groups as compared to the control monkeys. In 1965, Sinski, et al. demonstrated that aerosol and subcutaneous vaccination with formalin killed arthrospores and a Boivin-type fraction of the organism protected monkeys from subsequent aerosolized arthrospore challenge. They found that while pathologic tissue changes were the same in the vaccination groups, all six animals given subcutaneous spores and four of six given the Boivin antigen or aerosolized spores were protected from the disease. Infection was not prevented by any vaccine although each prevented deaths due to coccidioidomycosis. They indicated that skin hypersensitivity seemed to be a

better index of resistance than precipitin or complement-fixing antibody titers.

Kong and Levine (1967) reviewed the research literature concerning the experimental induction of immunity in the respiratory mycoses. They stated that although the epidemiological data has indicated that the defense mechanisms against these diseases are strong, they are little understood. They also suggested that vaccines should be developed for use against the respiratory mycoses, especially in areas of high endemicity.

Little is known about the protection afforded by prior experience of laboratory animals to aerosolized H. capsulatum mycelium. In addition, the relation of skin hypersensitivity in the resistance of the host when exposed to airborne reinfection is not clear. For these reasons preliminary experiments have been initiated to investigate some of these problems. Reported in this paper are the results of three experiments, conducted over a five year period, in which airborne mycelial particles of Histoplasma capsulatum have been used to sensitize guinea pigs. Differences in susceptibility to reinfection and in serological variations were evaluated with respect to dermal hypersensitivity.

CHAPTER II

MATERIALS AND METHODS

Many techniques remained the same for each experiment. These will be discussed first. Variations of techniques from experiment to experiment will be discussed separately.

In each of the following experiments the Scritchfield isolate of Histoplasma capsulatum was used for sensitizing and challenging guinea pigs. A culture filtrate (histoplasmin) of the mycelial phase grown in liquid asparagine medium (Smith, 1943) was employed as a complement-fixing (CF) antigen. Yeast phase cells of the isolate were used to prepare complement-fixing and agglutinating antigens. However, 0.1 ml of standardized H-42 histoplasmin, obtained from the U.S. Public Health Service in a 1:10 or 1:25 dilution, was injected intracutaneously for determination of skin hypersensitivity. The millimeters of erythema and induration were measured 48 hrs later.

The Henderson Apparatus, a modification of that described by Henderson (1952), was used to expose the animals to aerosols generated by a Collison nebulizer.

The total, non-circulating air flow through the apparatus was 28 liters/min of which the nebulizer contributed 8 liters/min of droplet particles of 10 microns or less. The cloud was assayed by sampling with Porton, all glass, short stem impingers (12 liters/min) which contained 10 ml of sampling fluid. The aerosol sampling period was 2 min in all sensitization and challenge runs. The animals were exposed for 10 min during sensitization or challenge followed by a 1 min sterile air wash. The ambient air temperature in the exposure chamber was 76-80 F and the relative humidity was maintained at 82%. Before returning the animals to their cages, their noses were swabbed with 70% ethanol and marked with dye to show that they had been exposed.

The formulae for determinations of spray factors, suspension concentrations and infective doses were those as elaborated by Elberg and Henderson (1948). Four impingers each were used to sample the aerosol before and after the animals were exposed. A sample was withdrawn from the nebulizer suspension before and after animal exposure. Serial ten-fold dilutions were made of those suspensions from which 0.5 ml amounts were spread on each of four petri plates of Modified Sabouraud's Agar (Bacto-Neopeptone 10g, Bacto-Dextrose 20g, Bacto-Agar 15g, distilled water 1 liter, pH 6.5) containing 40 units/ml of streptomycin and 20 units/ml of penicillin. Estimates were made of the total number

of mycelial particles or yeast cells by hemacytometer counts of suspensions to which 1 drop/ml of lactophenol cotton blue was added.

When the guinea pigs (Hartley strain) were obtained they were separated according to sex, weighed and sequentually placed, one at a time, into the cages beginning with the lightest pigs first. Then a heavier animal was placed in each of the cages, etc. By using this method the cage mean weights were approximately the same. After a period of 1 week for adjustment to their new environment, they were tatooed with an identifying number and assigned to treatment groups. Any sick or dead animals were replaced with others of equal weight of the same sex. However, once the treatments were assigned and started no replacement was made. At autopsy, separate spleens or lungs were placed in preweighed glass grinder tubes. The tubes were reweighed and 5 ml cysteine saline (L-cysteine 10g, NaCl 8.5g, distilled water 1 liter, pH 6.5) were added. The tissues were then homogenized with a teflon grinder. Dilutions of 10^{0}, 10^{-1}, 10^{-2}, and 10^{-3} were made of the tissue homogenates and 0.5 ml was plated on four plates of Modified Sabouraud's Agar, Mycosel (B. B. L.) or blood agar at each dilution. All animals dying or accidently killed during the experimental periods were autopsied and cultured as were the animals in that particular experiment. All plates in these experiments were incubated at room temperature for 3 weeks before the

colonies of H. capsulatum were counted. The isolation of at least one colony of H. capsulatum from an organ homogenate was considered as a positive test.

First Experiment: Sixty-nine male guinea pigs skin test negative to 1:10, H-42 histoplasmin and weighing an average of 469g (SD 77g) were randomly selected and distributed three pigs to a cage. The light, medium or heavy animal in each cage was marked, respectively, on the head, back or rump with 0.1% Bismarck Brown (in 95% ethanol).

The cages were randomly assigned to three sensitization groups; aerosol-viable mycelial particles, aerosol-killed mycelial particles and control. The first group was assigned seven cages and the other two groups were given eight. Within these groups, four cages of the aerosol-viable group and five cages each of the aerosol-killed and control groups were selected for subsequent challenge. The remainder of the animals were maintained as challenge controls. A five ml sample of blood was drawn by cardiac puncture from each guinea pig, and the serum was saved for future serologic studies.

The mycelial phase inoculum for sensitization was obtained by growing the Scritchfield isolate on Sabouraud's Dextrose Agar (Difco) slants for 3 weeks at room temperature. The growth was scraped off into cysteine-saline and broken up with a Teflon tissue grinder. The suspension was diluted in saline so that a total of one million viable particles

would be inhaled by the animals during the 10 min exposure period. The killed particle inoculum was made by heat killing one-half of the original suspension for 2 hr at 56 C. A sample was plated on Modified Sabouraud's Agar to test for complete killing of the mycelial fragments. The aerosol was sampled by impingement of the particles in enriched nutrient buffered gelatin (Larsh and Cozad, 1965) containing 4 drops of sterile olive oil.

Four weeks after sensitization exposure all of the animals were skin tested and bled again. The guinea pigs previously selected were challenged with 1 x 10^5 viable yeast cells. The yeast phase cells had been grown on blood agar slants (Bacto-Blood Agar Base 40g, distilled water 1 liter, human blood 40 ml) for 3 days at 37 C. The cells were harvested in cysteine saline, centrifuged (2400 rpm for 7 min), washed three times and diluted to the final concentration calculated to give the challenge dose.

Three weeks after challenge, the animals were skin tested and bled. Then they were sacrificed, their spleens removed, homogenized and plated on Modified Sabouraud's Agar.

Second Experiment: One hundred and four male guinea pigs skin test negative to 1:25 H-42 histoplasmin and weighing an average of 513g (SD 72g) were randomly selected and placed four in a cage. The cage number was tatooed on the ear. The head, back, rump or chest was stained on each

animal to correspond with the animals relative weight within the cages. Animals within the four weight groups were randomly assigned to four airborne sensitization regimens; a 50 viable particle group, a 2500 viable particle group, a 2500 killed particle group and a control group. One-half of each group was selected to be challenged. One-half of the challenge and control groups were autopsied at 3 weeks and the remainder at 18 weeks.

The mycelium for sensitization was grown at room temperature in 250 ml screw top Erhlenmeyer flasks containing 100 ml of Modified Sabouraud's Maltose Broth (Bacto-Neopeptone 10g, Bacto-Maltose 20g, distilled water 1 liter, pH 6.5) and agitated for 2 weeks at room temperature on a Eberbach platform shaker (approximately 100 excursions/min). The pellets of mycelial growth were centrifuged (3000 rpm for 15 min) and resuspended in saline. The mycelium was homogenized for 20 min in a stainless steel, water-jacketed Waring blendor using cold tap water circulating continuously in the jacket to prevent overheating. The homogenate was centrifuged (3200 rpm for 20 min) and washed three times with saline. The particles were finally resuspended in saline in a concentration estimated to provide the sensitization doses. The dead particle inoculum was prepared by splitting the 2500 live particle inoculum into four equal parts. Three of these were heat killed at 56 C for 2 hrs. The animals in the viable cell groups were exposed one time

to the sensitizing antigen in the Henderson Apparatus but the killed particle group was exposed for 3 successive weeks to the airborne, killed particles. The control group was exposed to aerosolized sterile saline.

Four weeks after sensitization (1 week after the last killed particle exposure) the pigs were skin tested and challenged with approximately 1 x 10^5 viable yeast cells. These cells were grown at 37 C for 3 days on blood agar in 250 ml Erhlenmeyer flasks. The cells were harvested and washed in cysteine saline and then diluted to contain the required challenge dose. After 3 and 18 weeks the animals were skin tested and autopsied. The spleens were plated on blood agar medium which contained penicillin and streptomycin. The plates were incubated for 3 weeks at room temperature before the colonies were counted.

Third Experiment: Forty-eight male and female guinea pigs (Hartley strain) skin test negative to 1:25, H-42 histoplasmin weighing an average of 450g (SD 31g) and 449g (SD 32g) were randomly selected and placed four to a cage. Each animal was identified within a sex group by an individual tatooed ear number. Cages were randomly assigned to four "sensitization" groups (control, aerosol-viable, aerosol-killed and subcutaneous-killed) of which one-half were to be challenged and the rest to be controls.

Mycelial phase cultures of the Scritchfield isolate were obtained by inoculating 250 ml Erhlenmeyer flasks of

yeast extract agar (Smith, 1964) which was modified by adding 0.1 ml of mineral salts concentrate (NH_4SO_4 2g, K_2HPO_4 14g, KH_2PO_4 6g, sodium citrate 1g, $MgSO_4 \cdot 7\ H_2O$ 90mg, distilled H_2O 1 liter, Smith, 1966) and inoculated with 0.5 ml saline suspension of 1×10^5 yeast phase cells. After 3 weeks incubation at room temperature, the growth was scraped off into saline. The suspension was homogenized in a Sorvall Omni-mixer (3 min at 8000 rpm) which was cooled in an ice water bath. The homogenate was filtered through 4 layers of sterile cheesecloth, centrifuged and washed three times in saline (3200 rpm for 15 min). For aerosolization, the suspension was diluted to the required concentration in glycerol-saline (glycerin 20ml, NaCl 8.5g, distilled water 1 liter, pH 6.5) and in saline for subcutaneous injection.

The inoculum for the aerosol-killed and subcutaneous-killed particle groups was obtained by adding 0.5% formalin to a portion of the suspension and incubating it at 37 C for 3 days. Then the formalin was removed by centrifuging and washing the particles in sterile saline (3200 rpm for 15 min). The aerosol-killed inoculum was finally restored to the original volume in glycerol-saline. The subcutaneous inoculum was diluted so that the same number of particles was contained in 0.2 ml of a saline suspension as was calculated to be inhaled by the aerosol inoculated animals.

At 0, 2, 4 and 10 weeks the aerosol-viable group was exposed to 5, 3, 10 and 110 viable particles respectively per animal (a total of approximately 1.8 million particles, living and dead, per animal). The aerosol-killed and subcutaneous-killed were inoculated weekly for 6 weeks and again at 10 and 11 weeks. Thus the total dosage of particles in the killed particle groups was approximately twice that of the aerosol-viable group.

The guinea pigs were skin tested with 1:25 H-42 histoplasmin at 0, 4, 8, 10, 12, 14, and 17 weeks. The weight of each animal was determined at 4 week intervals. Sera were obtained by cardiac puncture from all animals at the beginning of the experiment (0 week), prior to challenge (14th week), and prior to autopsy (17th week). Complement-fixation (CF) tests were performed on individual serum specimens using as antigens, heat-killed (2 hr at 56 C) Scritchfield yeast phase cells and the culture filtrate of asparagine medium (Smith, 1943). The culture filtrate antigen was prepared by growing the mycelium in asparagine broth at room temperature for 4 weeks after which it was killed with 1:10,000 merthiolate. After the mycelial pad was removed using cheesecloth, the filtrate was filtered through a Seitz filter. A 1:4 dilution of this material was used to determine CF titers. The yeast phase cells were grown on Enriched Cystine-Heart Agar (Difco) bottles for 3 days at 37 C. These were harvested in saline, washed

three times by centrifugation, and heat killed at 56 C for 2 hrs. A dilution of 1:250 of the packed cells was used for CF titer determinations. The capillary tube agglutination (CTA) test of Cozad and Larsh (1960) was used to determine agglutinin titers.

The challenge inoculum was prepared from Bacto-Cystine Heart Agar enriched with Bacto-Hemoglobin yeast cultures grown for 3 days at 37 C. The cells were harvested, filtered through 4 layers of sterile cheesecloth, centrifuged (2400 rpm for 7 min) and washed three times and suspended in glycerol-saline in a concentration necessary to deliver 1×10^5 viable cells per animal in the Henderson Apparatus.

Three weeks after challenge the guinea pigs were sacrificed. The lungs and spleen were removed and placed in separate preweighed grinder tubes. After homogenizing the tissues, 0.5 ml of the lung homogenate was spread on Mycosel Agar plates, (B. B. L.). The spleens were plated on Blood Agar to which streptomycin (40 units/ml) and penicillin (20 units/ml) had been added.

CHAPTER III

RESULTS

Experiment I. The dosage of viable mycelial particles of Histoplasma capsulatum inhaled by each guinea pig in the viable particle test group was calculated to be 80 per animal (or a total of approximately one thousand viable and dead particles). This is much less than the one million particles that had been planned to use to sensitize this group of animals. This reduction was due to the inefficient breaking up of the mycleium with the teflon grinder system into particles that could be aerosolized (10 microns or less).

Table 1 shows that despite this low dosage of aerosolized viable particles 64% (7/11) of the guinea pigs had a skin induration of 5 mm or more four weeks after one sensitization exposure. Only 8% (1/12) of the control group and 7% (1/14) of the killed mycelium group developed reactions of this size. The mean sizes of induration are also shown. In those animals that were challenged the viable particle group mean was 10.8 mm (SD 9.4 mm) as compared to 2.5 mm (SD 3.8 mm) and 1.2 mm (SD 1.6 mm) respectively in the

control and killed particle groups. In all guinea pigs, challenged and non-challenged, the average indurations were 13.0 mm (SD 9.3 mm), 2.1 mm (SD 3.1 mm), and 1.5 mm (SD 2.9 mm) respectively in the viable particle, control and killed particle groups. It should be noted that the comparatively small means in the latter two groups are due to a large number of non-reactors in these groups.

The challenge dose was 1.29×10^5 viable yeast cells. This compared favorably with the planned dosage of 1×10^5 and indicates the relative efficiency of aerosolization of the yeast phase of this organism when compared to the mycelium. Table 1 shows that those groups (control and killed particle) that had very few reactions as large as 5 mm induration had the most animals from whose spleens H. capsulatum could be recovered three weeks after challenge. The control and killed particle group spleens were, respectively, 67% (8/12) and 79% (11/14) culturally positive for the fungus. A positive culture was one in which at least one colony of H. capsulatum was recovered from a spleen. Only 27% (3/11) of the spleens from the animals in the viable particle group were positive. Table 2 shows that if all of the challenged and autopsied animals were pooled, the animals with a negative skin reaction (less than 5 mm induration) were most often associated with a positive spleen culture. The converse was true for those which were skin test positive. Skin tests of the challenged guinea pigs prior to autopsy

showed a change from negative to 100% positive (mean 12 mm) in all groups. This indicated that although the spleens of some animals were culturally negative, the animals had been exposed to the challenge aerosol.

There were no significant differences between treatment groups with respect to body or spleen weight. Complement-fixing antibodies (using a yeast cell antigen) were detected at less than 1:4 in all serum samples taken prior to challenge. In fact only 6 of the 56 animals had a titer of greater than 1:4 at autopsy. There was no association between serologic titer and sensitization group.

The organism was not recovered from any of the 13 animals that died or were accidently killed during the experiment. Also, none of the challenge controls (21 guinea pigs) were culturally positive.

Experiment II. Sensitization doses of 225 and 7500 viable particles were inhaled by the groups supposed to receive 50 and 2500 particles respectively. The total particle count (viable and non-viable) was estimated to be approximately 2.8 and 94 thousand. The killed particle group received the latter dosage for three consecutive weeks (a total of 2.82 million killed particles).

As shown in Table 1, 88% (7/8) of both viable particle sensitization groups reacted to histoplasmin skin tests with indurations equal to or greater than 5 mm induration. Despite the killed particle group having received at

least three times the total number of mycelial particles of the viable groups, less than 17% (2/12) of the guinea pigs had 5 mm or better indurations. There were two of seven animals (29%) in the control group with positive skin tests.

Four weeks after the beginning of the sensitization exposures, the previously selected animals were each challenged with 2.4×10^5 viable yeast cells. As in the previous experiment, those animals which developed positive skin test reactions (in both viable particle groups) had fewer infected spleens by culture. There were three positive spleens of the 18 in these viable particle groups. Meanwhile, 86% (6/7) of the control group and 92% (11/12) of the killed particle group had Histoplasma positive spleens. Table 2 shows that the animals which have negative skin tests, also were most likely to be infected. As in Experiment I, those with positive skin tests appear to have no culturable splenic H. capsulatum.

Table 3 shows the relation of induration size and the isolation of the fungus from the animal's spleen. This table shows that the larger skin test reaction is more likely to be associated with a negative spleen culture in the animal. A 5 mm induration was arbitrarily selected as a positive skin test but as can be seen from the table, a larger skin test is better in terms of association with negative spleen cultures. This is particularly true of those in the viable particle groups. However, there were a

few in the control and killed particle group which had positive skin tests and were infected.

Table 4 shows that there was no difference in those animals in the relatively light and heavy weight groups with regard to skin test size or the number of positive spleens. There was a difference in the number of positive spleens with regard to the time of autopsy after challenge. While 57% (20/35) of the challenge pigs had positive spleens at three weeks, none was positive in the eighteenth week autopsy group. This is suggestive of the ability of the animals to recover naturally from an infection with this isolate.

Table 5 shows the relation of pre-challenge skin tests with regard to time of autopsy and sensitization group. If the reactions of all of the animals are examined, it appears that there are differences in the sensitization groups but not in the groups to be autopsied at different times. However, as mentioned earlier, this difference is mostly due to the larger number of non-reactors in the control and killed particle groups. When one looks at those which react to a size greater than zero millimeters of induration, it can be seen that mean sizes between the sensitization groups become more alike. Thus those animals that do react, appear to react to the same magnitude regardless of the sensitization group.

Two animals accidently killed during the experiment and the 32 animals used as challenge controls within the various sensitization groups were culturally negative for H. capsulatum.

Experiment III. When small numbers of mycelial particles were used to sensitize guinea pigs by subcutaneous (SC) and respiratory routes, it took longer than in the previous experiments to sensitize guinea pigs. Figure 1 shows that it took 8 weeks to achieve at least 40% skin test positives in the SC and aerosol viable-particle groups. Even at challenge (14 weeks) the aerosol-killed particle groups had not reached this level. Although the figure shows a decline in percentage of positive reactors after 10 weeks, it is not due to loss of ability of the animals to react but rather to unscheduled deaths and accidental killing of animals which had become too large for the aerosol apparatus. Although the male and female group were essentially the same size at the beginning of the experiment, the males (mean 952g, SD 104g) grew to be significantly larger than the females (mean 829g, SD 104g). Therefore, the males were difficult to safely fit into the aerosol chamber holder because of their size.

The animals were challenged with 5.7×10^4 viable yeast cells 14 weeks after initial sensitization. Table 6 shows that there was no difference between the challenged guinea pigs of both sexes in the number of culturally

positive lungs or spleens with respect to sensitization route. While the lungs of all these animals were uniformly infected, the control group and subcutaneously sensitized group also had infected spleens. Only one of eight spleens was positive in the aerosol-viable sensitization group. The aerosol-killed group seemed to be in between the SC and control groups and the aerosol-viable group in regard to positive spleens.

Complement-fixation tests (CF) and Capillary Tube Agglutination tests (CTA) were run on pre-sensitization, pre-challenge and pre-autopsy sera. None of these tests detected antibodies in the pre-sensitization (control) sera. Table 7 shows the distribution of CF titers in pre-challenge and pre-autopsy sera. Not only did the subcutaneous group have the greatest percentage of positive skin tests, but they also appear to have more animals with a CF titer. There appears to be little difference in titer between pre-challenge (pc) and pre-autopsy (pa) sera even though the latter was drawn 3 weeks after a viable yeast challenge of some of the animals. The CTA titers were low and difficult to read. Although 1:1 and 1:5 titers were often found, there were very few that were higher. It was found that the best time for reading these tests was 4 hrs when testing guinea pig sera. The reactions tended to be weaker than with rabbit serum as a 2+ reaction was the rule although the same antigen reacted strongly (4+) with rabbit serum.

Table 8 shows the arbitrarily defined positive titers (CF and CTA) and positive skin test induration compared to isolation of the fungus from the lungs and spleens within each sensitization group. There was little difference in the four tests in this respect as, i.e. if the histoplasmin reaction is negative (less than 5 mm induration), the serologic titer is also negative (CF--less than 1:10, CTA--less than 1:5). However, positive induration or antibody titer is associated with positive spleen culture in the subcutaneously sensitized group. In the aerosol-viable group, any type of positive test is apparently associated with a negative spleen culture. It seems then that route of sensitization and the type of sensitizing antigen are important in interpreting these results.

Table 9 shows associations between skin test reactions and the serologic tests. The CTA and CF (yeast antigen) tests agree more closely with skin test sensitivity than does the CF (histoplasmin) test. However, all of the serologic tests are significantly associated in agreement with the histoplasmin skin reaction.

None of the animals accidently killed or dying during the experiment was culture positive. This has been true throughout all of these experiments. It is significant in that although some of the animals were sensitized with viable mycelial elements, none of these was found to have a disseminated infection.

Several methods were used, different in each of the three experiments, to produce the mycelial inoculum used in sensitization. Of these, I found that the best medium for producing the mycelium, especially for microconidia, was the one described by Smith (1964). This medium enhanced the production of microconidia by this isolate even though it did not produce many of these structures on Sabouraud's agar or Potato Dextrose agar. An inoculum prepared as described in Experiment III contained 95% or more of these small conidia. The water-jacketed Waring blender appeared to homogenize the mycelium, while maintaining viability better than the Omni-mixer and was superior, by far, to the glass tube and teflon pestle method.

CHAPTER IV

DISCUSSION

Since airborne mycelial elements are presumed to infect humans, and even cause disease in a few, studies of aerosol sensitization and infection of laboratory animals are warranted. A first step in this area was reported when Larsh (1960) showed that guinea pigs could be sensitized to the extent of displaying a positive histoplasmin skin test after aerosol exposure to yeast or mycelial phases of viable or non-viable Histoplasma capsulatum.

All three experiments included in this paper show that guinea pigs can be sensitized by airborne viable mycelial particles. However, a single exposure to 80 or more viable particles was more efficient in producing skin test hypersensitivity than were three, bi-weekly exposures of less than 10 viable particles. One must consider that those inocula were not prepared in the same manner. Therefore, the number of particles and the application of dosages are not the only differences between the sensitization inocula. Cozad and Furcolow (1953) have shown that the mycelium of many isolates produces an abundance of

microconidia of less than 5 microns in diameter. Brown, et al. (1950) have shown that particles larger than 5 microns rarely reach the alveoli of the lungs. It would seem logical then that an inoculum composed mostly of particles of this size, perhaps microconidia, would be most desirable. The medium described by Smith (1964) did stimulate microconidia production by the isolate (Scritchfield) used in this experiment. The microconidia may be separated by various methods of which filtration through layers of sterile cheesecloth seems to be the most practical.

When guinea pigs are challenged with airborne yeast, it is expected that the lungs of the animals will contain the organism if they are examined soon afterwards. The organism was recovered from almost all of the lungs (32/33) three weeks following challenge. However, the previously aerosol-sensitized animals had few isolations of H. capsulatum from the spleen as compared to the control or subcutaneously sensitized guinea pigs. The viable particles were by far more efficient than the killed particles in sensitizing the animals even though two to three times more killed particles were inhaled by some of the animals. Whether this is due to (1) multiplication of viable particles in the host, (2) release of antigenic metabolic by-products from the organism or, (3) stimulation of host cell by-products by a viable organism cannot be assessed by these experiments. It does appear though that the

organism is prevented from disseminating from the lungs in those animals sensitized by the respiratory route. This is not true for those subcutaneously sensitized.

The ability of the guinea pig to recover from an infection by this isolate within 18 weeks after challenge has been shown. This factor complicates the interpretation of the results. Whether this is a phenomenon with the Scritchfield isolate or occurs with other isolates, remains to be studied. Perhaps the use of another animal species i.e. hamster, monkey, etc would be better for these infection studies. Salvin (1953) has immunized mice intraperitoneally against parenteral challenge by H. capsulatum. Larsh and Cozad (1965) have infected mice with an aerosol challenge. However, serologic responses and skin hypersensitivity are relatively difficult to measure in this animal.

The arbitrary selection of 5 mm induration as a positive histoplasmin hypersensitivity reaction has been shown to be significant. The guinea pigs with such a reaction, produced by an aerosol of viable particles, react differently than do the other sensitization groups (control, subcutaneous, and aerosol-killed particle). Their spleens are culture negative if they have a positive skin test. Using viable particles to sensitize animals by other routes (i.e. subcutaneous, etc) may be necessary to show if this is a route or viability effect.

The relative weight or sex of the guinea pigs was not different with respect to the development of positive skin tests or susceptibility to infection. The respiratory volume has been found to be related to guinea pig weight as shown in the following equation (Guyton, 1947):

$$\text{Resp. Vol. (ml)} = 2.10\ (\text{Weight in grams})^{3/4}.$$

This indicates that although a larger animal will inhale more material, the material per unit weight will be the same. Thus male guinea pigs are not only larger than females at maturity, but they respire a larger volume. The volume per unit weight would be the same. Since there were no differences in weight or sex, female guinea pigs would be better for long-term airborne work because they remain smaller than males. At least nine males were killed by strangulation when putting them in the aerosol apparatus. No females were lost in this manner. The use of females would probably minimize these losses of experimental units.

Many factors remain to be studied. Among these are (1) the antigenic and pathogenic difference in other isolates, (2) sensitization by various routes using viable particles and (3) challenge of previously sensitized animals by various routes. Investigation should be made with regard to the cellular level of host immune response--especially in respect to the respiratory system. Electrophoretic studies of sera from animals respiratorily sensitized may be of value. These aspects were not within the scope of this paper.

CHAPTER V

SUMMARY

Three experiments were reported in which guinea pigs were sensitized and infected by aerosolization of the Scritchfield isolate of Histoplasma capsulatum.

In Experiment I, 67% (7/11) of the animals which inhaled 80 viable particles of the mycelial phase developed skin test reactions of 5 mm or greater within 4 weeks after exposure. Subsequent challenge with 1.29×10^5 viable yeast cells resulted in only 27% of the spleens of these animals being culturally positive for H. capsulatum. The control and killed particle groups had only 8% (1/12) and 7% (1/14) positive skin tests repectively. However, the percentage of spleen infections were respectively, 67% (8/12) and 79% (3/14) in these groups.

In Experiment II, 88% (14/16) of guinea pigs sensitized by 225 or 7500 airborne viable particles of mycelium became skin test positive. Challenged with 2.4×10^5 viable yeast cells, these animals had 18% (3/16) culturally positive spleens. The control and killed particle groups were 29% (2/7) and 17% (2/12) skin test positive while 86% (6/7) and

92% (11/12) of their spleens were culturally positive. The heavier guinea pigs (mean 543g) were not different from the lighter ones (mean 482g) with respect to skin test size or the number of positive spleen cultures.

In Experiment III, three, bi-weekly airborne exposures to 5, 3, and 10 viable particles and one to 110 viable particles at 10 weeks resulted in 56% (9/16) positive skin tests at 14 weeks. Killed mycelium given subcutaneously in eight doses (a total of approximately 3.6 million particles) produced 38% (8/21) positive skin tests. Aerosol infection with 5.7×10^4 viable yeast showed that while 97% (32/33) of the animal's lungs were culture positive, only 14% (1/7) of aerosol-viable particle group had splenic H. capsulatum. By contrast 78% (7/9) of the subcutaneously sensitized pigs were positive. Control and aerosol-killed particle animal groups had 67% (6/9) and 43% (3/7) positive spleen cultures. No differences between males and females were noted with respect to size and number of skin test reactions or number of isolations of the fungus from infected spleens. The data suggested that the interpretation of skin tests and serologic titers depends upon the method and route of sensitization when subsequent re-infections were examined.

Variations in techniques for producing aerosol inocula were discussed. Suggestions for further studies were listed.

Table 1. Isolation of Histoplasma capsulatum from Spleens And Skin Test Reactions in Challenged Guinea Pigs.*

Experiment	Sensitization Group	Skin Test** (Induration in millimeters)				Spleen Culture		Total
		Less than 5	5 or Greater	Mean	SD	Positive	Negative	
I	Control	11	1	2.5	3.8	8	4	12
	80 Living Mycelial Particles	4	7	10.8	9.4	3	8	11
	Killed Mycelial Particles	13	1	1.2	0.6	11	3	14
	Total	28	9			22	15	37
II	Control	5	2	2.4	4.6	6	1	7
	225 Living Particles	1	7	9.1	4.4	2	6	8
	7500 Living Particles	1	7	11.8	5.4	1	7	8
	Killed Mycelial Particles	10	2	1.1	2.6	11	1	12
	Total	17	18			20	15	35

*Composed of all guinea pigs challenged with approx. 1 X 10^5 viable yeast of H. capsulatum and autopsied 3 weeks after challenge.

**Injected intradermally with 0.1 ml of histoplasmin (1:10 in Exp. 1, 1:25 in Exp. II) of U. S. P. H. S. H:42 antigen and read at 48 hours.

Table 2. Comparison of Skin Test Size to Isolation of *Histoplasma capsulatum* From Spleens of Challenged Guinea Pigs*

Experiment	Spleen Culture	Skin Test** (Induration)		Total
		Less than 5 mm	5 mm or Greater	
I	Positive	20	2	22
	Negative	8	7	15
	Total	28	9	37
II	Positive	14	6	20
	Negative	3	12	15
	Total	17	18	35

*Composed of all pigs (pooled) challenged with approx. 1 X 10^5 viable yeast of H. *capsulatum* and autopsied 5 weeks after challenged.

**Injected intradermally with 0.1 ml of histoplasmin (1:10 in Exp. I, 1:25 in Exp. II) of U. S. P. H. S. H:42 antigen and read at 48 hours.

Table 3. Comparison of Histoplasmin Skin Hypersensitivity with H. capsulatum Isolation from Spleens of Challenged Guinea Pigs.

Experiment	Spleen Culture		NUMBER OF GUINEA PIGS							
			Induration (mm)						Total	Grand Total
			0	1-4	5-8	9-12	13-16	17+		
I*	Neg	V	1			2	1	4	8	
		K	2	1					3	15
		C	2	2					4	
	Pos	V	3						3	
		K	7	3	1				11	22
		C	4	3			1		8	
II**	Neg	V	2		2	3	5	1	13	
		K	1						1	15
		C			1				1	
	Pos	V				3			3	
		K	9		2				11	20
		C	5		1				6	

*Skin tested with 0.1 ml of 1:10 H-42 Histoplasmin (USPHS).
**Skin tested with 0.1 ml of 1:25 H-42 Histoplasmin (USPHS).

Neg = No colonies of H. capsulatum isolated.
Pos = At least one colony of H. capsulatum isolated.

V = Viable particle sensitization group (pooled), K = killed particle sensitization group, C = control group.

Table 4. Relative Guinea Pig Body Weight Compared with Respect to Time of Autopsy, Histoplasmin Hypersensitivity, and Splenic H. capsulatum Isolation.

Experiment II

Autopsy Week	Relative Weight* Group	Total Number Animals	Induration (mm) Mean	SD	Number Animals Challenged	Number Positive Spleens**	Per Cent Positive
3	Light	26	5.2	5.6	18	12	66.7
	Heavy	25	5.2	6.4	17	8	47.1
	Total	51	5.2	6.0	35	20	57.1
18	Light	25	5.2	5.6	17	0	0.0
	Heavy	26	5.7	6.8	18	0	0.0
	Total	51	5.5	6.2	35	0	0.0
Total	Light	51	5.2	5.6	35	12	34.3
	Heavy	51	5.4	6.5	35	8	22.9
	Total	102	5.3	6.1	70	20	28.6

*Light = 482 g (SD 77g), Heavy = 543 g (SD 49 g).

**At least one colony of H. capsulatum isolated.

Table 5. Comparison of Histoplasmin Skin Hypersensitivity of Guinea Pigs with Respect to Sensitization Group and Time of Autopsy

Experiment II

Autopsy Week	Sensitization Group	Total Number Animals	Induration Size			Induration Size	
			Total Mean(mm)	Total SD(mm)	Number of Reactors*	Reactor Means(mm)	Reactor SD(mm)
3	Controls	11	1.5	3.8	2	8.5	4.9
	225 Viable	12	10.0	4.0	11	10.9	2.5
	7500 Viable	12	9.5	6.2	9	12.9	2.8
	7500 Killed	16	0.8	2.3	2	6.5	1.5
	Total	51	5.2	6.0	24	11.0	3.2
18	Controls	12	1.3	3.2	2	8.0	2.8
	225 Viable	12	9.8	4.8	11	10.7	3.8
	7500 Viable	12	11.5	5.1	11	12.5	3.8
	7500 Killed	15	0.5	2.1	1	8.0	0.0
	Total	51	5.5	6.2	25	11.2	3.8
	Grand Total	102	5.3	6.1	49	11.1	3.5

*Induration of greater than zero millimeters.

Table 6. Number of Isolations of Histoplasma capsulatum from Guinea Pig Lungs and Spleens with Respect to Sex and Sensitization Route.

Experiment III

Sex	Organ	SENSITIZATION ROUTE								Total	
		Controls		Subcutane(K)		Aerosol(K)		Aerosol(V)			
		(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)
Males	Lungs	3	0	3	0	2	1	4	0	12	1
	Spleen	3	0	3	0	0	3	0	4	6	7
Females	Lungs	6	0	6	0	4	0	4	0	20	0
	Spleen	3	3	4	2	3	1	1	3	11	9
Total	Lungs	9	0	9	0	6	1	8	0	32	2
	Spleen	6	3	7	2	3	4	1	7	17	16

(+) At least one colony of Histoplasma capsulatum isolated.

(-) No colonies of Histoplasma capsulatum isolated.

(K) Killed mycelial particles.

(V) Viable mycelial particles.

Table 7. Complement-Fixation Titers of Guinea Pigs Sensitized Previously by Aerosolized and Subcutaneously Injected Mycelial Particles of H. Capsulatum.

Experiment III

	NUMBER OF SERA															
	Control								Subcutan(K)							
	Infected				Control				Infected				Control			
	H		Y		H		Y		H		Y		H		Y	
Titer	pc	pa	pc	pa	pc	pa	pc	pa	pc	pa	pc	pa	pc	pa	pc	pa
0	12	9	11	8	8	8	8	8	7	7	3	1	8	9	3	4
1:5			1										1			2
1:10										2	2	3			3	1
1:20				1					2		1	1			1	1
1:40									1			1		1	2	1
1:80											3	2				
1:160									1						1	
1:320											2	1				1
1:640																
1:1280																
1:2560																
Total	12	9	12	9	8	8	8	8	11	9	11	9	10	10	10	10

(K) Formalin-killed particles.
(V) Viable particles.
Infected=challenged with approx. 1 X 10^5 viable yeast cells of H. capsulatum.
H=Histoplasmin CF antigen
Y=Yeast cell CF antigen
pc=prechallenge
pa=preautopsy

Table 7. Continued

NUMBER OF SERA																
Aerosol(K)								Aerosol(V)								
Infected				Control				Infected				Control				
H		Y		H		Y		H		Y		H		Y		
pc	pa	pc	pa	pc	pa	pc	pa	pc	pa	pc	pa	pc	pa	pc	pa	
6	5	4	3	9	6	7	5	8	7	4	4	6	5	6	5	
1		2				1	1			1	1					
1	1		1									1	1			
	1		1			1				3						
								3	1	1	1			1	1	
		2									1					
			2							1						
										1	1					
8	7	8	7	9	6	9	6	11	8	11	8	7	6	7	6	

Table 8. Number of Isolations of Histoplasma capsulatum from Guinea Pig Lungs and Spleens with Respect to Sensitization Route, Skin Test Hypersensitivity, and Serology.

Experiment III

	SENSITIZATION GROUP															
	Control				Subcutane(K)				Aerosol(K)				Aerosol(V)			
	Lungs		Spleen		Lungs		Spleen		Lungs		Spleen		Lungs		Spleen	
	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)
Induration																
<5mm*	9	0	6	3	4	0	3	1	5	0	3	2	5	0	1	4
≥5mm	0	0	0	0	5	0	4	1	1	1	0	2	3	0	0	3
Histo-plasmin(CF)																
<1:10**	9	0	6	3	5	0	4	1	6	0	3	3	6	0	1	5
≥1:10	0	0	0	0	4	0	3	1	0	1	0	1	2	0	0	2
Yeast Cell(CF)																
<1:10	9	0	6	3	2	0	2	0	5	0	3	2	3	0	1	2
≥1:10	0	0	0	0	7	0	5	2	1	1	0	2	5	0	0	5
CTA																
<1:5	8	0	5	3	3	0	2	1	4	1	2	3	3	0	1	2
≥1:5	1	0	1	0	6	0	5	1	2	0	1	1	5	0	0	5
Total	9	0	6	3	9	0	7	2	6	1	3	4	8	0	1	7

*Defined as being at least 5mm for two consecutive test periods prior to challenge.

**Arbitrarily determined and representing titers prior to challenge.

(+) At least one colony of H. capsulatum isolated.
(-) No colonies of H. capsulatum isolated.

Table 9. Comparison of Histoplasmin Skin Hypersensitivity in Guinea Pigs with Respect to Serologic Titers.

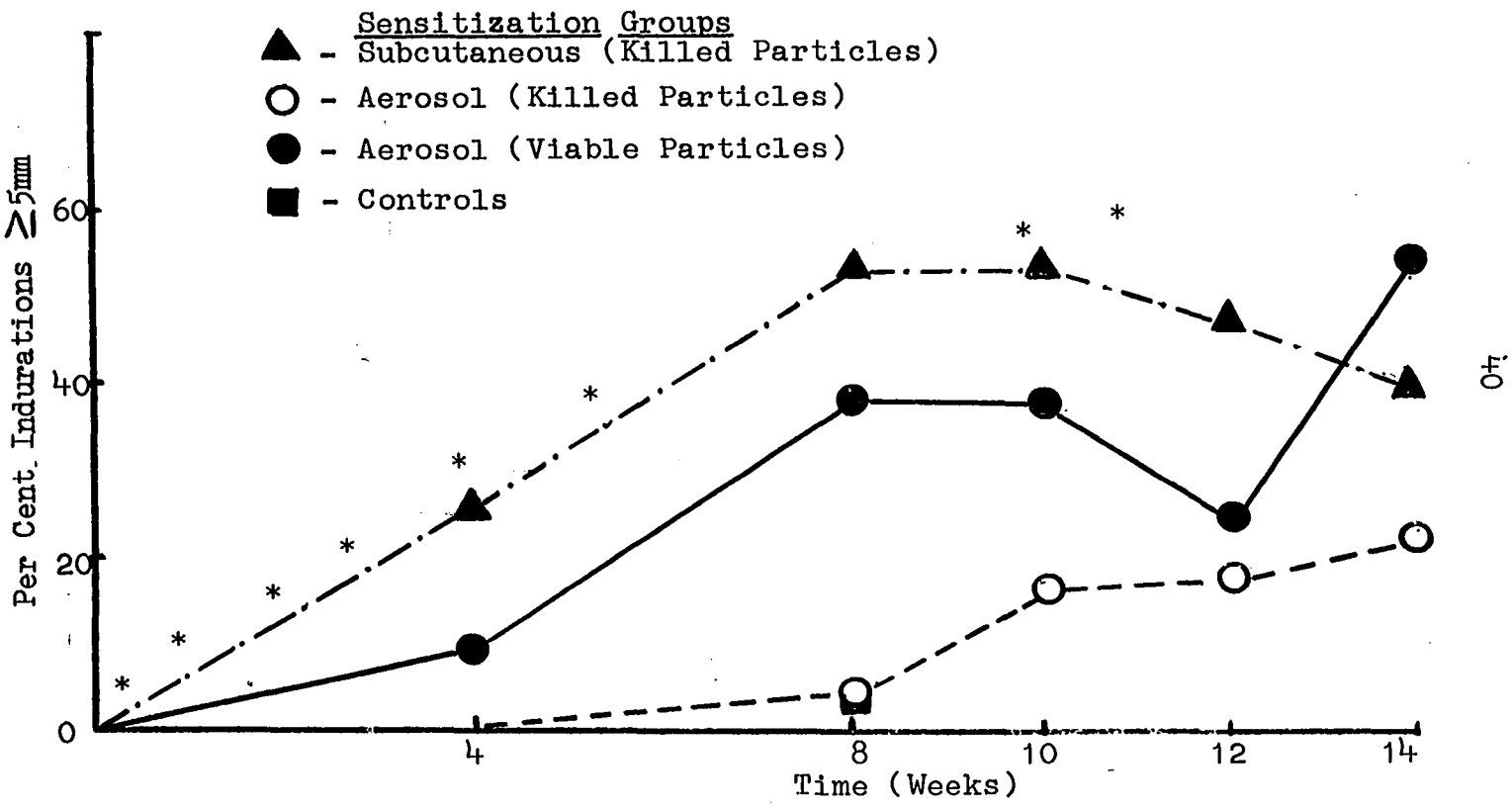
Experiment III

Titer	SKIN TEST HYPERSENSITIVITY					
	Less than 5mm Induration**	Equal or Greater than 5mm Induration	Total	Less than 5mm Induration	Equal or Greater than 5mm Induration	Total
Histoplasmin(CF)						
≤1:10	36*	10	46	31*	8	39
≥1:10	3	7	10	1	6	7
Total	39	17	56	32	14	46
Yeast Cells(CF)						
≤1:10	27	3	30	23	2	25
≥1:10	12	14	26	8	13	21
Total	39	17	56	31	15	46
CTA						
≤1:5	30	3	33	24	2	26
≥1:5	11	12	23	8	12	20
Total	41	15	56	32	14	46

*Does not include Control Group guinea pigs.
**Refers to Skin test reaction prior to challenge.

Figure 1. Percentage of Positive Histoplasmin Skin Tests in Guinea Pigs with Respect to Time and Sensitization Route.

Experiment III



*Indicates sensitization period

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