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# THE ROLE OF DELAYED HYPERSENSITIVITY IN BLASTOMYCOSIS OF MICE

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# THE ROLE OF DELAYED HYPERSENSITIVITY IN BLASTOMYCOSIS OF MICE

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# THE ROLE OF DELAYED HYPERSENSITIVITY IN BLASTOMYCOSIS OF MICE

## CHAPTER I

## INTRODUCTION

In recent years there has been an increase of interest in delayed or cellular hypersensitivity. It is now known that delayed hypersensitivity plays a critical role in autoimmune diseases, transplantation immunity, and host defense against infectious disease. In general, cellular defense mechanisms are the major participants in host defense against those pathogens capable of intracellular survival and proliferation. This defense involves resistance to a number of bacterial diseases, and may be extremely important in host defense against cancer.

It should be pointed out that there are types of infection in which humoral antibody is the predominant mediator of immunity. However patients with aggammaglobulinemia, still have good immunity to many diseases (31).

Delayed hypersensitivity is the result of a series of complex interactions between specialized cells involved in cell-mediated immunity. The primary cells involved are antigen-reactive lymphocytes and macrophages. The cells

involved in delayed hypersensitivity do not have abundant endoplasmic reticulum, although they contain many ribosomes and other structures necessary for protein synthesis. The lymphocytes, upon contact with specific antigen, produce soluble mediaters which influence other cells. If contact with antigen occurs intradermally in an appropriately sensitized individual, an inflammatory reaction develops which reaches its maximum in 24-48 hr. Histologically the initial cells to infiltrate a skin test site are polymorphonuclear leukocytes. The peak of this infiltration occurs 4-6 hr following antigen injection. At 8-12 hr after injection both lymphocytes and macrophages begin to infiltrate the site, and reach a maximum at 24-48 hr. Giant cells and epithelial cells may be observed in the site at this time and may persist for several days (3). This reaction consists mainly of erythema and induration. It must be differentiated from immediate hypersensitivity and arthus reactions, which occur within minutes if anaphylactic antibodies are present or after two hours if precipitin antibodies are present.

The type of immune response obtained in experimental animals and probably man is dependent, in part, on the manner in which the host is exposed to the antigen. Injection of antigen by the intravenous route seldom induces delayed hypersensitivity but may induce antibody synthesis, primarily in the spleen (5). The best induction of delayed

hypersensitivity results from incorporation of antigen into complete or incomplete Freund's adjuvant (30) and its injection intradermally into the footpads, or subcutaneously, into experimental animals (1, 2, 7).

The development of delayed-type hypersensitivity has many characteristics which are similar to antibody-mediated responses; both are functions of lymphocytes, are specific for a given antigen, and are increased by restimulation. There are however, some differences. A major breakthrough in the understanding of delayed hypersensitivity occurred when Landsteiner and Chase (14) reported that delayed-type hypersensitivities could be passively transferred with peritoneal exudate cells. More recently a unique difference in the reaction of antigen with cell-associated receptors was described. The specificity is apparently different from that described for humoral antibodies. Studies using synthetic polypeptides substituted with a hapten have shown that the determinant necessary to elicit a delayed reaction is larger than that required to react with humoral antibodies (25).

Although antibodies are produced by the host in response to foreign agents, in some diseases they are not involved in protection of the host. Studies with sera from animals containing antibodies to C. neoformans and H. capsulatum were unsuccessful in passive protection trials (15, 20). In contrast transfer of protection was

obtained by transferring cells. Although one-half of the Coccidioides-infected survivors and one third of the spherule vaccinated mice did not exhibit a footpad reaction, Kong et al. (13) have shown that transfer of spleens from immune to normal mice resulted in immunity to aerosolized challenge. In addition passive transfer of delayed sensitivity to tuberculin was achieved by transfer of mouse spleen cell suspensions (16).

Blastomyces dermatitidis is a dimorphic fungus which causes a chronic granulomatous and suppurative disease, thought to originate as a respiratory infection. After establishment in the lungs, the organisms may disseminate to other organs, causing a progressive disease which may be lethal. The lungs, bones, and skin are the predominate organs involved in the systemic form of the disease. Mild respiratory clinical forms of blastomycosis may occur (as in coccidioidomycosis and histoplasmosis) but their frequency has not been proved. Although delayed hypersensitivity in man seems to be a reliable index of previous exposure to dimorphic fungi, little is known of its relationship to protection. Most immunity in experimental mycotic infections may be determined in experimental animals by noting increased survival after challenge with a lethal dose, inhibition of fungal dissemination, enhanced cellular responses, or delayed hypersensitivity. A critical test for host immunity is the prevention of infection after

severe challenge, or an increased ability of the host to control the disease. Although severity of blastomycosis has been modified by prior infection, no data on fungal growth have been presented (11).

Patients with blastomycosis typically become hypersensitive. The most extensively used technique to detect this state is the intradermal injection of ground mycelial particles, yeast cells, or blastomycin (21). Blastomycin is almost exclusively used because of its stability while in storage, and its solubility. Smith (28) showed that delayed hypersensitivity was critical to the prognosis in patients with blastomycosis. Patients with recent pulmonary invasion or local skin lesions having a positive skin test and a negative complement fixation test (CF) had the best prognosis. Patients with more extensive infection with a positive skin test and a positive CF test in low titer responded to treatment better than those in which the skin test was negative and the CF test was positive with high titers.

Mice are susceptible to infection with B. dermatitidis and are often used for laboratory investigation and in clinical confirmation of suspected infection with this organism (6, 26, 27). Intraperitoneal inoculation of a saline suspension of the pathological material (to which antibiotics have been added if the specimen is sputum) usually results in many small abscesses in the liver, spleen

and mesentery in 3-4 weeks. Because of the relative difficulty of skin testing the mouse, delayed hypersensitivity in this animal may be detected systemically or by footpad inoculation. Salvin (23) and Box et al. (4) showed that mice could be experimentally induced to develop delayed hypersensitivity to H. capsulatum, a systemic fungal pathogen similar to B. dermatitidis. Following intravenous injection of living or dead H. capsulatum into sensitized mice, 90% of the mice died in 48 hr.

In recent years an in vitro technique has been developed for the detection of delayed hypersensitivity. This technique enables investigators to take advantage of the inhibition of macrophages that occurs when lymphocytes are stimulated by antigen to produce and release specific soluble inhibitors. Inhibition of cell migration was refined to give a reproducible and a quantitative test by George and Vaughan (10) in 1962. Peritoneal exudate cells (PE) cells have been most frequently employed in this test. It is quite sensitive and appears to be specific for delayed hypersensitivity (9). By culturing lymph node tissue from patients with a positive skin test to histoplasmin, Thor and Dray (29) were able to specifically inhibit the migration of human monocytes in the presence of 30 µg of histoplasmin per ml of medium.

The primary goal of this investigation was to determine the role of delayed hypersensitivity in blastomycosis

of mice. We wanted to determine if mice that had delayed hypersensitivity to B. dermatitidis would be protected from the lethal effects of intraperitoneal challenge. We wished to investigate induction of delayed hypersensitivity in mice with killed yeast cells and with viable cells of B. dermatitidis. We also wanted to determine the concentration of yeast cells necessary for induction of delayed hypersensitivity and for footpad sensitivity tests. Finally we sought to investigate two highly inbred mouse strains (C57BL/6J and CBA/J) for their value as experimental animals in delayed hypersensitivity experiments.

# CHAPTER II

## MATERIALS AND METHODS

Test animals:

Two-month-old C57BL/6J or CBA/J mice of both sexes were used. The mice were separated according to sex and given water and mouse chow ad libitum.

Organism:

The yeast phase of Blastomyces dermatitidis (isolate 242) used in this study was obtained from the University of Oklahoma stock culture collection. Yeast cultures were maintained on brain heart infusion (BHI) agar slants at 37 C.

Determination of $LD_{50}$:

To determine the 21 day intraperitoneal (ip) $LD_{50}$ for C57BL/6J mice, Blastomyces dermatitidis was grown on BHI agar slants for three days at 37 C. The cells were harvested by washing the slants with sterile physiological saline, collected with a sterile pipette, and placed in a sterile vaccine bottle. Hemocytometer counts were made to determine the number of cells/ml. The stock suspension of yeast cells was diluted to the desired concentration for

injection. One-tenth ml amounts of the diluted inoculum were spread on BHI agar plates for viability determinations. The plates were incubated at room temperature for three weeks; at this time the B. dermatitidis colonies were counted and recorded. Cells prepared in this manner were 15-40% viable. Each mouse was injected ip with 0.5 ml of the appropriate cell concentration and the number of survival days was noted. Studies were made with 120 mice to determine the $LD_{50}$. Calculations were performed according to the method of Reed and Muench (17).

Footpad tests:

To detect delayed hypersensitivity in mice, footpad sensitivity tests were done according to the procedure of Youmans and Youmans (34). The footpad tests were performed by injecting the appropriate antigen concentration contained in a volume of 0.03 ml into the right or left hind footpad and a similar volume of sterile physiological saline into the opposite footpad. A 1 ml syringe with a 27 gauge needle was used for the injections. Unless otherwise specified the thickness of each footpad was measured with a calipers calibrated to measure in tenths of a mm at 0, 6, 24, and 48 hr after injection. The difference in thickness between the saline-injected and the antigen-injected foot was calculated and used as a measure of the amount of swelling. The average difference for all mice in the test group was calculated and considered the mean increase in footpad

thickness. Before footpad testing the mice in each group were individually marked by notching their ears for recognition throughout the testing period.

Preparation of merthiolate-killed B. dermatitidis yeast cells:

Yeast cells, prepared by the method of Restrepo-Moreno, and Schneidau (18) were used in sensitization of mice with killed cells and in footpad sensitivity tests. Yeast phase cultures were prepared by inoculation of the flasks with 5 ml of a stock suspension of yeast cells and incubation of the flasks at 35 C with constant agitation on a gyrorotary shaker (New Brunswick Scientific Co., New Jersey) at 103 rotation per minute. The stock suspension was prepared by transferring the growth from a 3-day old yeast culture on BHI agar slants to 200 ml of a tryptic soy broth dialysate medium and incubating for 1 week on the gyrorotary shaker at 35 C. After 1 week the cultures were checked for absence of contamination and uniformity of growth. The cultures were pooled and merthiolate was added to a final concentration of 1:10,000. After 2 days at 5 C the cells were separated by centrifugation at 1500 rpm for 15 minutes and stored at 5 C in 1:10,000 merthiolate. In all experiments the cells were washed 3 times in sterile physiological saline before use.

Dry-weight determination of killed yeast cells:

Dry-weight determinations of killed B. dermatitidis yeast cells were made to standardize the inoculum. A stock suspension of merthiolate-killed yeast cells was washed by centrifugation in 50 ml graduated glass centrifuge tubes 3 times at 1500 rpm for 15 minutes. The packed cells were resuspended to a 15% suspension in sterile distilled water. One ml of this suspension was placed in each of 6 tared weighing bottles. The bottles were placed in a drying oven (Fisher Isotemp) at 100 C for 1 hr, cooled, and reweighed until a constant weight was obtained, at which time the weight of the cells was recorded. The average dry weight equivalent per ml of packed wet cells was calculated. Aliquots from a stock suspension of killed cells were used for dry weight determinations, sensitization experiments and for preparation of cells used in the footpad tests. For inoculation, cells were suspended in sterile physiological saline (0.85% NaCl). The yeast cell concentration referred to in experiments are based on the dry weight equivalent of packed wet cells. Emulsion of P-BSA or killed B. determatitidis yeast cells with incomplete Freund's adjuvant:

An incomplete Freund's adjuvant-yeast-cell emulsion was prepared to determine if a delayed-type hypersensitive response could be produced in mice with killed cells. The emulsion was prepared by continuous grinding, and 1 volume

of a suspension of killed cells (35 or 140 mg yeast cells/ml) was added a drop at a time into a mortar containing a mixture of 5 parts of marcole 52 (Humble Oil and Refining Co.) and 1 part Arlacel A (Hill Top Research Inc., Miamiville, Ohio). After all the cell suspension had been added, further emulsification was carried out by passing the material through an 18 gauge needle until the emulsion formed discrete drops on the surface of water. For sensitization each mouse was injected sc with 0.1 ml of this emulsion; this amount contained the equivalent of 2 mg of dry yeast cells.

Picryl BSA-incomplete adjuvant emulsion was prepared in a similar manner except that a solution containing either 0.35 or 1.75 mg of P-BSA was added to the marcole 52 and Arlacel A.

Inoculation of mice with picryl bovine serum albumin:

To determine if delayed hypersensitivity could be induced to a protein antigen in C57BL/6J or CBA/J mice, a protein-hapten conjugate was used. The protein was BSA (Fraction V, Pentex) modified by conjugation with picryl chloride (Eastman Organic Chemicals) following the procedure of Gell and Benacerraf (2). Seventy-five C57 mice were placed in 3 main groups. Twenty-five mice (5 per subgroup) in one group each received 2 subcutaneous (sc) injections in the inguinal region of 5 µg/0.1 ml of P-BSA emulsified in incomplete Freund's adjuvant. A second group

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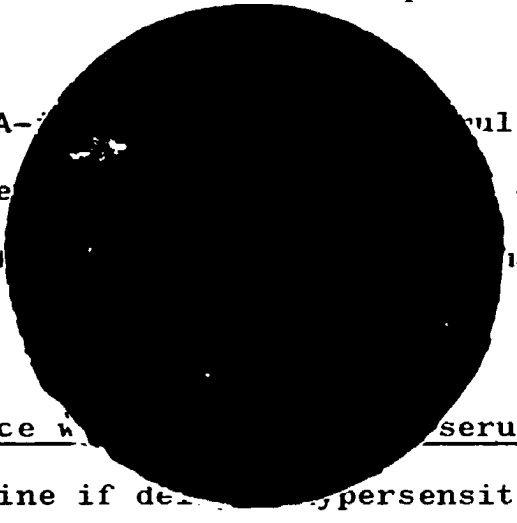
Picryl BSA-[redacted]ulsion was prepared in a similar manne[redacted] containing either 0.35 or 1.75 mg o[redacted]arcole 52 and Arlacel A.



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of 25 mice received 25 µg/0.1 ml in a similar manner. Twenty-five uninoculated mice (5 per subgroup) served as controls. Five mice from each P-BSA-treated group and 5 control mice were tested for footpad sensitivity to 50 µg of BSA on days 5, 15, 25, 35, and 40. A 1 ml syringe and a 25 gauge needle were used to make the injections. In a similar manner 10 CBA/J mice were inoculated and tested for footpad sensitivity at 15 and 35 days following initial injection. Ten CBA/J mice were used as controls.

Inoculation of mice with killed yeast cells:

To determine the time after vaccination with B. dermatitidis that delayed hypersensitivity could be detected by footpad inoculation and the most effective concentration of yeast cells to use for footpad sensitivity, 60 CS7 mice were separated into 2 groups. The first group consisted of 30 (5 per subgroup); all the mice in this group received 2 sc injections of merthiolate-killed yeast cells emulsified in incomplete Freund's adjuvant. The injections contained 2 mg of yeast cells/0.1 ml of emulsion and were given on day 0 and day 7. A 1 ml syringe with a 25 gauge needle was used for making the injections. The second group consisted of 30 uninoculated control mice (5 per subgroup). Both sc-inoculated mice and controls were tested for footpad sensitivity on days 5, 12, and 20. Two concentrations (40 or 50 µg) of killed B. dermatitidis

yeast cells were used for the detection of footpad sensitivity.

At each test period 10 mice were used for sc inoculation. The right rear footpads of 5 were each injected with 40 µg and those of the other 5 with 50 µg of yeast cells. The left footpads received an equal volume of sterile saline. The footpads of 10 control mice were each injected in a similar manner. Footpad measurements were recorded at 0, 6, 24, and 48 hr after injection and the mean increase in thickness was calculated.

Inoculation with viable yeast cells:

To investigate the ability of mice inoculated sc with viable cells to develop delayed hypersensitivity, 60 C57 mice were divided into 2 groups. The first group of 30 (5 per subgroup) received a single sc injection of viable B. dermatitidis yeast cells. Viable cells used in this study were prepared as described for the $LD_{50}$ studies. The cells were introduced with a 1 ml syringe and a 25 gauge needle. The second group consisted of 30 uninoculated control mice (5 per subgroup). Both sc-inoculated and control mice were tested for footpad sensitivity on days 3, 6, 9, 12, 15, and 18. At each test period five sc-inoculated mice and 5 controls were used. The right rear footpad of each mouse in both groups was injected with 45 µg of killed B. dermatitidis yeast cells. Footpad measurements were recorded at 0, 6, 24, and 48 hr and the mean increase in

thickness was calculated.

Protection tests:

To determine if sc inoculation of mice with viable cells of B. dermatitidis resulted in protection, 200 mice were separated into 2 groups of 100 each. Fifty in each group were sc injected with 0.1 ml of a yeast cell suspension that contained $3.9 \times 10^5$ cells/ml. Viable yeast cells for protection studies were prepared as described for $LD_{50}$ studies. Fifty uninoculated mice served as controls. Three days after sc injection 30 sc-inoculated mice and 30 controls were intraperitoneally (ip) inoculated with 0.5 ml of a yeast cell suspension that contained $3.9 \times 10^5$ cells/ml. Ten sc-inoculated mice and 10 controls were given an ip injection of an equal number of killed cells. The mortality of each group was recorded daily for 30 days. Ten sc-inoculated and 10 control mice were tested for footpad sensitivity by injection of 45 µg of killed yeast cells. In a similar manner 60 mice were challenged with 0.5 $LD_{50}$ on day 15 after sc inoculation. Mortality was observed and recorded daily for a 30 day period.

Migration inhibition test:

A study designed to determine if delayed hypersensitivity could be detected by inhibition of migration of peritoneal exudate (PE) cells was undertaken. One hundred and twenty C57 mice were divided into 2 groups of 60 each.

One group was injected sc with 0.1 ml of B. dermatitidis yeast cell suspension that contained $3.9 \times 10^5$ cells/ml; the other 60 mice were controls. The procedure for obtaining PE cells was basically that of Rowley and Whitsby (32). PE cells obtained by washing the peritoneal cavities of 20 sc-injected mice with 2-3 ml of Hanks balanced salt solution were pooled and placed in sterile siliconized centrifuge tubes. PE cells from 20 control mice were obtained and pooled in a similar manner. Control cells were maintained separately throughout the study. PE cells were centrifuged at 900 rpm for 5 minutes (PR-2, International Centrifuge), and resuspended in Eagles Minimal Essential Medium (MEM) (MEM-spinner base, Schwarz Bioresearch Inc.). This medium contained 15% calf serum (Grand Island Biological Co.), 85 units of penicillin, and 85 µg of streptomycin per ml of medium. The cells were washed once by centrifugation in MEM before use.

Mouse macrophage migration inhibition studies were based on the methods outlined by Anacker and Yamamoto (33). The packed cells were resuspended to a 10% suspension in MEM and drawn up in capillary tubes (1.1-1.2 mm internal diameter, Scientific Products). The capillaries were sealed on one end, placed in sterile tubes, and centrifuged for five minutes at 900 rpm. The capillaries were cut at the cell-fluid interphase and the piece containing the packed cells was attached to the bottom cover slip of a Sykes-Moore

tissue culture chamber (Bellco) with a small amount of sterile silicone. The chambers were assembled and filled with MEM with or without 30 µg/ml of blastomycin (242) and incubated at 37 C for 24 hr. Earlier experiments had shown that this concentration of blastomycin did not inhibit normal cell migration. The areas of migration of the cells from the open end of the capillaries were magnified and projected on a grid which previously had been marked and calibrated to measure the actual area of migration in $mm^2$. The % migration was calculated by dividing the area of migration of cells in the presence of 30 µg/ml of blastomycin multiplied by 100, by the area of migration of cells in the absence of blastomycin.

Suspensions of peritoneal exudate cells were assayed for the types of cells present; they were washed twice in MEM and resuspended in normal mouse serum. Smears from the suspensions were then stained with Wright's stain.

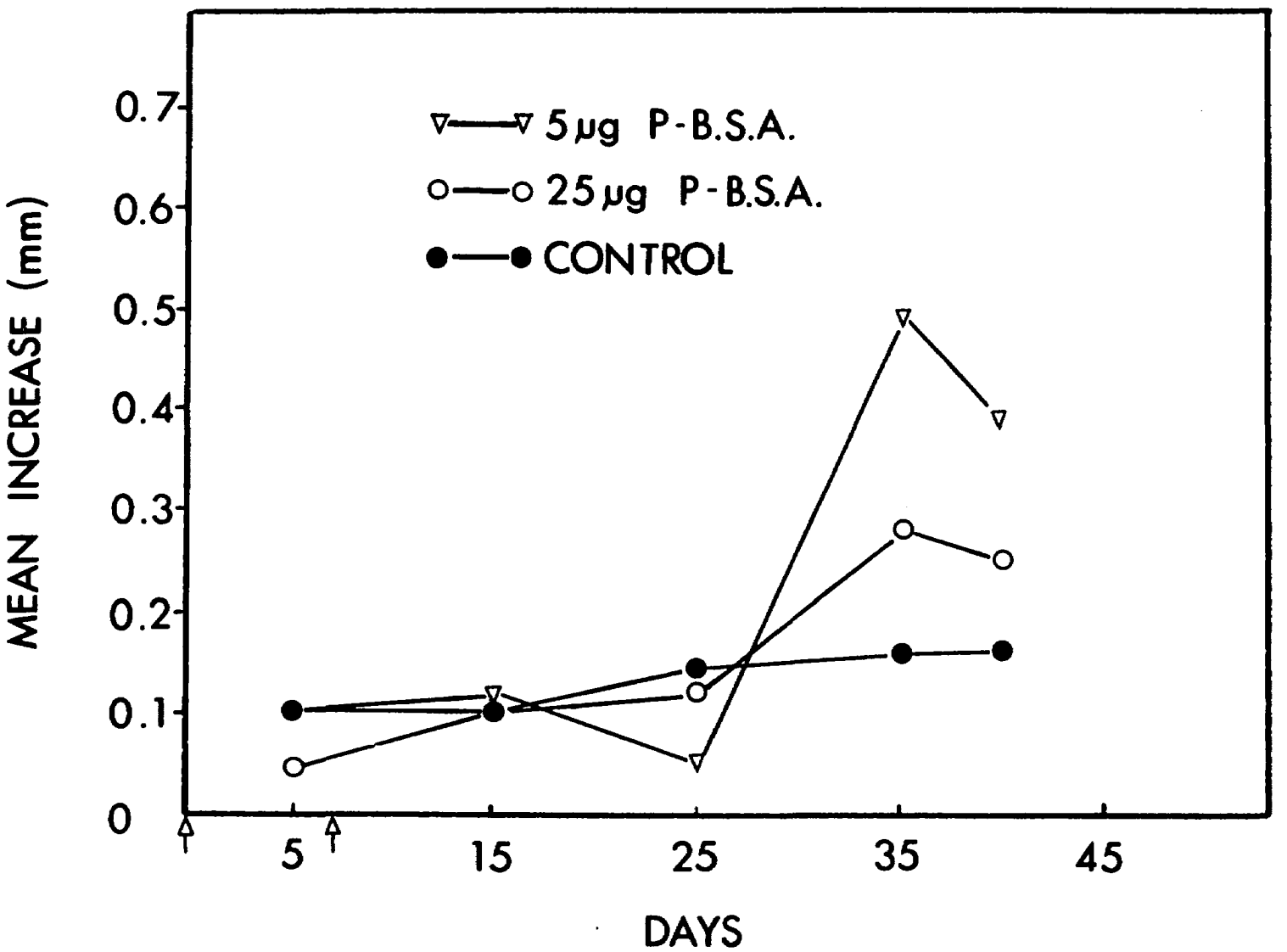
## CHAPTER III

## RESULTS

Inoculation of mice with P-BSA:

C57BL/6J mice inoculated sc with 5 or 25 µg of emulsified P-BSA were footpad tested with 50 µg of BSA after 5, 15, 25, 35, and 40 days. During the first 25 days sensitivity was not detected (48 hr reading) after footpad testing in the sc-inoculated or control mice (figure 1). At 35 and 40 days there was a considerable increase in the average 48 hr footpad thickness after testing in mice inoculated with either concentration of P-BSA, but not in the controls. An increase in footpad thickness was also noted in these mice 24 hr after footpad testing. The maximum increase in footpad thickness was obtained 48 hr after the 35 day footpad test in mice previously inoculated with 5 µg of P-BSA. At this time the mean increase in footpad thickness for these mice was 0.48 mm. At the 40 day footpad test the mean 48 hr increase in footpad thickness for the mice inoculated with 5 µg of P-BSA was 0.38 mm. The mice inoculated sc with 25 µg P-BSA responded to footpad testing in a similar manner but with a smaller 48 hr mean increase

Figure 1: Results of footpad sensitivity tests in C57BL/6J mice receiving picryl bovine serum albumin subcutaneously. Arrows indicate the days that the inoculations were made. The 48 hr mean increase after footpad test was plotted.


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
MEAN INCREASE (mm)
5 µg P-B.S.A.
25 µg P-B.S.A.
CONTROL
5
15
25
35
45
DAYS

in footpad thickness at the 35 and 40 day footpad test periods.

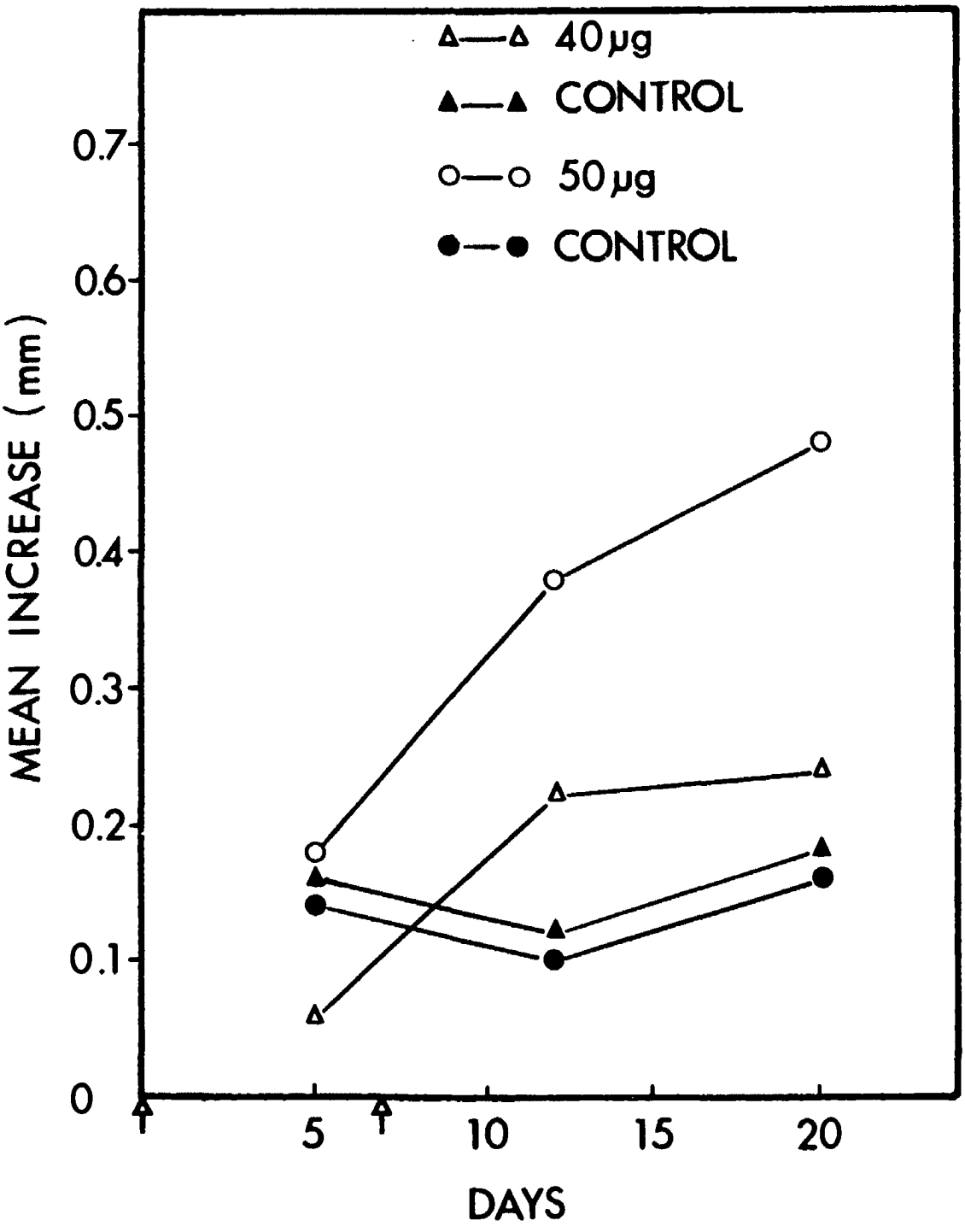
The footpads of CBA/J mice inoculated with 25 µg of the P-BSA emulsion were not sensitive to injection with 50 µg of BSA. No increase in footpad thickness at 48 hr after footpad testing was noted at either the 15 or 35 day test periods. These results showed that the CBA/J mice were not suitable for footpad testing; they were not a suitable animal for study of delayed hypersensitivity. Thus only the C57BL/6J mice were used in subsequent studies.

Inoculation of mice with killed cells of B. dermatitidis:

C57BL/6J mice inoculated sc with 2 mg of a yeast-cell emulsion of B. dermatitidis were footpad tested with 40 or 50 µg of killed yeast cells after 5, 12, and 20 days. As can be seen in figure 2, footpad injection with 40 or 50 µg at 5 days did not stimulate a 48 hr increase in footpad thickness of sc inoculated mice or control mice. However hyperreactivity at 48 hr to 50 µg was noted in sc inoculated mice at the 12 and 20 day test periods. The maximum 48 hr increase in footpad thickness occurred at the 20 day test period when mice were footpad tested with 50 µg. At this time the mean increase in footpad thickness for these mice was 0.48 mm, which was considerably larger than footpad thickness of control mice. The footpads of mice tested with 40 µg were not larger 48 hr after injection than those of control mice at either the 12 or 20 day test

Figure 2: Results of footpad inoculation with killed yeast cells of B. dermatitidis.

Arrows indicate the days that the subcutaneous inoculations were made. C57BL/6J mice were inoculated with 2 mg of killed yeast cells emulsified in incomplete Freund's adjuvant. The 48 hr mean increase after footpad injection was plotted.


40µg
CONTROL
50µg
CONTROL
MEAN INCREASE (mm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
5
10
15
20
DAYS

periods. It was determined later however, that 45 µg of killed cells elicited a hypersensitive response in sensitized mice but did not elicit a response in normal mice. Therefore 45 µg was used as the footpad testing concentration of killed yeast cells in future experiments. Additional experiments have shown that 5 or 25 µg of killed cells do not elicit a footpad response in mice inoculated with the yeast cell emulsion and footpad tested in a similar manner. Other experiments have shown also that mice can be sensitized with either 0.5 or 2 mg of the yeast-cell emulsion. An increase was not seen in the 48 hr footpad thickness of control animals following footpad testing. It was shown by results of this study that C57 mice can be sensitized to killed B. dermatitidis yeast cells and the mice become hypersensitive by 12 days after sc inoculation.

Mice inoculated sc with 2 mg of emulsified yeast cells were footpad tested at 12 days with 50 µg of killed yeast cells. Following footpad injection their footpads were measured at 0, 6, 24, and 48 hr and the mean increase in footpad thickness was calculated for each period (table 1). At 6 hr following footpad injection the footpad thickness of mice inoculated sc and controls was increased. By 24-48 hr however, the average footpad thickness of mice inoculated sc had increased while those of controls had decreased. For this reason, 48 hr was chosen as the best time to read the results of footpad

Table 1: Mean increase in footpad thickness of C57BL/6J mice following footpad injection with 50 µg of killed cells 12 days after sc inoculation with 2 mg of killed cells of B. dermatitidis emulsified in incomplete Freund's adjuvant.

| Material injected* | Mean increase (mm) Hours after injection | | | |
|---|---|---|---|---|
| | 0 | 6 | 24 | 48 |
| killed cells | 0.06** | 0.22 | 0.54 | 0.38 |
| none | 0.08 | 0.20 | 0.12 | 0.10 |

* A 2 mg amount (dry weight equivalent) in incomplete Freund's adjuvant was injected sc on day 0 and 7.

** Average of 5 mice.

sensitivity tests. In this paper all future references to the mean increase in footpad thickness will be referring to a 48 hr reading unless otherwise stated.

Inoculation of mice with viable yeast cells:

C57 mice were inoculated sc with viable yeast cells of B. dermatitidis and footpad tested with 45 µg of killed yeast cells at 3, 6, 9, 12, 15, and 18 days. Figure 3 shows that no appreciable difference was noted at 3 days in the average footpad thickness of mice inoculated sc with viable cells or in control mice. Footpad sensitivity tests in mice inoculated sc at days 6 and 9 produced a moderate increase in footpad thickness. Two animals in the sc-inoculated group had an increase in footpad thickness of 0.8 mm (one on day 6 and one on day 9). A marked increase in the mean footpad thickness of mice inoculated sc was noted at the 12, 15, and 18 day footpad test periods. The mean increase in footpad thickness of these mice on these test days was 0.48, 0.58, and 0.40 mm, respectively. The increase in footpad thickness of all control animals footpad tested on days 12, 15, and 18 was less than 0.2 mm. However, 10 out of 15 mice inoculated sc and footpad tested on these days had a mean increase of 0.72 mm (range-0.4-1.4).

Mice inoculated sc with viable cells, and control animals were footpad tested with 45 µg of killed cells on day 15 after inoculation and measured at 6 hr and then daily for 7 days (figure 4). The maximum increase in

Figure 3: Results of footpad sensitivity tests for C57BL/6J mice that received viable B. dermatitidis yeast cells. The single-sensitizing dose was $3.9 \times 10^4$ yeast cells

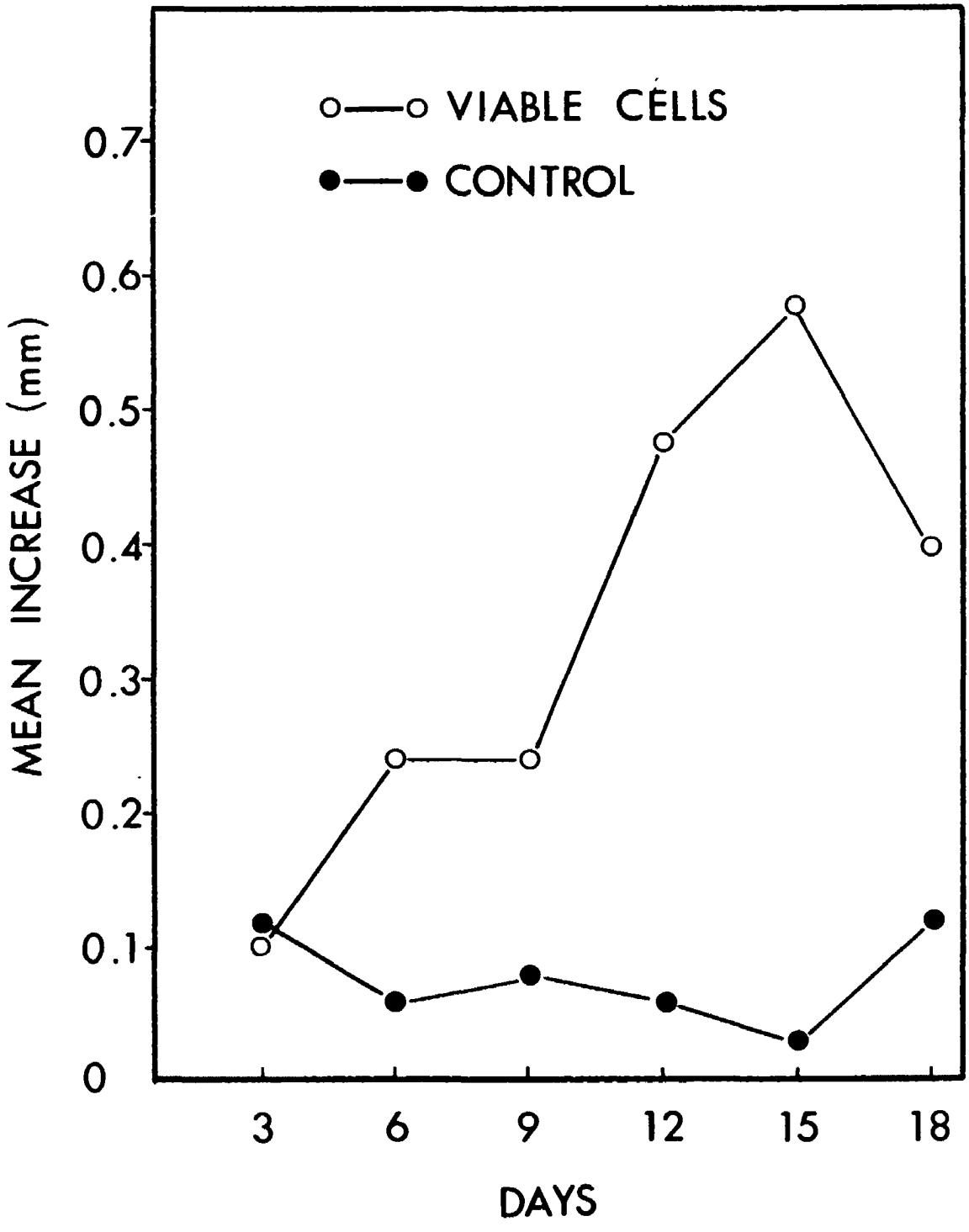
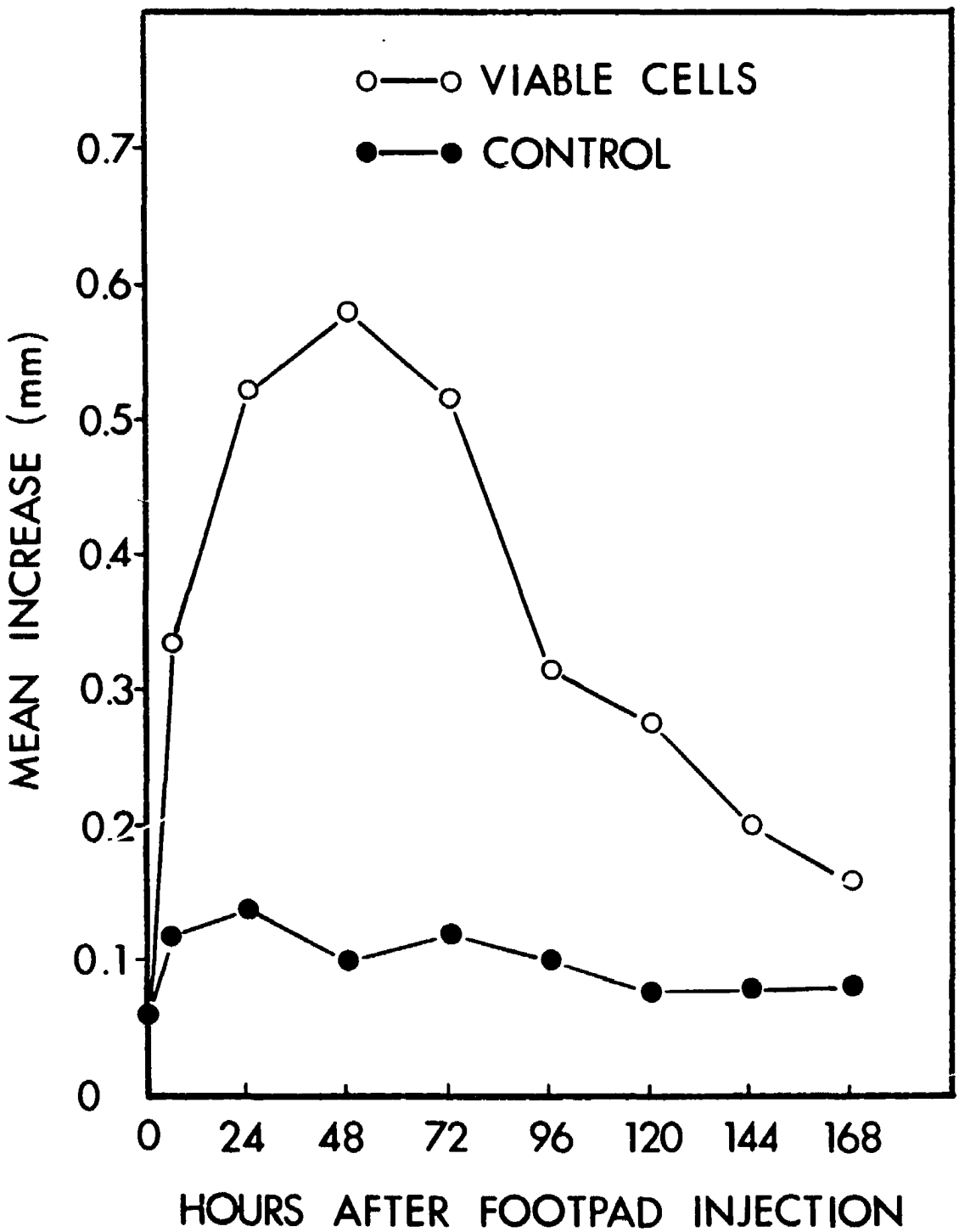

VIABLE CELLS
CONTROL
MEAN INCREASE (mm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
3
6
9
12
15
18
DAYS

Figure 4: Results of footpad sensitivity tests for mice that received viable B. dermatitidis yeast cells. The footpads of C57BL/6J mice inoculated subcutaneously and control mice were injected on day 15 and measured at 6 hr and then daily for 7 days.


VIABLE CELLS
CONTROL
MEAN INCREASE (mm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
24
48
72
96
120
144
168
HOURS AFTER FOOTPAD INJECTION

footpad thickness occurred at 48 hr. After this the mean footpad thickness subsided slowly until it was almost back to that of the controls after 7 days.

Visible lesions developed at the site of inoculation in C57 mice approximately 9-12 days after sc inoculation of viable cells. The lesions were swollen and inflamed subcutaneous areas which occasionally broke through the surface of the skin. Impression smears were made from the exudate material in this lesion. When the mice were autopsied at 20 days after inoculation these smears revealed the characteristic budding cells of B. dermatitidis. Its presence was confirmed by culture of the exudate on BHI agar plates. In most mice a large amount of exudate material was found just below the skin at the injection site.

From this study it was evident that mice inoculated sc with viable B. dermatitidis cells became hypersensitive to footpad injection of killed yeast cells 12 days after inoculation. It was determined also that the maximum increase in footpad thickness of mice inoculated sc and footpad tested at 15 days with 45 μg of killed cells occurred at 48 hr after footpad injection. In addition it was noted that mice were infected as evidenced by our ability to microscopically observe yeast cells in impression smears and to culture B. dermatitidis cells from the lesions at the injection site.

Protection tests:

C57Bl/6J mice inoculated sc with viable yeast cells and control mice were challenged ip with 0.5 $LD_{50}$ of B. dermatitidis at 3 and 15 days following the sc inoculation. This was just before and just after animals would have become hypersensitive. The 21 day $LD_{50}$ for C57 mice was $3.9 \times 10^5$ cells/ml. Additional C57 mice inoculated sc and control mice were tested for footpad sensitivity to 45 µg of killed cells of B. dermatitidis and were given an ip inoculation of a concentration of killed cells equal to 0.5 $LD_{50}$ on day 3 and 15 following the sc inoculation. The first deaths in control mice challenged at 3 days occurred at day 6 and 90% of the mice were dead by day 40 (figure 5). In mice inoculated sc and challenged with the same inoculum at 3 days, the first death was on day 11 and 96% of these mice were dead at 40 days.

Footpad-sensitivity tests performed on 10 mice inoculated sc and 10 control mice did not stimulate hypersensitivity detectable by the footpad test 3 days after sc inoculation. Inoculation of mice ip with a concentration of killed cells equal to 0.5 $LD_{50}$ did not result in death or any signs of distress in either the mice inoculated sc or in the controls.

After the 15-day ip challenge (figure 6) the first control mice died on day 11 and 93% were dead at 40 days. After the 15-day ip challenge no mice inoculated sc died until day 20 and only 36% were dead at 40 days.

Figure 5: Percent mortality in C57BL/6J mice inoculated subcutaneously with $3.9 \times 10^4$ viable cells of B. dermatitidis and intraperitoneally challenged 3 days later with 0.5 $LD_{50}$.

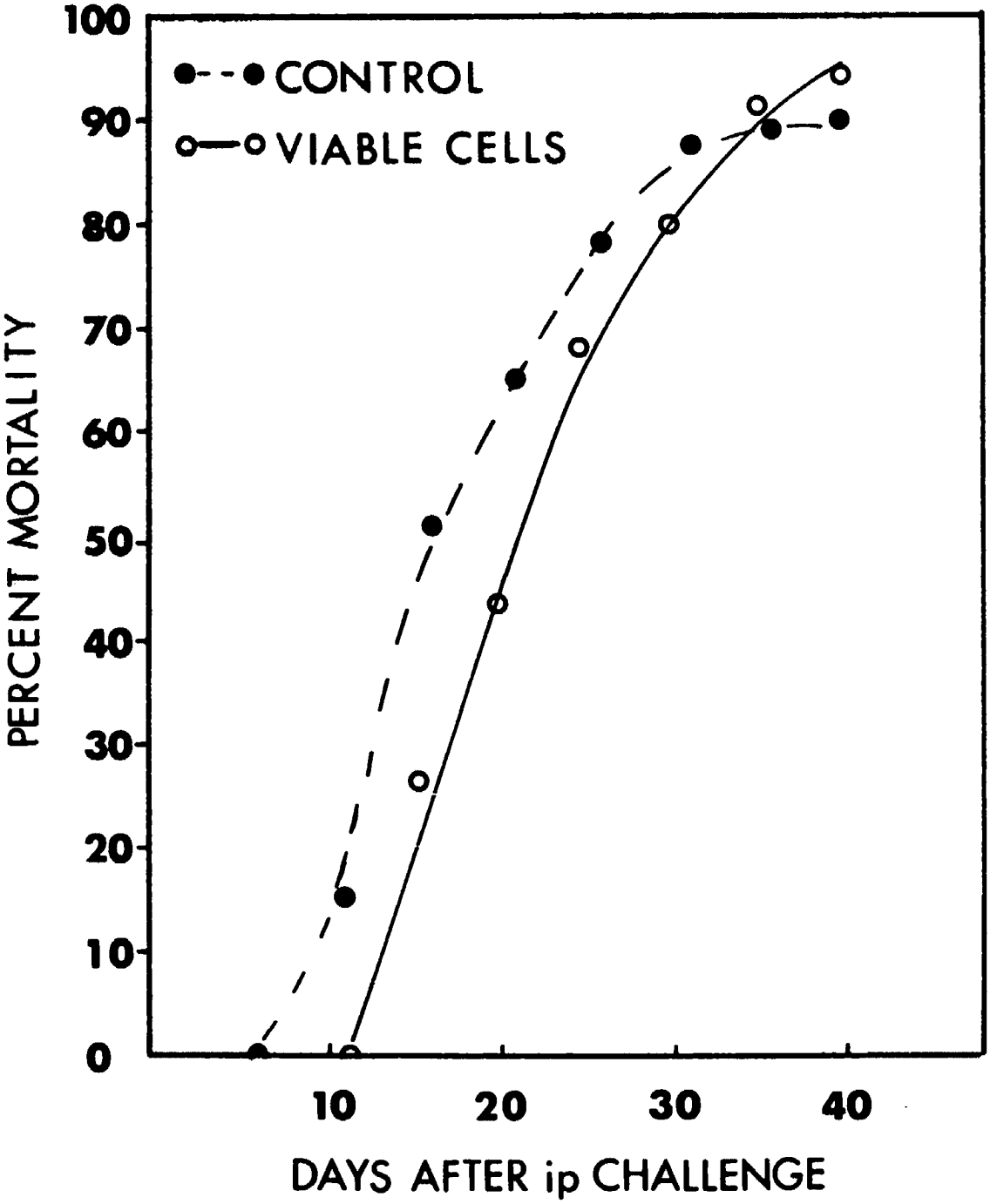
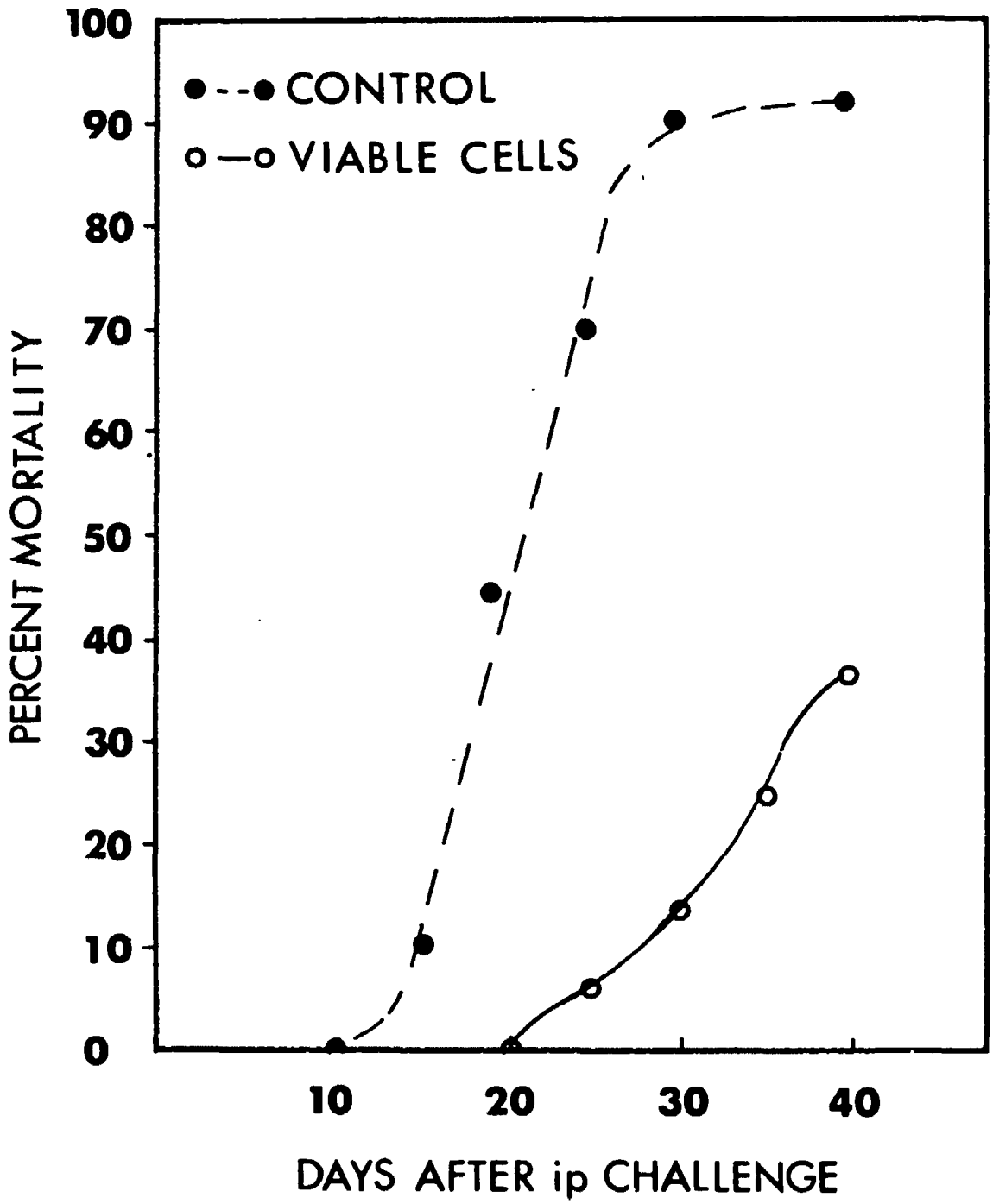

100
90
80
70
60
50
40
30
20
10
0
CONTROL
VIABLE CELLS
PERCENT MORTALITY
10
20
30
40
DAYS AFTER ip CHALLENGE

Figure 6: Percent mortality in C57BL/6J mice inoculated subcutaneously with $3.9 \times 10^4$ viable cells of B. dermatitidis and intraperitoneally challenged 15 days later with 0.5 $LD_{50}$.


100
90
80
70
60
50
40
30
20
10
0
CONTROL
VIABLE CELLS
PERCENT MORTALITY
10
20
30
40
DAYS AFTER ip CHALLENGE

Footpad tests performed on 10 control and 10 mice inoculated sc at 15 days showed that the sc inoculated mice had a marked increase in their mean footpad thickness while the footpad thickness of controls was not increased. The data in table 2 shows the mean footpad thickness of these mice tested with 45 μg of killed cells 15 days after sc inoculation with viable cells. As can be seen in this table the mean footpad thickness of mice inoculated sc was much larger at 24-48 hr than those of control mice. The standard deviation in thickness of the yeast-cell injected foot of these mice was larger at 24 and 48 hr because the range in footpad thickness during this time was 1.9-4.0 mm. In contrast the range in footpad thickness of control mice during this time which was 2.1-2.4 mm. Controls and mice inoculated sc were challenged at 15 days with a concentration of killed cells equal to 0.5 $LD_{50}$. Neither mice inoculated sc or control mice showed any signs of distress.

The results of this study show that sc inoculation of viable B. dermatitidis cells, stimulates footpad sensitivity to 45 μg of killed cells and also induces significant protection against lethal effects of viable B. dermatitidis. Mice inoculated sc and challenged at 3 days (before becoming hypersensitive) were not protected. Mice inoculated sc and challenged at 15 days (after becoming hypersensitive) were protected.

Table 2. Footpad sensitivity of C57BL/6J mice to killed yeast cells of B. dermatitidis 15 days after subcutaneous injection of $3.9 \times 10^4$ viable cells.

| Hours after footpad inoculation | Mean footpad thickness (mm) | | | |
|---|---|---|---|---|
| | control | | sc injected | |
| | right* | left | right | left |
| 0 | 2.17±0.06** | 2.08±0.07 | 2.08±0.10 | 2.03±0.12 |
| 6 | 2.37±0.15 | 2.15±0.11 | 2.50±0.31 | 2.13±0.16 |
| 24 | 2.29±0.18 | 2.12±0.09 | 2.66±0.50 | 2.01±0.13 |
| 48 | 2.19±0.14 | 2.07±0.10 | 2.87±0.61 | 1.99±0.11 |

*Right foot was injected with 45 µg of killed yeast cells; the left foot was injected with saline.

**Average foot thickness of 10 mice plus the standard deviation.

Inhibition of migration of peritoneal exudate cells:

Peritoneal exudate cells were obtained from mice inoculated sc with $3.9 \times 10^4$ viable cells of B. dermatitidis and from control mice. The ability of these cells to migrate in the presence of 30 µg of blastomycin was tested on days 3, 15, and 20. No inhibition of PE cell migration in the presence of 30 µg of blastomycin was noted at 3 days when the cells were obtained from either mice inoculated sc or from control mice (table 3). At 15 and 20 days however, PE cells obtained from mice inoculated sc were inhibited in their migration 13 and 21%, respectively. PE cells from control mice were not inhibited from migrating by 30 µg blastomycin at either of these test days.

Table 4 presents data representing the actual area of migration of the PE cells. The average migration of normal cells in the absence of blastomycin, using the mean migration for each test period, was $2.39 \pm 0.22$ mm$^2$. The average migration of normal cells in the presence of 30 µg of blastomycin was $2.36 \pm 0.24$ mm$^2$; the average migration of PE cells from animals inoculated sc with no blastomycin was $2.15 \pm 0.16$ mm$^2$; and in the presence of 30 µg of blastomycin it was $1.91 \pm 0.23$ mm$^2$. The standard deviation in migration within a single test was usually less than 0.34 mm$^2$; however, in one test a standard deviation of 0.54 mm$^2$ was observed. This occurred at the 3-day test period with normal cells in the absence of blastomycin. PE cells from

Table 3. Inhibition of peritoneal exudate cells obtained from C57BL/6J mice at various intervals after subcutaneous inoculation with viable B. dermatitidis cells. Percent migration in the presence of 30 µg of blastomycin.

| Days after injection of viable yeast cells | Percent migration of PE cells | |
|---|---|---|
| | control* | sensitized |
| 3 | 102** | 105 |
| 15 | 97 | 87 |
| 20 | 98 | 79 |

* Controls consisted of PE cells obtained from normal mice.

**Average percent migration from 4 capillaries representing 20 mice per group.

Table 4. Migration of peritoneal exudate cells from $4$ C57BL/6J mice inoculated subcutaneously with $3.9 \times 10^4$ viable cells of B. dermatitidis and control mice in the presence or absence of 30 µg of blastomycin.

| Days after sc injection of viable cells | Normal cells | | Sensitized cells | |
|---|---|---|---|---|
| | blastomycin concentration | | | |
| | none | 30 µg | none | 30 µg |
| 3 | 1.92±0.54* | 1.96±0.29 | 1.48±0.14 | 1.56±0.11 |
| 15 | 2.90±0.24 | 2.82±0.27 | 2.49±0.02 | 2.19±0.34 |
| 20 | 2.36±0.17 | 2.31±0.17 | 2.49±0.33 | 1.98±0.24 |

* Mean actual area of migration in $mm^2$

control C57 mice migrated 100% in the presence of 30 µg of blastomycin/ml. Other experiments had shown that 50 or 100 µg of blastomycin did cause inhibition of PE cells from control mice.

Suspensions of peritoneal exudate cells from mice inoculated sc and normal mice contained about 60% macrophages, 30% lymphocytes, and 8% segmented cells. The majority of the sc-inoculated mice developed lesions at the injection site after 12-15 days, but did not develop a fatal infection during the time of observation.

PE cells from mice inoculated sc with viable cells of B. dermatitidis are inhibited from migrating in the presence of 30 µg of blastomycin. This inhibition was noted at 15 and 20 days after sc inoculation of viable cells.

# CHAPTER IV

## DISCUSSION

Gell and Benacerraf (2) have shown that immunication with hapten-carrying proteins in guinea pigs is followed by the appearance of delayed hypersensitivity to the protein carrier. Antibodies against the carrier protein cannot be detected at this time although antibodies are present against the haptenic group. In addition, delayed hypersensitivity to the haptenic group was not detected in this study. It was our intention to minimize the immunological injury mediated by antibody reactions and to elicit only delayed hypersensitivity in the P-BSA sensitized mice. We found that delayed hypersensitivity to BSA in C57 mice was obtained 35 days after initial injection of P-BSA. Our results agree with those of Crowle and Hu (7) who obtained the maximum response from mice exhibiting hypersensitivity to BSA 3-4 weeks after treatment with BSA in incomplete Freund's adjuvant. C57 mice inoculated with P-BSA were found to be hypersensitive to footpad inoculation with BSA while CBA mice were not. For this reason the C57 mice were used for footpad sensitization studies.

The B. dermatitidis yeast cells used in this study induced delayed hypersensitivity. This sensitivity was detected by footpad inoculation with 45 or 50 µg of killed yeast cells. Although an increase in footpad thickness could be observed when footpads of sensitized mice were challenged with 40 µg of yeast cells, this response was not larger than the control mice. Additional experiments had shown that 5 or 25 µg of killed yeast cells were not sufficient to elicit a delayed footpad response in sensitized mice. In our studies uninoculated mice responded with a mild inflammatory footpad response when injected with 50 µg of killed cells, but in most mice this reaction had subsided by 24 hr and the yeast-cell injected foot appeared the same as the saline injected foot. Higher concentrations of yeast cells however, resulted in a toxic reaction which was characterized by redding, swelling, and induration which persisted for several days. The reaction resembled that observed when yeast cell concentrations not toxic for control mice were injected into the footpads of a sensitized mouse. Therefore it is necessary that uninoculated mice be used as controls in this type of investigation. The observation that B. dermatitidis yeast cells contain a substance toxic for mice had previously been reported in the literature. Salvin (24) found that a saline extract of acetone-dried yeast cells of this organism contained a substance which was able to produce death in mice within 48 hr.

Footpad sensitivity to killed yeast cells was noted also following sc inoculation with viable cells. Development of footpad sensitivity in these mice, tested every 3 days for 18 days, occurred between the 9 and 12 day test periods; twelve days was the time a notable increase in footpad thickness was observed in mice inoculated with killed cells. Mice can be sensitized by sc inoculation with either living or dead yeast cells of B. dermatitidis and this sensitization occurs approximately 12 days after initial inoculation. These results support investigations of hypersensitivity using other systemic fungal pathogens. It has been determined that hypersensitivity to H. capsulatum is produced in mice 9-14 days after inoculation (4, 23) or to C. immitis 15 days after injection with either spherules or coccidioidin contained in incomplete Freund's adjuvant (13).

If the footpad sensitivity observed in this investigation was due to delayed hypersensitivity, the migration of peritoneal exudate (PE) cells from sensitized mice should have been inhibited in the presence of blastomycin (242). Other investigators (12) have reported that the yeast phase and mycelial phase have common antigens. Blastomycin is a broth filtrate of the mycelial phase. In the present investigation evidence of delayed hypersensitivity was obtained when PE cells harvested from sc-inoculated mice were inhibited in their migration in the presence of 30 µg

of blastomycin. Inhibition of PE cell migration from mice inoculated sc although not observed 3 days after inoculation was 13% by fifteen and 21% by twenty days after inoculation. Although this inhibition was moderate it was notable, since PE cells from control mice were not inhibited. This inhibition occurred at a time when mice inoculated in a similar manner had developed considerable footpad sensitivity to killed yeast cells. Additional experiments had shown that higher concentrations (50 or 100 µg) of blastomycin inhibited the migration of PE cells from control mice 50-70%. In our investigations it was noted that PE cells from uninoculated BALB/cJ mice were inhibited in their migration up to 50% in the presence of 30 µg of blastomycin; however 100% migration of PE cells from these animals was obtained in the presence of 20 µg of blastomycin. These results point out that the blastomycin concentration used in mouse macrophage migration-inhibition studies is critical and must be carefully controlled. The sensitivity of PE cells from different strains of highly inbred mice may reflect the animals ability to control experimental infection with B. dermatitidis. Thus normal cells from BALB/cJ mice were inhibited in their migration in the presence of 30 µg of blastomycin and normal cells of C57 mice were uninhibited in their migration at this same concentration. In summary tissues of mice from different highly inbred mouse strains exhibit differing degrees of sensitivity to migration-inhibition toxic factors of fungi.

The results of the footpad tests and the PE inhibition studies have shown that these mice had acquired delayed hypersensitivity to B. dermatitidis after 12 days of immunization. The results of the protection showed that mice with delayed hypersensitivity were protected from a lethal challenge. When the mice inoculated sc and control mice were given a lethal challenge 3 days after sc inoculation, there was no evidence of protection; however, by 15 days the test mice had acquired a marked increase in their footpad size and were protected. It is evident that sc inoculation of viable B. dermatitidis yeast cells leads to the type of immune response that protects mice in this disease. We believe this response to be primarily a cell-mediated type of immunity.

In the present investigation we found that the manner of exposure of the host to the pathogen was critical in the production of delayed hypersensitivity. Subcutaneous inoculation of living or dead cells in adjuvant produced a far better state of delayed hypersensitivity than intraperitoneal inoculation. Mice inoculated intraperitoneally with viable cells did not develop delayed hypersensitivity as detected by footpad inoculation and were unable to control the progressive disease. In contrast, in this investigation mice inoculated sc with viable cells developed a considerable increase in footpad size and at autopsy 20 days after inoculation there was no evidence of dissemination.

Roberts (19) compared the immune response of rabbits inoculated intravenously or intratracheally with viable cells of H. capsulatum. The results of this investigation show that the route of inoculation determines the type of response induced in rabbits to H. capsulatum. Seventy percent of the rabbits inoculated intratracheally with this organism, developed delayed hypersensitivity to histoplasmin, had few clinical symptoms, and the infection was not fatal. In contrast, only 14% of the rabbits inoculated intravenously developed delayed hypersensitivity, most had severe clinical symptoms, and a number of these animals died. These results imply that rabbits which have delayed hypersensitivity to H. capsulatum are more protected from the progressive disease.

# CHAPTER V

## SUMMARY

C57BL/6J mice rendered hypersensitive to B. dermatitidis were protected from the lethal effects of a blastomyces infection. This protection was observed following a lethal intraperitoneal (ip) challenge with viable cells 15 days after subcutaneous (sc) inoculation of the mice with $3.9 \times 10^4$ viable cells. It is important to note that no protection was observed in mice following a lethal ip inoculation of viable cells 3 days after receiving the sc inoculation. At 3 days after sc inoculation the mice had not become hypersensitive to killed yeast cells of B. dermatitidis, as indicated by footpad testing, and were not protected; however by 15 days after the sc inoculation the mice were hypersensitive and were protected.

Delayed hypersensitivity was produced in C57 mice by subcutaneously inoculating them with two injections of either 0.5 or 2 mg of merthiolate-killed yeast cells in adjuvant or with a single injection of $3.9 \times 10^4$ viable cells. It was found that footpad sensitivity could be detected in these mice by footpad inoculation of 45 or 50 µg of killed yeast cells 12 days following the initial

injection. Lower concentrations of killed cells failed to elicit an increase in footpad thickness 48 hr following footpad injection of sensitized mice.

The route of inoculation was critical in the induction of hypersensitivity. Subcutaneous inoculation of living cells or killed cells in adjuvant produced a far better state of delayed hypersensitivity than ip inoculation.

Evidence of delayed hypersensitivity was noted also when peritoneal exudate cells from mice inoculated sc with viable cells were inhibited from migrating in the presence of 30 µg of blastomycin. Peritoneal exudate cells from mice that received a single subcutaneous injection of viable cells were inhibited 13% at fifteen days and 21% at twenty days following injection. Peritoneal exudate cells from control mice were not inhibited in the presence of 30 µg of blastomycin at any test period.

C57BL/6J mice were found to be more suitable for footpad sensitization studies than CBA/J mice. No increase in footpad thickness in either mouse strain was seen 15 days after inoculation with 25 µg of picryl bovine serum albumin, but by 35 days the footpads of the C57 mice inoculated with 50 µg of BSA were notably larger at 48 hr than those of control mice. The footpads of CBA mice inoculated in a similar manner and tested at 35 days were not larger than those of the control mice. Thus the C57 mice were used for studies of delayed hypersensitivity.

PE cells from uninoculated BALB/cJ mice were inhibited in their migration in the presence of 30 µg of blastomycin, but PE cells from C57 mice were not. Blastomycin concentrations used in mouse macrophage migration-inhibition studies is critical and also the mouse strain employed is of prime importance.

From the results of this study delayed hypersensitivity or cellular immune factors play a major role in host protection against blastomycosis in mice.

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