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Role of Delayed Hypersensitivity in Blastomycosis of Mice

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C57BL/6J mice rendered hypersensitive to *Blastomyces dermatitidis* were protected from the lethal effects of a blastomyces infection. This protection was observed following a lethal intraperitoneal challenge with viable cells 15 days after subcutaneous inoculation with 3.9×10^4 viable cells. Delayed hypersensitivity was induced in C57 mice by two injections of Merthiolate-killed cells in adjuvant or by a single injection of viable cells. Development of hypersensitivity was determined at appropriate intervals by footpad injection with killed yeast cells.

Although delayed hypersensitivity (DH) to *Blastomyces dermatitidis* in man and animals frequently develops as a result of previous contact or infection with this fungus, little is known of its relationship to host defense. DH in patients with blastomycosis is usually observed as a strongly positive skin reaction to intradermal blastomycin. The question of whether this type of cell-mediated immunity contributes to the ability of the host to arrest the progress of the infection or serves to enhance the disease process is still not resolved.

Smith (15) in 1949 recognized the importance of DH to patients with blastomycosis and observed that the prognosis in these patients was dependent on the nature of the immune response by the host. In general those patients with DH to killed yeast cells or blastomycin had the better prognosis. However, he felt that patients with DH should be desensitized before treatment with iodides and those with neither positive skin tests nor complement-fixing antibodies should be actively immunized with killed yeast cells.

Laboratory animals may be used to investigate the role of DH in mycotic diseases. Several investigators have reported that mice develop DH to proteins (4), mycobacterial cells (C. M. S. Pass and H. Friedman, Bacteriol. Proc., p. 55, 1965), and fungal antigens (2, 6, 12). The extent of immunity in systemic fungal diseases may be determined in mice by noting increased survival after challenge with a lethal dose, suppression of fungal dissemination, or modification in the course of reinfection (7). Although a number of laboratory animals are susceptible to infection with *B. dermatitidis* (3, 14), mice are readily infected with this fungus and may be used for experimental studies (7, 8).

Studies of DH to other fungal pathogens in mice have been reported in the literature. Salvin (12) and Box and Briggs (2) have shown that inoculation of sensitized mice with large concentration of *Histoplasma capsulatum* yeast cells may be fatal within 48 hr. Kong et al. (6) have reported that mice immunized with killed spherules of *Coccidioides immitis* developed DH, as detected by footpad inoculation, to spherulin or mycelial extracts.

The primary goal of this investigation was to determine the role of DH in blastomycosis of mice, to determine if mice that were hypersensitive to *B. dermatitidis* would be protected from the lethal effects of intraperitoneal challenge, to investigate the induction of DH with killed yeast cells and with viable cells of *B. dermatitidis*, and to determine the concentration of killed cells necessary to elicit footpad sensitivity in sensitized mice.

MATERIALS AND METHODS

Animals. Two-month-old C57BL/6J mice of both sexes were used. All mice were maintained in air-conditioned quarters and given food and water ad libitum.

Organism used. *B. dermatitidis* originally isolated from a human case of blastomycosis was used. Yeast cultures were maintained on brain heart infusion (BHI) agar slants at 37 C.

Determination of LD_{50}. One hundred-twenty C57 mice were used to determine the 21-day intraperitoneal (ip) mean lethal dose (LD_{50}) for the culture of *B. dermatitidis* used. Inocula were prepared from organisms grown on BHI agar slants for 3 days at 37 C. The yeast cell growth

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was harvested, washed, and suspended in a sterile physiological saline solution (PSS). Organisms were counted directly in a hemocytometer (aggregates of 2 to 3 organisms were counted as one), and the inoculum was adjusted to contain the desired yeast cell numbers in 0.5 ml. In addition, serial 10-fold dilutions were plated on BHI agar for determination of the viable count. Cells prepared in this manner were 15 to 40% viable.

Each mouse was injected ip with 0.5 ml of the appropriate cell concentration, and the number of survival days was noted. Calculations were performed according to the method of Reed and Muench (9).

Footpad tests. To detect DH in mice, footpad sensitivity tests were done according to the procedure of Youmans and Youmans (16). The 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 PSS into the opposite footpad. The thickness of each footpad was measured with calipers at 0 (immediately before) and at 6, 24, and 48 h after injection. The difference in thickness between the antigen-injected and the saline-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 for recognition throughout the testing period.

Preparation of the killed-cell inoculum. *B. dermatitidis* yeast cells, prepared by the method of Restrepo-Moreno and Schneidau (10), were used for sensitization of mice with killed cells and in footpad sensitivity tests. Briefly, yeast cultures were prepared by inoculating 200 ml of a tryptic soy broth dialysate medium (10) with 5 ml of a stock suspension and incubating for 1 week at 35 C and 103 rpm on a gyratory shaker (New Brunswick Scientific Co., N.J.). The original stock suspension was prepared by transferring the growth from a 3-day-old yeast culture on BHI agar slants to 200 ml of the tryptic soy broth medium and was incubated in a similar manner. After incubation the cultures were checked for 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 1,500 rpm for 15 min, resuspended in 50 ml of sterile PSS with 1:10,000 Merthiolate, and stored at 5 C. In all experiments the cells were washed three times in PSS before use.

The concentration of yeast cells for induction of hypersensitivity and for footpad tests was standardized by determining the dry weight equivalent of packed wet cells.

Emulsion of yeast cell antigen with incomplete Freund adjuvant. A *B. dermatitidis* yeast cell-incomplete Freund adjuvant was prepared to determine if a DH response to Merthiolate-killed yeast cells could be induced in mice. The emulsion was prepared by continuous grinding while 1 volume of a suspension containing 35 or 140 mg of yeast cells was added a drop at a time into a mortar containing a mixture of five parts of marcole 52 (Humble Oil and Refining Co.) and one part arlacel A (Hill Top Research Inc., Miamiville, Ohio). After all the 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 with 0.1 ml of the emulsion: this amount contained the equivalent of 0.5 or 2 mg of dry yeast cells.

Inoculation of mice with killed B. dermatitidis yeast cells. To determine the time after inoculation with killed yeast cells that DH could be detected in C57 mice and to evaluate two concentrations of yeast cells for use in footpad testing, 60 mice were separated into two groups. The first group consisted of 30 mice: all mice in this group received two subcutaneous (sc) injections of Merthiolate-killed yeast cells emulsified in incomplete Freund adjuvant. The injections contained 2 mg of yeast cells/0.1 ml of emulsion and were given on days 0 and 7. The second group consisted of 30 uninoculated control mice. One-half of the 30 mice inoculated sc and one half of the 30 control mice were footpad tested with 50 μg of killed cells. The rest were footpad tested with 40 μg. Five mice from each group were tested for footpad sensitivity on days 5, 12, and 20. Footpad measurements were recorded, and the mean increase in footpad thickness was calculated.

Inoculation with viable B. dermatitidis yeast cells. To investigate the induction of DH in C57 mice inoculated sc with viable cells, 60 mice were divided into two groups of 30 each. The first group of 30 received a single sc injection of 3.9×10^4 yeast cells. Viable cells used in this study were prepared as described for the LD_{50} studies. The second group consisted of 30 uninoculated control mice. Five mice inoculated sc and five control mice were tested for footpad sensitivity on days 3, 6, 9, 12, 15, and 18.

Protection tests. To determine if DH induced by sc inoculation of mice with viable cells resulted in protection, 200 mice were placed in two groups of 100 each. Fifty in each group were injected sc with 3.9×10^4 yeast cells. The viable cells used in protection studies were prepared as previously described for LD_{50} studies. Fifty uninoculated mice were used as controls. At 3 and 15 days after the sc injection, 30 mice inoculated sc and 30 controls were challenged ip with a one-half LD_{50} of *B. dermatitidis* yeast cells. Ten mice inoculated sc and 10 control mice were tested for footpad sensitivity by footpad inoculation at each challenge period. In addition, 10 mice inoculated sc and 10 controls were inoculated ip with a concentration of killed yeast cells equal to one-half LD_{50}. The mortality of each group was recorded daily for 40 days.

RESULTS

Inoculation of mice with killed yeast cells of B. dermatitidis. C57 mice inoculated sc with a yeast cell-incomplete Freund adjuvant emulsion containing 2 mg of Merthiolate-killed yeast cells were tested by footpad inoculation with 40 or 50 μg of killed yeast cells after 5, 12, and 20 days. At the 5-day test period footpad injection with 40 or 50 μg of killed yeast cells did not elicit an increase at 48 h in the footpad thickness of mice inoculated sc or control mice. However when the footpads of sc inoculated mice were injected with 50 μg at both the 12- and 20-day test periods a considerable mean increase in footpad thickness was noted. The maximum mean increase in footpad thickness occurred at the 20-day test period. At this time the mean increase in footpad thickness of sc inoculated mice was 0.48 mm compared to 0.12 for control mice. The mean increase in footpad thickness of mice tested with 40 μg was not significantly larger than that of the control mice at either the 12- or 20-day test periods. It was also determined that the mice could be sensitized with 0.5 mg of the yeast-cell emulsion.

Mice inoculated with 2 mg of emulsified yeast cells were footpad tested at 12 days with 50 μg of killed cells. Following footpad injection, their footpads were measured at 0 (before injection), 6, 24, and 48 h, and the mean increase in footpad thickness was calculated for each period (Table 1). At 6 h following footpad injection the mean footpad thickness of both sc inoculated and control mice was increased. By 24 and 48 h, however, the mean increase in footpad thickness of mice inoculated sc had increased whereas that of the controls had decreased. For this reason the 48-h period was chosen as the best time to read the results of footpad sensitivity tests. In this paper future reference to the mean increase in footpad thickness will refer to the 48-h reading. Inoculation of 50 μg of killed cells into the footpads of control mice resulted in an inflammatory response which usually lasted 6 to 12 h, however, a few footpads of control mice were still swollen at 24 h. In a later experiment it was found that 45 μg of killed cells would detect DH in sensitized mice but produced a minimal inflammatory reaction in control mice. Therefore 45 μg of killed cells was used as the footpad antigen concentration in all subsequent footpad tests.

Inoculation of mice with viable cells of B. dermatitidis. C57 mice were inoculated sc with 3.9×10^4 viable yeast cells and tested for footpad sensitivity with 45 μg of killed yeast cells at 3, 6, 9, 12, 15, and 18 days. The data in Fig. 1 show that the mean footpad thickness of mice inoculated sc and control mice was essentially the same at 3 days. A moderate increase in the mean footpad thickness of mice inoculated sc was observed on days 6 and 9. At these test periods two mice inoculated sc 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 test periods. The mean increase in footpad thickness on these test days was 0.48, 0.58, and 0.40 mm, respectively.

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 adjuvant*

Material injected	Mean increase (mm) after injection			
	0 h	6 h	24 h	48 h
Killed cells[a]	0.06[b]	0.22	0.54	0.38
None	0.08	0.20	0.12	0.10

[a] A 2-mg amount (dry weight equivalent) in incomplete Freund adjuvant was injected sc on days 0 and 7.

[b] Average of five mice.

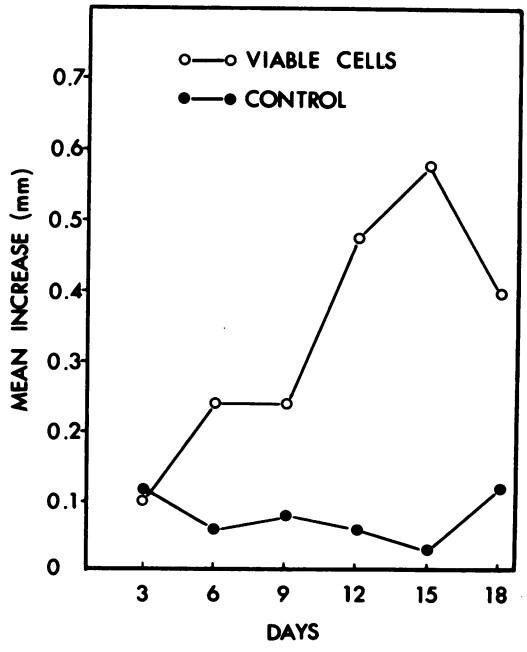

FIG. 1. *Results of footpad sensitivity tests for C57BL/6J mice that received viable B. dermatitidis yeast cells. The single-sensitizing dose was 3.9×10^4 yeast cells.*

Mice inoculated sc with viable cells and control mice were footpad-tested with 45 μg of killed cells on day 15 and measured at 0 (before injection) and 6 h and then daily for 7 days (Fig. 2). The increase in mean footpad thickness of the sc inoculated mice peaked at 48 h and then slowly decreased until it was almost that of the controls after 7 days.

Visible lesions developed at the site of inoculation in C57 mice approximately 9 to 12 days after sc inoculation of viable cells. The lesions were swollen and inflamed areas which occasionally ruptured through the skin surface. Mice were killed and autopsied 20 days after inoculation, and impression smears were made from the exudate in the lesions. These smears revealed the characteristic budding cells of *B. dermatitidis*. Its presence was confirmed by culture of this fungus from the exudate. Although a large amount of exudate material was observed just below the skin at the injection site, no lesions were seen in the internal organs, nor could the fungus be cultured from these organs, suggesting that dissemination had not occurred.

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 mean footpad thickness of mice inoculated sc with viable cells and tested by footpad inoculation 15 days later with 45 μg of killed cells occurred 48 h after footpad injection.

LD_{50} determination. Because of the difference in virulence among isolates of *B. dermatitidis* (G. A. Hill and S. Marcus, Bacteriol. Proc., p. 87, 1959), it was necessary to determine the virulence of the particular isolate used. Based on a 21-day period, the ip $LD_{50/21}$ in C57 mice for the isolate used in this study was 3.9×10^5 cells/ml. Future reference to LD_{50} in this paper will refer to a yeast cell concentration of 3.9×10^5 cells/ml.

Protection tests. Studies were done to determine if DH induced by viable *B. dermatitidis* yeast cells resulted in protection from the lethal effects of ip challenge. C57 mice inoculated sc with 3.9×10^4 viable yeast cells and control mice were challenged with 0.5 LD_{50} of *B. dermatitidis* at 3 days (before sensitization) and at 15 days (after sensitization) following the sc inoculation. Additional mice inoculated sc with viable cells and control mice were tested for their footpad sensitivity to 45 μg of killed yeast cells, and for their response after receiving an ip inoculation of killed cells equal to 0.5 LD_{50} on days 3 and 15 following the sc inoculation.

The first deaths of control mice challenged at 3 days occurred 6 days after ip challenge, and 90% of the mice were dead by day 40 (Fig. 3). The first deaths of mice inoculated sc and challenged

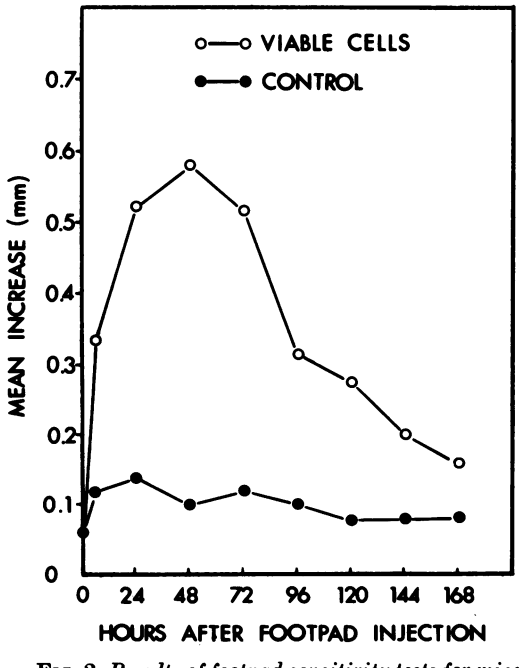

FIG. 2. *Results of footpad sensitivity tests for mice that received viable B. dermatitidis yeast cells. The footpads of C57BL/6J mice inoculated subcutaneously and control mice were injected on day 15 and measured at 0 and 6 h and then daily for 7 days.*

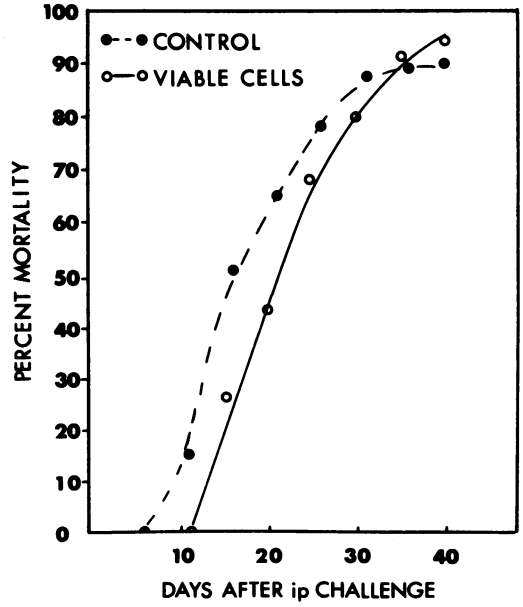

FIG. 3. *Percent mortality in C57BL/6J mice inoculated subcutaneously with 3.9×10^4 viable cells of B. dermatitidis and intraperitoneally challenged 3 days later with 0.5 LD_{50}.*

with the same inoculum occurred 11 days after the ip challenge, and 96% of these mice were dead at 40 days. Footpad sensitivity to 45 μg of killed cells was not observed in mice inoculated sc or in controls when tested at 3 days, nor did ip inoculation of killed cells result in death or any signs of distress in either group.

After the 15-day ip challenge (Fig. 4) the first control mice died on day 11 and 93% were dead at 40 days. Mice inoculated sc with viable cells 15 days earlier did not die until 20 days following the ip challenge, and only 36% were dead at 40 days. Footpad tests performed on mice inoculated sc with viable cells and controls at 15 days showed that mice inoculated sc had a mean increase in their footpad thickness of 0.88 mm, whereas that of the controls was 0.12 mm. Neither mice inoculated sc nor control mice showed any signs of distress when challenged ip at 15 days with a concentration of killed cells equal to 0.5 LD_{50}.

The results of this study showed that sc inoculation of viable *B. dermatitidis* cells stimulated footpad sensitivity to 45 μg of killed cells and also induced significant protection against the lethal effects of ip challenge with viable cells. Mice inoculated sc and challenged at 3 days (before sensitization) were not protected. However, those inoculated sc and challenged at 15 days (after sensitization) were protected.

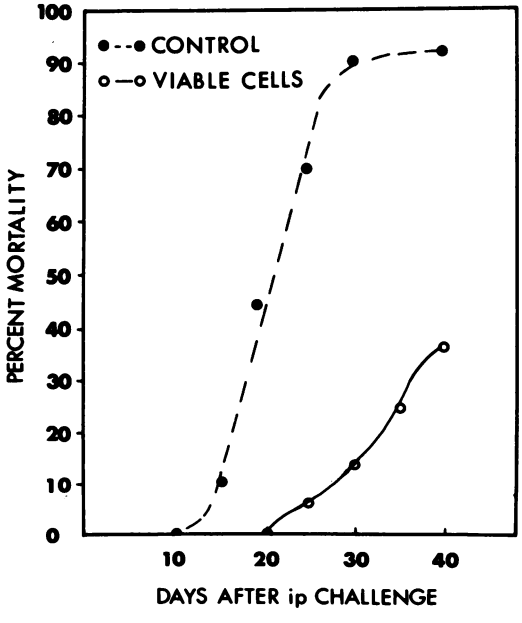

FIG. 4. *Percent mortality in C57BL/6J mice inoculated subcutaneously with 3.9 × 10⁴ viable cells of B. dermatitidis and intraperitoneally challenged 15 days later with 0.5 LD_{50}.*

DISCUSSION

Delayed hypersensitivity to *B. dermatitidis* was detected in C57BL/6J mice following sc injection with viable yeast cells or with killed cells in adjuvant. This hypersensitivity was detected by footpad inoculation with killed yeast cells. In the present study the earliest detection of DH occurred 12 days after the initial sc inoculation with either viable cells or killed cells in adjuvant. These results are similar to those reported for studies of DH with other systemic mycotic diseases. Investigators have reported that DH to *H. capsulatum* develops in mice 9 to 12 days after injection of viable cells (2, 12) and to *C. immitis* 15 days following injection of spherules in incomplete Freund adjuvant (6). Although individual mice varied in the magnitude of their footpad response, the mean increase in footpad thickness was similar in sensitized mice whether the sensitizing antigen was viable cells or killed cells in adjuvant.

Although the footpads of control mice responded initially with an inflammatory response when injected with 45 or 50 μg of killed cells, in most mice this reaction was minimal at 24 h and absent at 48 h. Footpad concentrations of yeast cells greater than 50 μg resulted in an inflammatory reaction for both inoculated and control mice. This reaction persisted for several days and appeared similar to that observed when yeast cell concentrations not toxic for control mice were injected into the footpads of sensitized mice. These results emphasize the importance of control mice for making accurate interpretations of footpad test results. It has been reported that *B. dermatitidis* yeast cells contain a substance which is toxic for mice. Salvin (11) found that a saline extract of acetone-dried yeast cells would cause death in mice within 48 h.

It is important to note that sc inoculation of either viable cells or killed cells in adjuvant produced a better state of DH than ip inoculation. Mice inoculated ip with viable cells did not develop DH as detected by footpad testing; however, mice inoculated sc with viable cells developed considerable footpad sensitivity.

The results of the protection studies suggest that mice with DH to *B. dermatitidis* are protected from a lethal ip challenge. When mice inoculated sc and control mice were challenged 3 days after sc inoculation, no protection was noted in the inoculated mice; however, by 15 days the mice inoculated sc had developed hypersensitivity to footpad inoculation with killed yeast cells and were protected. The results of this study suggest that DH or cell-mediated immunity may be important factors in host protection against blastomycosis in mice.

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