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Cell-Mediated Immunoprotection in Blastomycosis

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Delayed hypersensitivity can be induced in C57BL/6J mice by two subcutaneous injections of Merthiolate-killed *Blastomyces dermatitidis* yeast cells in Freund incomplete adjuvant. Development of delayed hypersensitivity peaked at the day 18 post-primary antigen-emulsion injection, as determined by footpad sensitivity tests. Mice rendered hypersensitive to *B. dermatitidis* were protected from the lethal effect of a blastomyces infection. The protection effects were shown both in mortality tests and in data from organ cultures as expressed by indices of resistance. Data from this study show that there is a close parallel relationship between host resistance and the prevailing level of delayed hypersensitivity.

It is generally accepted that delayed hypersensitivity (DH), or cell-mediated immunity, plays a part in the mechanism of resistance in the host. The relationship between antimicrobial resistance and DH relates exclusively to infections caused by facultative intracellular parasites (10).

Live cells of *Blastomyces dermatitidis*, *Coccidioides immitis*, *Cryptococcus neoformans*, and *Histoplasma capsulatum* can effectively induce immunity as shown by an increase in 50% lethal dose (LD_{50}) in experimental animals (3, 9, 11, 13, 15). Agreement on the efficacy of live vaccines by different workers does not always extend to killed vaccines. With killed *B. dermatitidis*, the intravenous (i.v.) route of vaccination in the mouse system has been used and has been reported to be ineffective (G. A. Hill and S. Marcus, Bacterial. Rev., p. 87, 1959). Landay, Hotchi, and Soores (4) reported that white ICI mice given an intraperitoneal (i.p.) injection of killed *B. dermatitidis* suspension were protected against lethal challenge with a spore-mycelium suspension. However, when immunized animals were challenged with yeast cells, protection was not observed.

Studies by Spencer and Cozad (15) showed that DH can be induced by a single injection of viable cells or by two subcutaneous injections of Merthiolate-killed yeast cells of *B. dermatitidis* incorporated in Freund incomplete adjuvant. Footpad testing was used to determine hypersensitivity. They also reported that C57BL/6J mice, rendered hypersensitive to *B. dermatitidis* by subcutaneous (s.c.) inoculation of viable yeast cells, were protected against a lethal challenge of *B. dermatitidis* yeast cells.

The present investigation constitutes a continuation of this study and gives further evidence of the importance of cell-mediated immunity in resistance to blastomyces infections.

In fungus infections, a sensitive measure of immunity is obtained by determining the extent of suppression of parasite multiplication in host tissue; the wane or enhancement of immunity may be demonstrated by determining relative changes in the numbers of viable cells. With this in mind, the "index of resistance," as described by Blanden et al. (2), was employed as a means of showing the relationship between host resistance and prevailing level of DH, as determined by footpad hypersensitivity.

MATERIALS AND METHODS

Culture. The yeast phase of *B. dermatitidis*, isolate 242, originally obtained from a human case of blastomycosis, was used in all studies. The organism was maintained on brain heart infusion (BHI) agar slants incubated at 37°C.

Test animals. Ten- to thirteen-week-old inbred C57BL/6J mice of both sexes were used. This strain was originally obtained from Jax Laboratories, Bar Harbor, Maine, and bred in the animal facilities of the Department of Botany and Microbiology, University of Oklahoma. Mice were separated by sex and given water and mouse chow (Ralston Purina) ad libitum.

Preparation of Merthiolate-killed *B. dermatitidis* yeast cells. For preparation of the Merthiolate-killed yeast cells, the methods described by Restrepo-Moreno and Schneidau (12), and by Spencer and Cozad (15), were followed.

The yeast cell inoculum for induction of hypersensitivity and for footpad testing was standardized by determining the dry-weight equivalent of packed wet cells.

Emulsion of yeast cell antigen with Freund incomplete adjuvant. The antigen-emulsion (Ag-emulsion) was prepared by continuous grinding, while 1 volume of a suspension containing 40 mg/ml (dry-weight equivalent) of Merthiolate-killed yeast cells was added dropwise into a mortar containing an equal volume of Freund incomplete adjuvant (Difco Laboratories, Detroit, Mich.).

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.

Physiological saline solution-Freund incomplete adjuvant emulsion (PSS-emulsion) was prepared by mixing 1 volume of 0.85% PSS to an equal volume of Freund incomplete adjuvant in the same fashion.

Determination of hypersensitivity time patterns. To determine the DH time patterns, 84 mice were divided into two groups. On days 0 and 7, in the first group of 42 mice, each animal was inoculated subcutaneously in the inguinal area with 0.1 ml of Ag-emulsion which contained 2 mg of Merthiolate-killed yeast cells. For the second group of 42 mice, each animal received two injections of 0.1 ml of PSS-emulsion on days 0 and 7. These served as controls.

Three mice from each group were footpad tested for DH at 3-day intervals during the first 30 days of the experiment, then on day 35, and thereafter at weekly intervals up to day 56.

Footpad hypersensitivity tests were done by the procedure described by Youmans and Youmans (16). The tests were carried out by injecting 45 μg of dry-weight equivalent of Merthiolate-killed whole yeast cells contained in 0.03 ml into the right hind footpads and a similar volume of PSS into the left hind footpads. Footpads were measured with dial gauge calipers at time 0 (immediately before) and 48 h after challenge. The mean differences in thicknesses between the right and left hind footpads served as the measure of DH. Before footpad testing, each mouse was marked individually for recognition throughout the testing period.

Preparation of inocula for mouse infection. Inocula were prepared from organisms grown on BHI agar slants for 3 days at 37°C. The yeast cells were harvested from the surface of the slants with cold 0.1% cysteine–PSS, and hemacytometer counts were made to determine the numbers of cells. It should be noted that the cells tended to aggregate; aggregates of two to four cells were counted as one.

For viability determinations, serial 10-fold dilutions were prepared from the suspensions used for inoculations. Cell suspensions of 0.1 ml containing 30, 300, and 3,000 cells per ml were plated in triplicate on BHI agar plates containing 20 U of penicillin and 40 μg of streptomycin per ml (BHI antibiotic plate). All suspensions were spread with sterile bent glass rods and incubated at room temperature for 21 days before the colonies were counted. The average viability for all inocula was 64%.

Effects of DH on resistance of mice to *B. dermatitidis* infection. A group of 168 mice was divided into two groups. In the first group of 84 mice, each animal was inoculated with Ag-emulsion, and in the second group of 84, each mouse was inoculated with PSS-emulsion on days 0 and 7.

Six mice from each group were selected at random at intervals after primary and secondary inoculation. Each mouse was intravenously inoculated with 0.5 ml of the cell suspension containing 1.5×10^5 cells through the lateral tail vein, using a 27-gauge needle. At 1 and at 48 h after inoculation, three mice from each group were killed by etherization, immersed in 95% alcohol, and placed in a bacteriological safety hood for autopsy. Approximately 0.1 ml of blood from the axillary fold of each mouse was removed with a sterile Pasteur pipette and spread on BHI antibiotic plates individually. The lungs, liver, and spleen from each mouse were removed aseptically and placed in vaccine bottles. To obviate the possible "contamination" of the yeast cells attached to the surface of the organs, each organ was washed two times with 0.1% cysteine–PSS (pH 6.5) before being ground. The lungs and livers were then placed into individual sterile grinder tubes containing 1 to 2 ml of cold sterile 0.1% cysteine–PSS (pH 6.5) and ground separately with a motor-driven Teflon glass homogenizer. The homologous organ homogenates from each group were pooled and placed into a sterile, calibrated centrifuge tube, and the total volume was brought up to 10 ml with 0.1% cysteine–PSS. The spleens from each group were ground in one sterile grinder tube containing 2 ml of 0.1% cysteine–PSS. Two dilutions were made on lung homogenates and one dilution on liver homogenates. The spleen homogenates remained undiluted. One-tenth milliliter of each dilution and the pooled homogenates, except those of the lungs, were plated onto BHI antibiotic plates. All platings were done in triplicate. Cultures were spread with sterile bent glass rods and incubated at room temperature for 21 days before being read. The index of resistance was calculated, using the method of Blanden, Lefford, and Mackaness (2).

Protection tests. To investigate whether DH, induced by s.c. inoculation of Merthiolate-killed yeast cells in Freund incomplete adjuvant, could protect mice from i.v. and i.p. challenge with viable *B. dermatitidis* yeast cells, 160 mice were placed in two groups of 80 each. The Ag-emulsion was injected into one group and the PSS-emulsion in the other by the same fashion as described in the section of determination of hypersensitivity time patterns. On days 3, 9, 18, and 35, after the primary inoculation, 20 mice from each group were selected at random. Half of each group was challenged with 1.95×10^5 yeast cells which is half of a 21-day LD_{50} dose given i.p. (15); the other half was given an i.v. challenge with 1.95×10^2 yeast cells which is half of a 21-day LD_{50} dose given i.v. Numbers of dead mice were recorded daily for 50 days after challenge.

RESULTS

Hypersensitivity time patterns. C57BL/6J mice inoculated with Ag-emulsion were footpad tested with 45 μg of killed whole yeast cells at 3-day intervals during the first 30 days and at 35, 42, 49, and 56 days of the experiment. The data in Fig. 1 show that the mean footpad thickness increase of the Ag-emulsion-inoculated and the control mice was essentially the same at the third day. A moderate increase in the mean footpad thickness of mice inoculated with Ag-emulsion was observed on days 6, 9, 12, and 15. The mean increase in footpad thickness peaked at the day 18 post-primary Ag-emulsion inoculation (0.48 mm) and then waned slightly. At day 56 of the experiment, the mean footpad thickness increase was 0.26 mm.

Recovery of *B. dermatitidis* from blood and organs. Only a small number of *B. dermatitidis* were occasionally recovered from mouse blood collected from the axillary fold 1 h after i.v. inoculation. Organisms could not be recovered 48 h after inoculation.

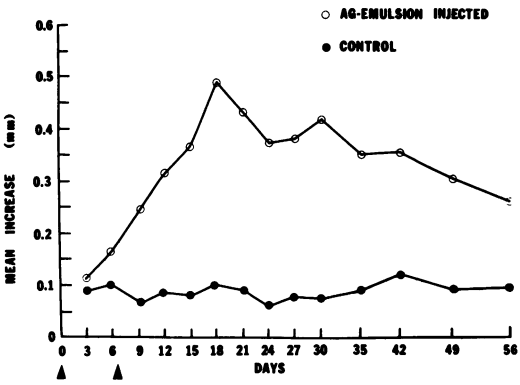

FIG. 1. *Hypersensitivity time patterns in C57BL/6J mice to killed B. dermatitidis yeast. Arrows indicate the days that the s.c. inoculations were made. Mice were inoculated with Ag-emulsion containing 2 mg of Merthiolate-killed B. dermatitidis yeast. Each point is an average of three mice. The 48-h mean increase after footpad testing with 45 μg of killed yeast cells was plotted.*

Table 1 shows the mean viable counts of *B. dermatitidis* recovered from lungs, livers, and spleens of mice 1 and 48 h after i.v. inoculation with 1.5×10^5 viable yeast cells. From this table, it is evident that most of the organisms inoculated were recovered from the lungs. Only a small number were recovered from the livers and spleens.

Effect of DH on resistance of mice to *B. dermatitidis* infections. The levels of host resistance to *B. dermatitidis* in the experimental groups were estimated by comparing the increase in the number of cells of *B. dermatitidis* during a 2-day period in the lungs, livers, and spleens of control mice with the corresponding increase found in Ag-emulsion-inoculated mice. For each group the log of mean *B. dermatitidis* counts obtained at 1 h (x_1 or y_1) was subtracted from the corresponding 48-h value (x_2 or y_2) to give the geometric increase, ($x_2 - x_1$) or ($y_2 - y_1$), during the 2-day period. The geometric increases calculated for mice of the Ag-emulsion-injected groups were then separately subtracted from the corresponding values to provide a measure of host resistance, $(x_2 - x_1) - (y_2 - y_1)$.

TABLE 1. *Mean viable B. dermatitidis counts recovered from organs of control mice and Ag-emulsion-injected mice 1 and 48 h after i.v. challenge with 1.5×10^5 viable yeast cells of B. dermatitidis*

Day[a]	Group[b]	Count after 1 h:			Count after 48 h:		
		Lungs[c]	Liver	Spleen	Lungs	Liver	Spleen
3	I	33,889	56	0	216,667	267	0
	II	33,000	33	0	243,334	144	0
6	I	41,111	33	0	307,778	156	4
	II	39,111	89	0	250,000	300	0
9	I	10,333	44	0	97,778	344	54
	II	8,111	67	0	45,556	378	14
12	I	50,111	11	0	308,889	300	18
	II	43,667	44	9	135,556	589	37
15	I	10,444	11	9	167,778	178	89
	II	9,000	11	0	52,667	33	0
18	I	12,222	78	0	334,444	456	45
	II	10,333	89	0	58,667	122	4
21	I	19,333	67	0	275,556	589	67
	II	15,778	156	4	74,444	344	18
27	I	35,889	11	0	387,778	300	45
	II	32,778	33	0	185,556	522	9
30	I	17,000	67	0	257,778	378	14
	II	15,667	117	0	92,222	322	4
35	I	12,778	78	0	135,556	267	4
	II	10,556	89	4	48,222	222	4
42	I	25,333	33	0	226,667	289	0
	II	23,667	22	0	135,556	89	0
49	I	12,778	56	0	67,444	122	18
	II	9,667	33	0	38,000	144	9
56	I	10,667	33	0	172,222	256	4
	II	17,556	11	0	168,889	78	4

[a] Days of challenge post-first Ag-emulsion injection.
[b] Group I, normal control; group II, Ag-emulsion injected.
[c] Average of three mice.

These values refer to the intercept ratio of each organ. The progressive changes of the index of resistance ot *B. dermatitidis* infection in lungs, livers, and spleens of Ag-emulsion-injected mice are shown in Fig. 2. From this figure, it is obvious that there was no protection in the Ag-emulsion-inoculated mice during the third day of the experiment. The intercept ratio for the lungs, livers, and spleens increased progressively thereafter and peaked on day 18 in the lungs and livers with log units of 0.67 and 0.83, respectively. Resistance to *B. dermatitidis* developed more slowly in the spleen; it peaked on day 21 with 1.17 log units.

The intercept ratio fell rapidly after reaching the maximum value; however, a low level of resistance persisted until day 49.

Protection tests. Mice, when challenged with i.v. and i.p. inoculation of sublethal doses of viable *B. dermatitidis* yeast cells 3 days post-primary Ag-emulsion inoculation, showed no protection against the challenge doses (Fig. 3). The protective effect was obvious when the challenge doses were given 9 days post-Ag-emulsion inoculation (Fig. 4) and reached maximum on day 18 (Fig. 5). A low level of protection persisted to day 35 post-primary Ag-emulsion inoculation (Fig. 6).

DISCUSSION

The induced immunity to fungi is influenced strongly by the morphological attributes of the vaccine preparation and the dose of vaccine, as well as the routes of vaccination and challenge (3). The extent of immunity induced by killed *H. capsulatum* (13, 14) and *C. immitis* (6, 8) was found to be dose dependent. The importance of dose of killed *C. neoformans* cells was emphasized by Abrahams and Gilleran (1) when they reported that doses either above or below a certain range were suboptimal for protection. Therefore, it is possible that the inefficacy of the yeast-phase vaccine of *B. dermatitidis* used by Hill and Marcus (Bacteriol. Rev., p. 87, 1959) was related, in part, to the dose used.

The vaccine route appeared to be of major importance in fungal immunity induced by non-

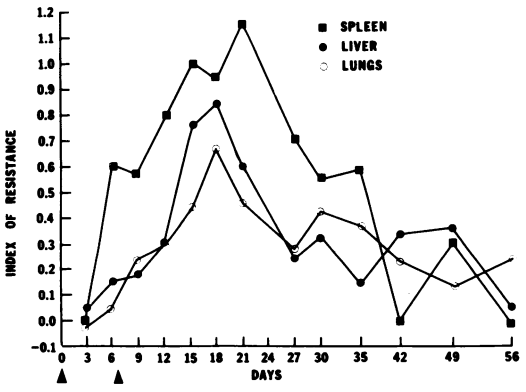

FIG. 2. *Progressive changes of the index of resistance to B. dermatitidis infection in lungs, liver, and spleen of Ag-emulsion-injected mice. Arrows indicate the days that the s.c. inoculations were made. Each point was obtained from data representing 12 mice.*

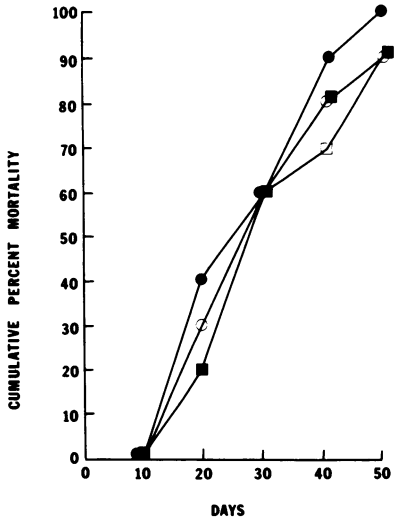

FIG. 3. *Cumulative percent mortality in C57BL/6J mice receiving half of a 21-day LD_{50} challenge 3 days post-first Ag-emulsion injection; 10 mice per group. (●), i.v. Ag-emulsion injected; (○), i.v. control; (■), i.p. Ag-emulsion injected; (□), i.p. control.*

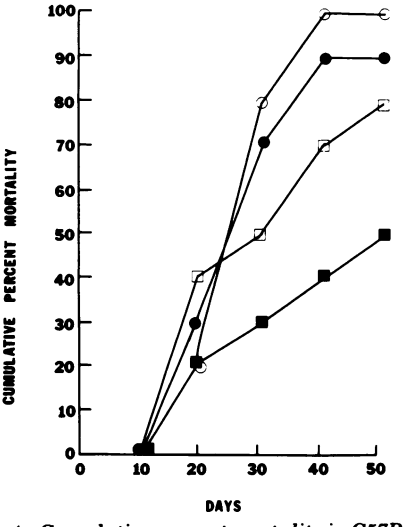

FIG. 4. *Cumulative percent mortality in C57BL/6J mice receiving half of a 21-day LD_{50} challenge 9 days post-first Ag-emulsion injection; 10 mice per group. (●), i.v. Ag-emulsion injected; (○), i.v. control; (■), i.p. Ag-emulsion injected; (□), i.p. control.*

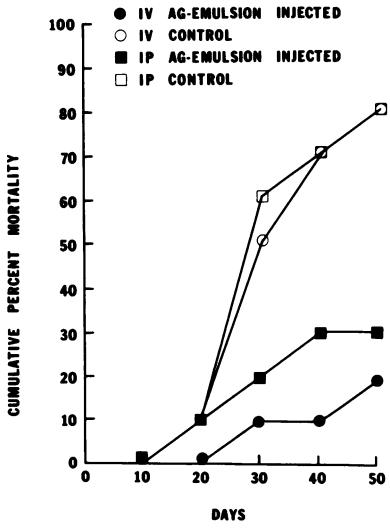

FIG. 5. *Cumulative percent mortality in C57BL/6J mice receiving half of a 21-day LD_{50} challenge 18 days post-first Ag-emulsion injection; 10 mice per group.*

living vaccines. The intranasal route was less effective than the s.c., and the weakest immune response followed i.v. vaccination (7). The ineffectiveness of vaccination with killed *B. dermatitidis*, as reported by Hill and Marcus (Bacteriol. Rev., p. 87, 1959), was also possibly due to the route of injection.

The ineffectiveness of protection against *B. dermatitidis* yeast cell challenge as reported by Landay et al. (4) was probably due to either too high a challenge dose or the low level of immunity induced in the mice.

B. dermatitidis was shown to elicit a minimal immunogenic response in experimental animals (3). The yeast-phase *B. dermatitidis* antigen, however, when incorporated into adjuvants, leads to the production of DH or to its increase. Our work provides further evidences to support the findings of Spencer and Cozad (15) that DH can be induced in mice when inoculated twice with Merthiolate-killed *B. dermatitidis* yeast cells incorporated into Freund incomplete adjuvant.

B. dermatitidis was recovered only in small numbers from mouse blood collected from the axillary fold 1 h, but not 48 h, after i.v. inoculation. This suggests that *B. dermatitidis* yeast cells inoculated i.v. in mice are rapidly filtered by organs and tissues.

The fact that the greatest number of *B. dermatitidis* cells was recovered from the lungs suggests that this organ is of extreme importance in resistance to blastomycosis. Larsh and Cozad (5) found the liver to be the organ of greatest predilection in histoplasmosis.

The small number of organisms recovered from livers and spleens suggests that either these organs can clear the organisms more effectively or only a small number of organisms can reach these organs by the time of experiment.

The intercept ratio in different organs correlates well with the footpad hypersensitivity time pattern. From the data of protection tests, it is clear that no protective effect can be detected in mice when challenged i.v. and i.p. 3 days post-primary Ag-emulsion inoculation. Mice when challenged 9 days post-primary inoculation, however, showed significant protection against the challenge doses. The protective effect peaked day 18 post-primary Ag-emulsion inoculation and then decreased but persisted until day 35 post-primary inoculation. These findings again provide evidences showing the close parallel relationship which exists between host resistance and the prevailing level of GH.

Spencer and Cozad (15) found that it takes 1.0×10^3 more organisms to kill an equal number of mice in the same length of time when the organisms are given i.p. than when the organisms are given i.v. The present data in protection tests also confirm those findings.

The defense mechanisms of mice in blastomycosis infection have previously been little understood. It is probable that the "angered" macrophages in the sensitized mice killed or sup-

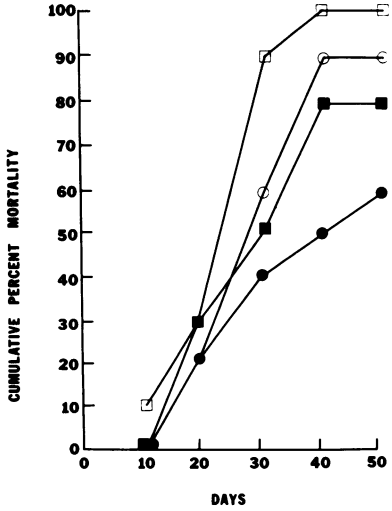

FIG. 6. *Cumulative percent mortality in C57BL/6J mice receiving half of a 21-day LD_{50} challenge 35 days post-first Ag-emulsion injection; 10 mice per group.* (●), *i.v. Ag-emulsion injected;* (○), *i.v. control;* (■), *i.p. Ag-emulsion injected;* (□), *i.p. control.*

pressed *B. dermatitidis* multiplication more effectively than those of the normal mice.

Although antibodies arising during the course of human mycoses have diagnostic and prognostic value, their protective effect, if any, has not been demonstrated (3). No attempts were made to determine the mouse sera antibody titers in the present study.

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