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Protection Against Pulmonary Blastomycosis: Correlation with Cellular and Humoral Immunity in Mice After Subcutaneous Nonlethal Infection

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A model of pulmonary blastomycosis in the mouse, in which the portal of entry is the same as natural human infection, was used to study resistance to challenge after subcutaneous infection. One week after subcutaneous infection, mice were partially resistant to pulmonary challenge, and mice challenged two weeks after infection were resistant. Measurement of cellular and humoral immune responses to *Blastomyces dermatitidis* antigens after subcutaneous infection showed the following. (i) Delayed-type hypersensitivity appeared 1 week after infection, and responses increased for 3 weeks thereafter. (ii) Proliferative responses in vitro appeared in spleen cells at 1 week and in contralateral lymph node cells at 3 weeks. (iii) Serum antibody, detected by an enzyme-linked immunosorbent assay, appeared 1 week after infection and then increased in titer. (iv) Peritoneal macrophages were activated to inhibit replication of *B. dermatitidis* in vitro by the first week after infection. Prior subcutaneous infection also resulted in rapid clearing of a second subcutaneous challenge, as well as resistance to a lethal intraperitoneal challenge. This resistance was associated with the development of cell-mediated and humoral immune responses. These data provide a chronological framework for selective transfer experiments.

The relative contributions of various elements of host defenses to resistance to fungal infections are currently an area of active investigation. The contribution may be different depending on whether the fungus is primarily intracellular or extracellular and depending on the route of infection. *Blastomyces dermatitidis*, a thermally dimorphic fungus, is a pulmonary pathogen in man, mice, and other mammals (15). The development of a model of pulmonary blastomycosis in the mouse, with desirable quantitative features (10) (and one in which the portal of entry is the same as natural human infection), makes the study of elements of resistance to this pulmonary pathogen possible.

In previous studies of blastomycosis, it was shown that mice infected subcutaneously (s.c.) developed delayed-type hypersensitivity (DTH) and resisted intraperitoneal (i.p.) challenge (16). Further studies indicated that mice injected s.c. with killed *B. dermatitidis* developed DTH and resisted i.p. and intravenous challenges (7). The correlation of the chronological appearance of DTH and resistance to infection was used in the latter study to argue for the primacy of cell-mediated immune reactions in resistance. However, transfer experiments or selective ablation experiments would provide more information on the relative roles of host elements in defense than would temporal correlations. Recently, we have presented evidence that activated macrophages may play a role in resistance to *B. dermatitidis* (3, 4).

In the present study, several elements of host immunity were studied chronologically after s.c. infection. Several antigen preparations were used to study cell-mediated immunity. The immune functions assayed were correlated with resistance to infection by the pulmonary route and other routes. Results from these experiments were used to plan additional studies involving passive or adoptive transfer of resistance to pulmonary challenge with *B. dermatitidis*.

MATERIALS AND METHODS

Mice. Healthy 8- to 12-week-old male BALB/cByJ mice were obtained from the Jackson Laboratory (Bar Harbor, Maine). Mice were fed mouse chow (Wayne Lab-Blox; Allied Mills, Chicago, Ill.) and acidified water ad libitum.

Culture and challenge procedures. *B. dermatitidis* strain ATCC 26199 was used in previous studies (9, 10). Replicate subcultures of yeast-phase organisms grown at 37°C on brain heart infusion slants were

stored at 4°C under sterile water until used. The organism was grown at 37°C in a chemically defined liquid medium and then transferred to blood agar plates before quantitation for in vivo challenge (10). Pulmonary challenge by the intranasal (i.n.) route was accomplished by techniques described previously (9, 10). We performed i.p. infection with 0.3-ml inocula. Mice were kept for 8 weeks after challenge.

The s.c. infection of mice which were to be subsequently studied in immunological assays or challenged was accomplished by injection of 20,000 colony-forming units (CFU) in 0.1 ml into each of two flank skin sites.

Antigens and mitogens. Four antigen preparations derived from *B. dermatitidis* (ATCC 26199) were used in these experiments. A killed whole-cell antigen (WKC) was produced by growing yeast cells in a chemically defined liquid medium at 37°C for 3 days on a gyratory shaker (10). The cells were pelleted by centrifugation, and the culture supernatant was retained for use as the antigen in the enzyme-linked immunosorbent assay for antibody. The cells were killed by the addition of Merthiolate (thimersol) (1:1000, vol/vol) for 4 days at 4°C, washed twice with sterile saline, and resuspended in phosphate-buffered saline (pH 7.2). The antigen was quantitated in terms of the equivalent dry weight of cells in suspension.

Two soluble antigens were used by us as previously described (3). The alkali-soluble, water-soluble blastomyces antigen (B-ASWS) (6), was kindly provided by Rebecca A. Cox (San Antonio State Chest Hospital, San Antonio, Tex.). A blastomyces urea lysate antigen (B-Ly) was kindly provided by John E. Bennett (National Institutes of Health, Bethesda, Md.). It was produced by extracting yeast cells with concentrated urea (11.7 M) in borate buffer (pH 9) for 72 h at 4°C. The supernatant was dialyzed against saline, filter sterilized, and adjusted to pH 8 with borate (J. E. Bennett, personal communication).

Candida albicans antigen was a preservative-free extract obtained from Hollister-Stier Laboratories, Berkeley, Calif. Concanavalin A, used as a mitogen in the lymphocyte proliferation assay, was obtained from Sigma Chemical Co., St. Louis, Mo.

Skin testing. Skin testing was performed as described previously (1, 2, 13). In brief, mice used for skin testing were prepared the day before the test by shaving the test area with electric animal clippers and applying a foam depilatory. A 0.01-ml amount of antigen was injected intradermally with a 26-gauge needle on a tuberculin syringe and a semiautomatic injector (Stepper; Indicon, Inc., Brookfield, Conn.). After 24 h, the diameter of induration was measured in two directions at 90° with a millimeter ruler, and the mean was recorded. Correlations of this reaction with DTH have been previously described (1, 2, 13).

Lymphocyte proliferation assay. Lymphocyte proliferative responses of spleen cells and lymph node cells to the fungal antigens and mitogens were assayed under optimal conditions previously determined (5). Briefly, 5×10^5 lymphocytes were cultured in 0.2 ml of medium (RPMI 1640, 5% horse serum, 5×10^{-5} M mercaptoethanol, 100 U of penicillin and 100 μg of streptomycin per ml) in round-bottom Microtest plate (Limbro; Flow Laboratories, Inc., Hamden, Conn.) wells at 37°C in 5% CO_2–95% air. At 24 h before harvest, cultures were pulsed with 1 μCi of [^{3}H]methylthymidine (specific activity, 6 Ci/mmol; Schwarz/Mann, Orangeburg, N.Y.). Mitogen-stimulated cultures were harvested on day 3, and antigen-stimulated cultures were harvested on day 6.

Optimal concentrations of each antigen were determined in preliminary experiments using cells from uninfected mice and mice recovered from s.c. infection, deriving a concentration which maximally stimulated cells from the latter without producing nonspecific stimulation. These concentrations were (final concentration in lymphocyte culture): B-ASWS, 100 μg/ml; B-Ly, 1:50; concanavalin A, 5 μg/ml; and candida antigen, 1:50.

The development of the lymphoproliferative response during the course of s.c. infection was followed by testing pooled lymphocytes from groups of three mice infected for varying periods of time, as described below.

Preliminary experiments showed that lymph node cells from nodes adjacent to sites of s.c. infection were unsuitable for lymphocyte proliferation assays because of high background counts. For this reason, contralateral nodes were used as sources of cells for these assays. The lymph nodes draining the sites of s.c. infection and spleens taken from mice after s.c. infection were cultured for fungi; these cultures were negative. At necropsy, there was no evidence of gross dissemination from the s.c. sites to other body sites.

Antibody to blastomyces. Antibody to blastomyces antigens in pooled sera was detected by an enzyme-linked immunosorbent assay procedure previously described (13), with slight modifications. In brief, a culture filtrate from *B. dermatitidis* ATCC 26199 grown in a chemically defined medium (SAAM,F) for 7 days (diluted 1:32) was used to sensitize polystyrene microtiter plates. Fetal calf serum (10%) was tested by us as a blocking agent and found to be more effective than 4% bovine serum albumin (18) in reducing nonspecific antibody reactions at low serum titers in this assay. Absorption of serum with a titer of 1:40 from mice previously infected s.c. with WKC reduced the titer to <1:10, equivalent to the titer of serum from uninfected mice, thus demonstrating specificity.

Assay of macrophage inhibition of replication of blastomyces. The ability of peritoneal macrophages to become activated and capable of inhibiting the replication of extracellular *B. dermatitidis* in vitro has been reported previously (4). This macrophage inhibition assay was used to follow the course of macrophage activation in s.c. infected mice. Briefly, resident peritoneal macrophages collected by lavage with RPMI 1640 were selected by adherence to flat-bottom Micro Test II plate (Falcon no. 3040; Falcon Plastics, Oxnard, Calif) wells (ca. 500,000 macrophages per well) for a 3-h incubation period. Adherent cells were challenged with 500 CFU of *B. dermatitidis* in 0.2 ml of medium (RPMI 1640, 10% fetal calf serum, 100 U of penicillin and 100 μg of streptomycin per ml). After 24 h at 37°C in 5% CO_2–95% air and then lysis of the macrophages in distilled water, the number of CFU per well was determined by plating, and the percent inhibition of replication was determined by the following formula: [1 − (CFU in cocultures/CFU in medium)] × 100. We have previously shown that changes in CFU in this assay correspond to changes in fungal cells per well, indicating effects on replication and not clumping (3, 4).

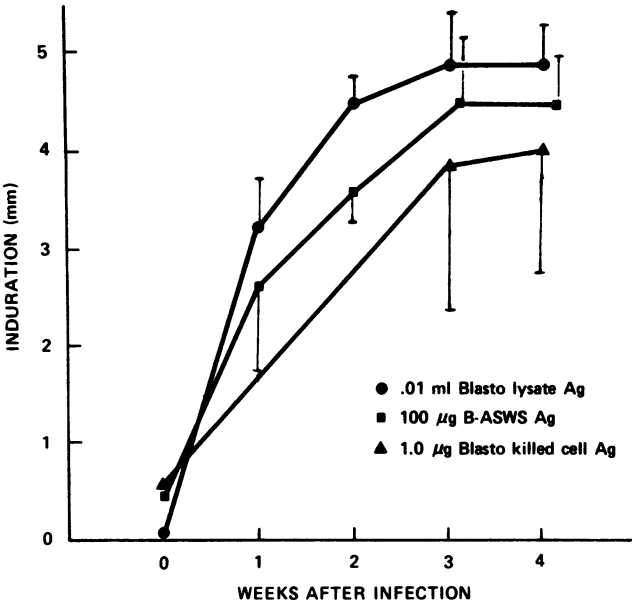

FIG. 1. Development of DTH after s.c. infection. Blasto, *Blastomyces*; Ag, antigen. The lysate antigen was used undiluted. The means ± the standard deviations are shown.

Testing of development of immune functions. The development and time course of the immune functions of DTH and lymphocyte proliferation, antibody production, and activation of macrophages to inhibit replication of blastomyces were delineated by sequentially infecting groups of mice s.c. at weekly intervals and testing all groups simultaneously. In other experiments, large groups of mice were infected s.c., and small subgroups were tested at intervals after infection, as noted. Skin testing was performed with three mice from a group, with blastomyces antigens and a control antigen (candida). Subgroups of three mice were bled to obtain serum for antibody measurement and were used to provide spleens and lymph nodes for the lymphoproliferation assay or to provide macrophages for the inhibition-of-replication assay or both. Serum or cells from normal (noninfected) mice of the same age were assayed as controls in each test.

Cultures of s.c. lesions. The lesions produced by s.c. inoculation of *B. dermatitidis* into normal mice and in mice previously infected s.c. were removed in toto and cultured quantitatively as previously described (3, 10). Two abscesses were produced on each mouse and were cultured separately. In brief, the tissue was minced, ground in saline in a glass tissue grinder, and agitated in a Vortex mixer to produce a fine suspension. Tenfold dilutions were cultured on blood agar plates in triplicate at 37°C, and CFU were enumerated.

The clearance of s.c. infection was compared by sacrificing three normal and three previously infected mice at weekly intervals and culturing the s.c. lesions.

Statistical analysis. Experiments were analyzed by the relevant statistical test (8), as indicated in the text.

RESULTS

Development of skin test responsiveness to *B. dermatitidis*. No early (4-h) reactions (Arthus type) were made by s.c. infected mice to either soluble or particulate *B. dermatitidis* antigens. The skin test reactions in infected mice peaked at 24 h, decreased slightly at 48 h, and subsided after 72 h. When mice infected s.c. 2 weeks previously were tested simultaneously in the flank skin and footpad (14) with 1.0 μg of WKC, significant reactions were made in the flank skin (4-mm induration) and the footpad (0.11 mm, increased swelling). Due to the ease of measuring indurated skin reactions compared with footpad swelling and due to the similarity of the former to indurated DTH reactions in guinea pigs and humans, the former was used in these studies.

Skin test reactivity to three antigens made from *B. dermatitidis* developed over the course of 4 weeks after s.c. infection (Fig. 1). The dose of each antigen that produced maximal induration in infected mice and minimal induration in normal mice was used. No nonspecific reactions occurred in noninfected mice with B-Ly antigen, as opposed to slight induration with 100 μg of B-ASWS antigen (mean, 0.4 mm) and 1.0 μg of the WKC antigen (mean, 0.5 mm). Higher concentrations of these latter two antigens produced increasingly larger nonspecific reactions in noninfected mice without significantly increasing reactions in infected mice above those obtained with the optimal concentrations (data not shown).

Significant responses to B-ASWS and B-Ly were observed 1 week after infection and rose progressively over the next 3 weeks (Fig. 1). In

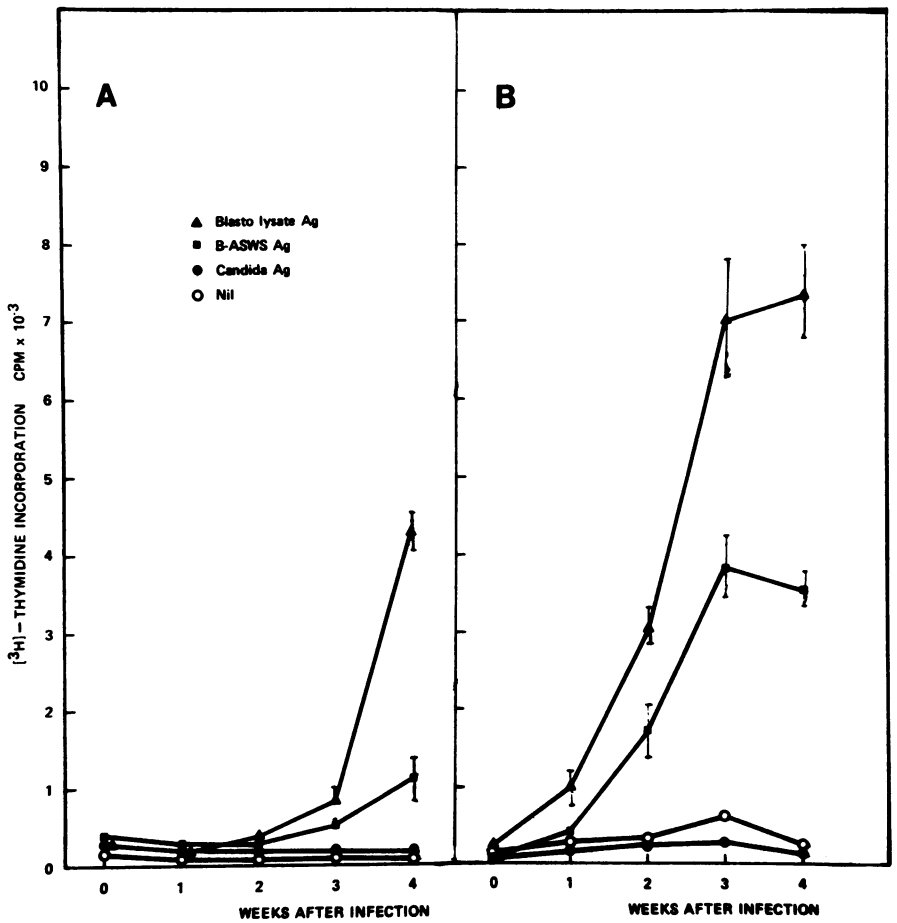

FIG. 2. Development of lymphoproliferative responses after s.c. infection. (A) Lymph node cells. (B) Spleen cells. Nil, Control (no antigen); other abbreviations are as in the legend to Fig. 1. The means ± standard deviations are shown.

other experiments, s.c. infected mice showed positive skin test reactions to WKC comparable to those elicited by the soluble antigens (Fig. 1) but did not show reactions to killed *C. albicans* (1.0 μg). The three antigens elicited comparable responses at 3 and 4 weeks postinfection. However, the B-Ly antigen gave a slightly higher mean induration than the B-ASWS antigen, which in turn was slightly more sensitive than WKC, but the differences did not appear significant.

Development of antigen-induced lymphoproliferative response of spleen cells and lymph node cells. Spleen cells and lymph node cells from s.c. infected mice developed antigen-induced lymphoproliferative responsiveness to two blastomyces antigens, the B-Ly antigen and the B-ASWS antigen (Fig. 2). Responsiveness in spleen cells to antigen began 1 week after infection and was highly significant ($P < 0.001$) 3 to 4 weeks postinfection (Fig. 2B). Lymph node cells from contralateral nodes, however, were found to be minimally responsive to blastomyces antigens at 2 weeks after infection (Fig. 2A). A significant ($P < 0.001$) increase in the responsiveness of these cells occurred 4 weeks after infection. The lysate antigen elicited a greater reaction than the B-ASWS antigen with both lymph node ($P < 0.01$) and spleen cells ($P < 0.01$), particularly at 4 weeks after s.c. infection. These responses were specific for blastomyces antigens in that no significant responses were made to an unrelated antigen (candida). The concentrations of candida antigen used here elicited proliferative responses in lymph node cells from mice immunized with candida (unpublished data).

Responsiveness of lymph node cells and spleen cells from s.c. infected mice to a nonspecific mitogen (concanavalin A) did not differ

TABLE 1. Mitogenic responses[a] of spleen cells and lymph node cells after s.c. infection

Source of cells[b]	Wk after infection	[^{3}H]thymidine incorporation[c]
Spleen	0	111,050 (7,700)
	1	104,890 (3,710)
	2	117,560 (1,480)
	3	117,170 (1,690)
	4	112,330 (2,860)
Lymph nodes	0	114,060 (7,820)
	1	128,210 (3,080)
	2	132,420 (11,800)
	3	152,200 (3,480)
	4	130,710 (2,880)

[a] Cells were stimulated with 5 μg of concanavalin A per ml for 72 h.

[b] Cells were pooled from three mice infected at 1, 2, 3, or 4 weeks before harvest.

[c] Results, expressed as counts per minute, are of triplicate cultures. Numbers in parentheses are standard deviations.

significantly from corresponding cells from untreated mice (Table 1). Thus, s.c. infection did not result in nonspecific suppression or enhancement of T-cell responsiveness.

In other experiments, both spleen and lymph node cells demonstrated significant antigen-specific responses and undiminished mitogen responsiveness 6 weeks after s.c. infection (2 weeks after resolution of s.c. infection) (data not shown).

Formation of antibody to *B. dermatitidis*. Antibody to *B. dermatitidis* after s.c. infection was initially detected in sera 1 week after infection, and the titer rose progressively until 4 weeks after infection (Fig. 3). The antibody titer appeared to plateau between 4 and 6 weeks after infection and to gradually decline after 8 to 12 weeks in other experiments (data not shown).

Development of macrophage inhibition of blastomyces replication. Peritoneal macrophages from mice infected s.c. 1, 2, 3, 4, or 6 weeks previously were tested simultaneously by a previously described assay to quantitate the inhibition of replication of blastomyces by murine macrophages (3, 4). Significant ($P < 0.001$) inhibition of blastomyces replication by peritoneal macrophages (CFU per well ± standard deviation, 4,505 ± 780 in medium versus 1,815 ± 200 in presence of macrophages) was observed as early as 1 week after infection (Fig. 4). Inhibition of replication reached a peak of 67%

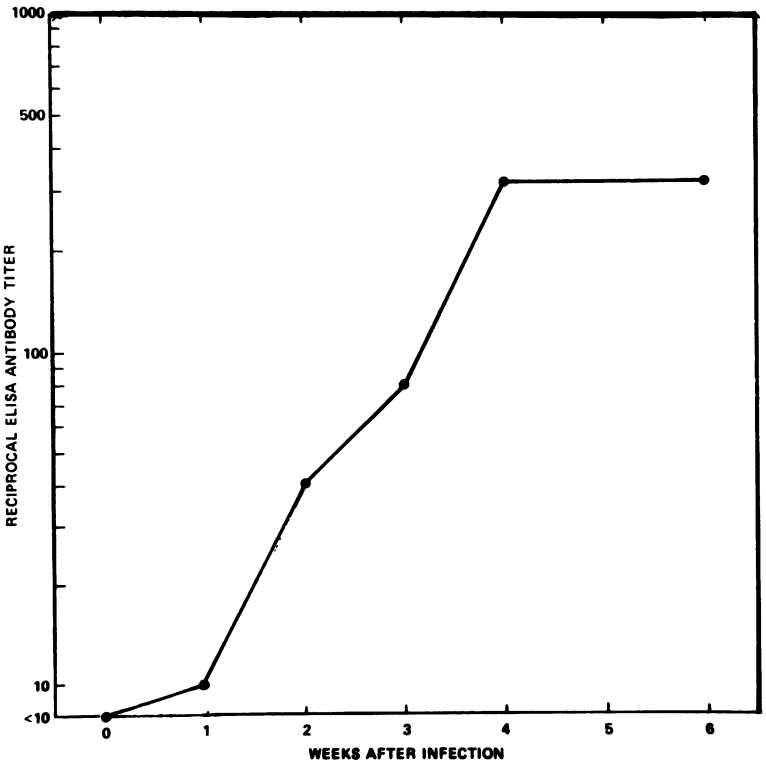

FIG. 3. Antibody response after s.c. infection, measured by the enzyme-linked immunosorbent assay (ELISA).

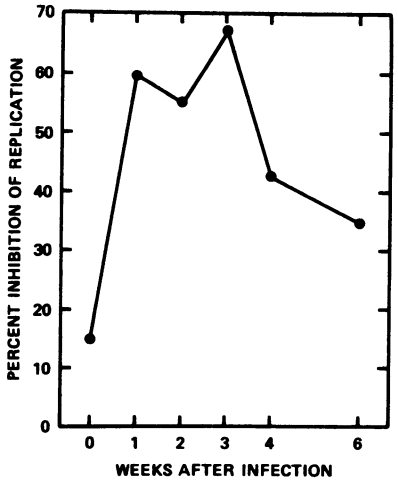

FIG. 4. Inhibition of blastomyces replication in vitro by peritoneal macrophages after s.c. infection.

at 3 weeks and fell progressively at 4 and 6 weeks after infection. However, even after 6 weeks of infection, the inhibition (35%) was still significantly greater than that produced by macrophages from uninfected (week 0) mice ($P < 0.01$ by the Student t test).

Effect of previous infection on recovery of blastomyces from s.c. lesions. To determine the effect of previous infection with blastomyces on the course of a subsequent s.c. infection, abscesses at a second, adjacent s.c. infection site were cultured quantitatively at various times after reinfection with 8,000 CFU. These results were compared with the recovery from normal mice cultured at the same time periods after an identical s.c. infection (Fig. 5). These results clearly indicated marked differences in the number of CFU recovered from the abscesses of mice previously infected 4 weeks before reinfection, as compared with those of normal mice. In normal mice, blastomyces replicated sevenfold during the first week of infection and thereafter declined until no viable yeasts were cultured (4 weeks). In contrast, blastomyces did not replicate in previously infected mice; CFU declined >10-fold during the first week of infection, and the infection was completely cleared by the second week (Fig. 5).

Development of protection against lethal i.n. challenge. Preliminary experiments testing the

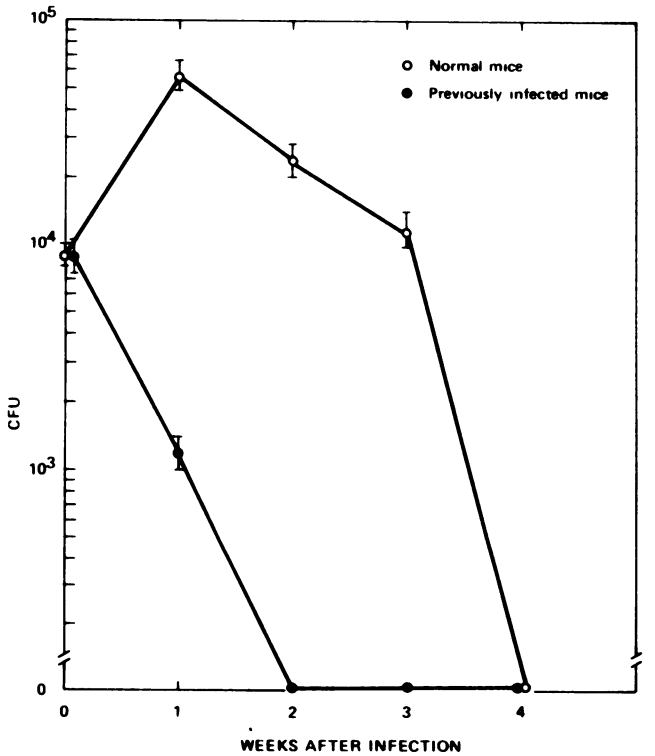

FIG. 5. Effect of prior s.c. infection (4 weeks earlier) on the recovery of blastomyces from abscesses resulting from a second adjacent s.c. challenge. CFU, CFU of blastomyces recovered per abscess. The mean ± the standard error of the mean for three absecesses is shown for each assay point.

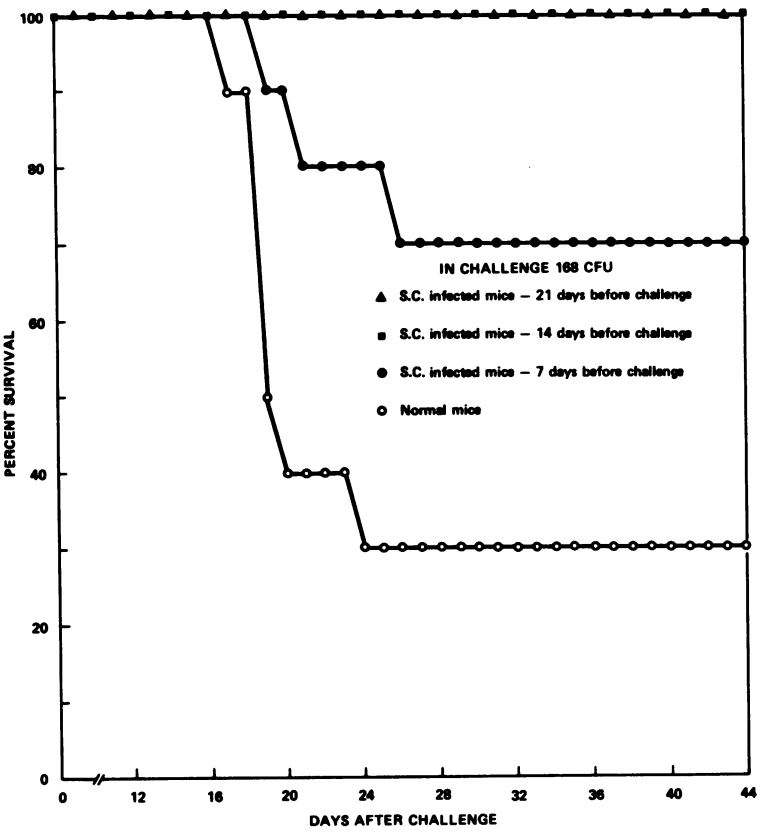

FIG. 6. Effect of prior s.c. infection on survival after i.n. challenge with blastomyces. Normal mice had no previous s.c. infection.

effect of s.c. infection against subsequent i.n. challenge with virulent blastomyces indicated that significant protection was present 4 weeks after the s.c. infection. None of 13 mice infected s.c. 4 weeks before i.n. challenge with 160 CFU died, compared with 8 of 14 normal mice (P = 0.0027 by the 2-tail Fisher exact test) (data not shown).

To demonstrate the development of the protective effect of s.c. infection to i.n. challenge, groups of 10 mice were infected s.c. with 40,000 CFU 7, 14, and 21 days before i.n. challenge with 168 CFU (Fig. 6). This i.n. challenge was lethal for 70% of the normal mice, compared with only 30% of the mice which had been s.c. infected 1 week before i.n. challenge. Even more striking was that none of the mice infected s.c. 2 or 3 weeks before i.n. challenge died ($P <$ 0.02, by chi-square analysis).

In this same experiment (data not shown), protection against an i.n. challenge of 1,008 CFU (100% lethal dose by day 35 after challenge) was also demonstrated. Of mice which received this challenge 2 weeks after s.c. infection, 70% survived, as did 80% of mice receiving this 100% lethal dose challenge 3 weeks after s.c. infection. When mice were challenged i.n. with this 100% lethal dose inoculum 4 weeks after s.c. infection, full protection was afforded (100% survival).

Protection of s.c. infection against i.p. challenge. To determine whether prior s.c. infection also afforded the same degree of protection to mice challenged i.p., as well as i.n., 12 mice infected s.c. 4 weeks previously were challenged i.p. with 421 CFU (Fig. 7). This challenge was lethal to 73% of the normal mice (equivalent to the lethality of 168 CFU used for i.n. challenge). Again, as was seen with the i.n. challenge, none of the mice previously infected s.c. died with this i.p. challenge (P = 0.0007 by 2-tail Fisher test).

DISCUSSION

Initial studies with *Blastomyces dermatitidis* indicated that the organism produced a minimal immunogenic response in experimental animals (11). Later studies, however, indicated that i.p. injection of killed organisms could protect

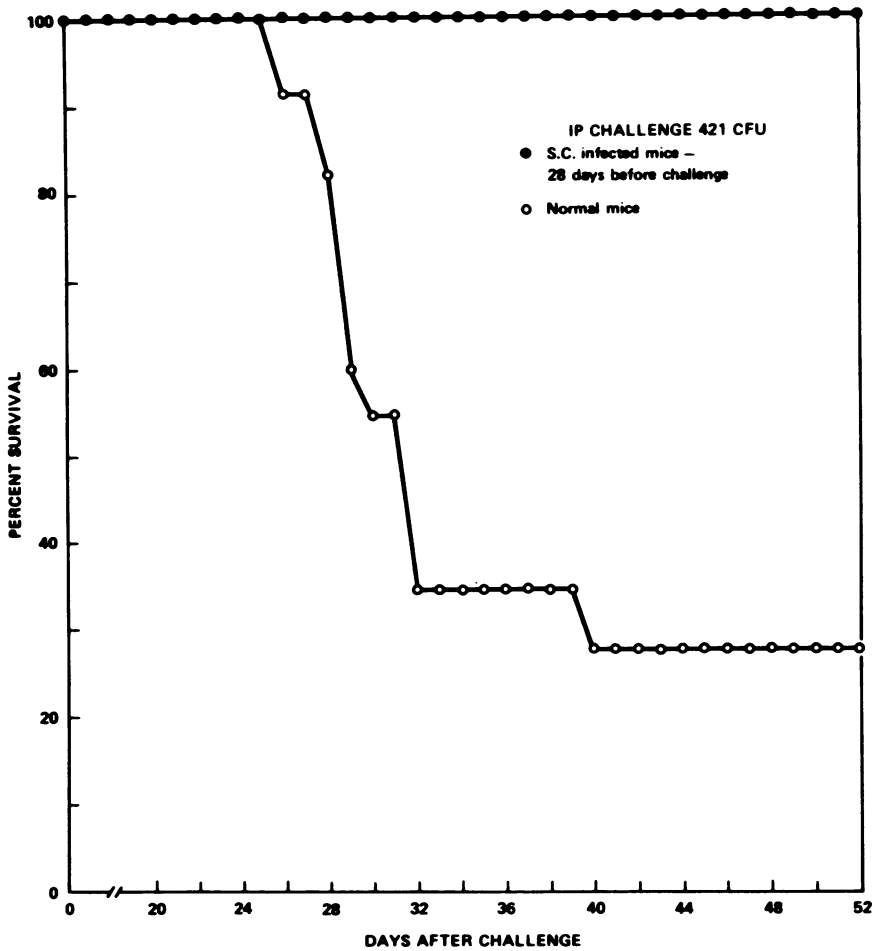

Fig. 7. Effect of prior s.c. infection on survival after i.p. challenge with blastomyces.

against intravenous challenge (12), s.c. infection could protect against subsequent i.p. challenge (16), and injection of killed organisms s.c. could protect against i.p. or intravenous challenge (7). Information on the protection against pulmonary challenge, the natural portal of entry and the most relevant route, by these procedures has not been published. We report here that after, and even during, the resolution of a s.c. infection, highly significant resistance to this pathogen was established in the pulmonary compartment. How the host deals with *B. dermatitidis* is of special interest because in vivo (10) and in vitro (4) studies have suggested that the parasitic-phase organism, unlike, for example, *Histoplasma capsulatum*, is largely extracellular. Of note, resistance to pulmonary coccidioidomycosis has been demonstrated after s.c. presentation of parasitic-phase coccidioidal antigen (17).

The development of resistance to a 70% lethal dose i.n. challenge beginning 1 week after s.c. infection and complete resistance after 2 weeks (Fig. 6) parallels the accelerated clearance data in s.c. abscesses (Fig. 5), as well as development of significant skin test reactivity to blastomyces antigen (Fig. 1), lymphoproliferative response of spleen cells (Fig. 2), antibody against blastomyces (Fig. 3), and activation of macrophages to inhibit the replication of blastomyces (Fig. 4). A similar correlation between development of antigen-specific DTH and lymphocyte reactivity after s.c. infection and protection against i.p. challenge has been reported (16) and was confirmed in the present study. The new immunological observations reported here raise the possibility that similar events take place in the lung after pulmonary challenge, and this is currently under investigation.

Our data expand the complexity of the association drawn previously between temporal correlations of DTH and resistance to reinfection (7). Several other potential components of host de-

fenses are shown to be stimulated at the time that resistance to reinfection occurs. This indicates that temporal correlations cannot be relied on to pinpoint a single defense mechanism as responsible for resistance. Our data provide a chronological framework for assessing the relative importance of the components of defenses against blastomycosis and for selecting appropriate elements for study in passive or adoptive transfer experiments. Results from such studies will be reported subsequently.

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