INFECTION AND IMMUNITY, Apr. 1991, p. 1423–1433
0019-9567/91/041423-11$02.00/0

Vol. 59, No. 4

T Cell-Mediated Immunity in the Lung: a *Cryptococcus neoformans* Pulmonary Infection Model Using SCID and Athymic Nude Mice

GARY B. HUFFNAGLE,[1,2] JUDY L. YATES,[2] AND MARY F. LIPSCOMB[1,2]*

Immunology Graduate Program[1] and Department of Pathology,[2] University of Texas Southwestern Medical Center at Dallas, 5323 Harry Hines Boulevard, Dallas, Texas 75235-9072

Received 21 August 1990/Accepted 18 January 1991

T cells are important in systemic anticryptococcal defenses, but a role in controlling an initial pulmonary infection has not been demonstrated. A murine model with intratracheal inoculation was developed to study the acquisition and expression of pulmonary T cell-mediated immunity against *Cryptococcus neoformans*. Infections with four strains of *C. neoformans* (305, 68A, 613D, and 52D) in two strains of mice (BALB/c and C57BL/6) were examined. Unencapsulated strain 305 and slowly growing strain 68A were readily controlled apparently by nonimmune pulmonary defenses, and no extrapulmonary dissemination was detected. Strain 613D grew progressively in the lungs and disseminated to the brain and spleen. Strain 52D initially grew rapidly in the lungs and disseminated to the spleen, but a clearance mechanism developed in the lungs after day 7 postinfection and in the spleen after day 28. SCID and athymic nude mice were unable to clear a strain 52D pulmonary infection, and a lethal disseminated infection occurred. Pulmonary clearance could be adoptively transferred into SCID mice infected with strain 52D by use of immune T cells from the spleen and lungs and hilar lymph nodes of infected immunocompetent donors. Furthermore, pulmonary clearance was almost 100-fold better in SCID mice that received immune T cells from the lungs and hilar lymph nodes than in those that received immune T cells from the spleen, even though equivalent levels of delayed-type hypersensitivity were transferred by both cell populations. These adoptive transfer studies suggested that the lung and hilar lymph node T cells from immune animals either are enriched in such a way as to mediate protective immunity or home to the lungs better than do splenic T cells.

Cryptococcosis is an infection which is usually manifested as meningitis, but the respiratory tract is the likely portal of entry for the etiologic agent, *Cryptococcus neoformans* (12, 37). Intact T cell-mediated immunity (CMI) is important for protection against this infection. Indeed, the majority of affected patients have defects in CMI, as exemplified by patients with AIDS, in whom cryptococcosis is one of the leading life-threatening secondary infections (12, 25, 36, 37). Primary lung infections without central nervous system (CNS) involvement are more likely to occur in immunocompetent individuals and usually resolve without therapy (3). Thus, it is likely that an important role for CMI is to control growth of the organism in the lungs and prevent dissemination to the CNS. However, it is uncertain how the pulmonary immune mechanism(s) operates to provide lung defense.

Mice provide an excellent model to study cryptococcal infections, but this model has not been used to assess the role of CMI in cryptococcal defenses in the lungs (8). Intranasal (1, 27, 40) and inhalation (23, 42) inoculations probably best mimic the natural acquisition of cryptococci and could potentially assess this role. However, a precise dose at a definable deposition time cannot be readily achieved with these routes. The objective of the current report was to develop a murine model of pulmonary cryptococcal pneumonia in which the development of CMI was required to resolve the infection. The model could then be used to define mechanisms by which CMI controls pulmonary cryptococcosis and subsequent dissemination. Results of these studies would likely be important in understanding mechanisms by which immunity contributes to the resolution of other pulmonary infections in which T cells are considered critical, e.g., pneumocystis and *Mycobacterium avium-M. intracellulare* infections (28, 43).

* Corresponding author.

MATERIALS AND METHODS

Mice. C57BL/6 mice were obtained from Jackson Laboratory (Bar Harbor, Maine). Normal BALB/c and athymic nude (BALB/c *nu/nu*) mice were obtained from Simonsen Laboratories, Inc. (Gilroy, Calif.). C.B-17 (BALB/c strain congenic for C57BL/6 immunoglobulin heavy chain) and SCID (C.B-17 *scid/scid*) mice were bred in specific-pathogen-free facilities in the animal resource center at the University of Texas Southwestern Medical Center at Dallas. SCID (severe combined immunodeficiency) mice have a defect in the recombinase system for the antigen receptor genes which results in a lack of mature B and T cells (6, 13, 41). Both male and female mice were used in the experiments, and the sex was recorded. There was no clear correlation between the sex of the mice and yeast virulence in these experiments. Mice were 8 to 12 weeks old at the time of infection and housed in cages with filter tops within a filtered-air-environment tent. Sterile food and water were given ad libitum. During the course of the experiments, mice did not show any overt signs of secondary infection, i.e., lethargy, ruffled or lost fur, or labored breathing. In addition, randomized mice were tested at the conclusion of the experiments and found to be negative for serum antibodies to mouse hepatitis virus, Sendai virus, and *Mycoplasma pulmonis*.

Organisms and culture conditions. All four yeast strains were obtained from the American Type Culture Collection (Rockville, Md.). In vitro generation times for the four strains were determined for growth in Sabouraud dextrose

broth (SDB; 1% neopeptone, 2% dextrose) at 23°C on a shaker (data not shown). Strain 305 (ATCC 52816) (21) is unencapsulated and has an in vitro generation time of 3.3 h. Strain 68A (ATCC 24064) (22) is encapsulated, is serotype A, and has an in vitro generation time of 6.0 h. Strains 613D (ATCC 36556) (26) and 52D (ATCC 24067) (22) are encapsulated, are serotype D, and have in vitro generation times of 3.1 and 2.6 h, respectively. Stock cultures of each strain were grown on Sabouraud dextrose agar (SDB with 1% agar) slants at room temperature and stored at −70°C. Working cultures were prepared monthly by thawing stock cultures and inoculating fresh slants. The working cultures were stored at 4°C, and unused cultures were discarded at the end of the month. For preparation of an infecting inoculum, organisms were inoculated from a working culture slant into SDB and grown on a shaker at room temperature until the culture was confluent (36 to 72 h). An aliquot of the culture was collected, washed three times in sterile nonpyrogenic saline (Travenol, Deerfield, Ill.), counted on a hemacytometer, and diluted to the appropriate concentration.

Route of infection. Mice were infected with $10^{3.5}$ CFU of *C. neoformans* in nonpyrogenic saline by surgical intratracheal (i.t.) inoculation. Mice were anesthetized with methoxyflurane (i.t.) vapor (Metofane; Pitman-Moore, Washington Crossing, N.J.) and restrained on a small board. A small incision was made through the skin over the trachea. The underlying tissue was separated, and a bent 30-gauge needle (Becton Dickinson, Rutherford, N.J.) attached by polyethylene tubing (Intramedic; Clay Adams, Parsippany, N.J.) to a tuberculin syringe (Monoject, St. Louis, Mo.) was inserted into and parallel with the trachea. A 50-μl inoculum was dispensed into the lungs. Following inoculation, the skin was sutured. Mice recovered with minimal visible trauma. At periodic intervals during the procedure, representative mice were euthanized and their lungs were removed to assay the number of CFU delivered i.t. Aliquots (50 μl) were collected at the beginning and at the end of the procedure to assay the total number of CFU contained in the inoculum to determine the percent deposition.

Assay for organ CFU. Mice were euthanized by an anesthetic overdose at various times after infection. Their lungs, brain, and spleen were removed and placed in plastic sample bags (Tekmar, Cincinnati, Ohio) each filled with 2 ml of sterile water. The samples were homogenized in the bags by crushing, and duplicates of 50 μl of the homogenates were plated on Sabouraud dextrose agar plates containing 50 μg of chloramphenicol (Remel, Lenexa, Kans.) per ml. Dilutions of the original homogenates were made in sterile water as necessary. Plates were incubated inverted at room temperature until colonies developed (6 days for 68A and 3 days for 305, 613D, and 52D). Colonies were counted, multiplied by the dilution factor, and expressed as $\log_{10}$ CFU ± standard deviation (SD) per organ. Organs without detectable CFU were included in the analysis as $\log_{10}$ CFU = 0. For comparing the results of infections with the different combinations of cryptococcal and mouse strains, the change in pulmonary CFU with time was termed "pulmonary clearance" whether clearance occurred for all combinations or not.

DTH assay. Preparation of cryptococcal filtrate antigen (CneF Ag) was adopted from a procedure described previously (39). In brief, 20 ml of formaldehyde (Sigma, St. Louis, Mo.) was added to a confluent 1,000-ml culture of strain 52D in SDB and the culture was placed on a shaker for 1 h. Previous studies have shown that CneF Ag cross-reacts with all strains of *C. neoformans* (38). The culture was harvested and centrifuged at 7,300 × *g*, and the supernatant was collected. The supernatant was filtered through a 0.45-μm-pore filter (Corning, Corning, N.Y.) and concentrated 50-fold in an Amicon (Danvers, Mass.) ultrafiltration unit with a PM-30 membrane (Amicon). The concentrated supernatant was dialyzed overnight at 4°C against phosphate-buffered saline (PBS). The sample was filter sterilized through a 0.22-μm-pore filter (Millipore, Bedford, Mass.), divided into aliquots, and stored at −20°C until used. The protein concentration, as determined by the Lowry assay (28a), was 8 mg/ml. A control sample was prepared from uninoculated SDB exactly as described above. Mice were challenged by footpad injections of 30 μl of CneF Ag and 30 μl of control in the right and left hind footpads, respectively, with a Tridak Stepper (Analytic Lab Accessories, Rockville Center, N.Y.) equipped with a 30-gauge needle and a tuberculin syringe. Footpad thickness was measured in millimeters 48 h later with a micrometer (Lux Scientific Instrument Corp., New York, N.Y.). The thickness of the right footpad minus that of the left footpad represented footpad swelling.

To measure delayed-type hypersensitivity (DTH) to sheep erythrocytes (sRBC; Colorado Serum Co., Denver, Colo.), we first sensitized mice by tail vein injection of 10^6 washed sRBC in 0.5 ml of PBS and then challenged them 5 days later with 6×10^7 washed sRBC in 20 μl of PBS in one footpad and 20 μl of PBS only in the other footpad. Footpad swelling was measured 48 h later as described above.

Preparation of MAb. Monoclonal antibodies (MAb) GK1.5 (anti-CD4 [11]) and YTS-169.4 (anti-CD8 [9]) were raised as ascites in SCID mice primed with 0.5 ml of pristane (2, 6, 10, 14-tetramethylpentadecane; Sigma) intraperitoneally (i.p.) 1 week before i.p. injection of approximately 10^7 hybridoma cells. The hybridoma cells were initially grown at 37°C in 7% CO_2 in RPMI 1640 medium (Inland Labs, Fort Worth, Tex.) supplemented with 10% fetal calf serum (GIBCO, Grand Island, N.Y.), 0.2 mM L-glutamine (GIBCO), 0.1 mM nonessential amino acids (Whitaker MA Bioproducts, Walkersville, Md.), 1 mM sodium pyruvate (Whitaker), 100 U of penicillin (GIBCO) per ml, and 100 U of streptomycin (GIBCO) per ml (RPMI complete medium). The ascites was collected, centrifuged, and stored at −20°C. MAb and normal rat serum were purified on a protein A-Sepharose affinity chromatography column (Bio-Rad, Rockville Center, N.Y.) and dialyzed against PBS, and the protein concentration was determined by measuring the A_{280} (an A_{280} of 1.45 = 1 mg of protein per ml). The MAb were diluted to 0.5 mg/ml in nonpyrogenic saline, filter sterilized, divided into aliquots, and stored at −20°C.

In vivo T cell depletion. Mice were given two i.p. injections of 150 μg of MAb, 150 μg of rat immunoglobulin G, or an equal volume of sterile PBS dialysate (to control for lipopolysaccharide contamination) 2 days apart. Depletion was maintained by repeated i.p. injections of 100 μg of MAb or control every 7 to 10 days following the initial injections. Depletion was monitored by staining of unfractionated spleen and/or unfractionated lung leukocytes with GK1.5, YTS-169.4, isotype-matched rat immunoglobulin, 30H12 (anti-Thy-1.2), and rat antimouse immunoglobulin (Jackson Immunoresearch, Avondale, Pa.).

Reconstitution of SCID mice. Spleen and/or lung lymphocyte cell suspensions from C.B-17 mice were prepared 21 days after i.t. infection with strain 52D as follows. Spleens were removed and placed in sterile nylon mesh tubing (Bally Ribbon Mills, Bally, Pa.) which was sealed at one end. The tubing was crushed with a rubber stopper into RPMI complete medium, creating a single-cell suspension. The cells

were pelleted, and the erythrocytes were lysed by resuspension in NH_4Cl buffer (0.829% NH_4Cl, 0.1% $KHCO_3$, 0.0372% Na_2EDTA [pH 7.4]). The cells were washed twice and plated overnight at 37°C in 7% CO_2 in RPMI complete medium to remove adherent cells, and the medium additionally contained 2 μg of amphotericin B (Sigma) per ml to reduce the number of yeast cells in the sample. Nonadherent cells were collected, pelleted, resuspended in warm RPMI 1640 plus 10% fetal calf serum and antibiotics, loaded onto a prewarmed nylon wool column (Robbins Scientific, Mountainview, Calif.), and incubated at 37°C for 1 h. The nylon wool-nonadherent cells were slowly eluted with warm medium, pelleted, counted, and resuspended in sterile PBS at an appropriate concentration. For lung lymphocytes, the lungs and hilar lymph nodes were removed, minced, and digested for 60 to 90 min in digestion buffer (RPMI 1640 plus 10% fetal calf serum, antibiotics, 0.7 mg of collagenase [Boehringer Mannheim Biochemicals, Chicago, Ill.] per ml, and 30 μg of DNase [Sigma] per ml). The mixture of cells and undigested fragments was passed through nylon mesh tubing, the fragments were further dissociated by crushing with a rubber stopper, and the resulting cell suspension was washed and pelleted. The sample was treated with NH_4Cl buffer and fractionated as described for the spleen cells.

For removal of T cells from the samples, the cells were resuspended at 2×10^7 cells per ml in RPMI complete medium, incubated at 4°C for 30 min with 25 μl of affinity-purified stock GK1.5, YTS-169.4, or rat immunoglobulin per 2×10^7 cells, washed of excess MAb, resuspended at 2×10^7 cells per ml in a 1:8 dilution of baby rabbit complement (Pel Freeze, Brown Deer, Wis.) in RPMI complete medium, and incubated at 37°C for 45 min. The treated cells were pelleted, washed, and resuspended to one mouse equivalent (the number of cells equivalent to that recovered from one donor mouse) per 0.5 ml. Cells were adoptively transferred into SCID mice by tail vein injection of a 0.5-ml inoculum.

Statistics. Statistical significance was analyzed with the *t* test, the Bonferroni method for multivariance (14), and the UTSTAT statistical package on the VAX 8800 in the computer resource center at the University of Texas Southwestern Medical Center at Dallas.

RESULTS

Development of a murine model of acquired pulmonary resistance. Three parameters for the cryptococcal i.t. infection model were considered: strain of *C. neoformans*, strain of mice, and inoculum size. With the i.t. inoculation procedure, greater than 90% of the infecting inoculum was recovered from the lungs 10 min following deposition in the majority of experiments (data not shown). Four strains of *C. neoformans* were compared to include strains of both serotypes A and D as well as a strain without a capsule (305) and a strain that grew more slowly than the others (68A). The cryptococcal strains and their properties are described in Materials and Methods. Two strains of mice were also compared: BALB/c mice ($H\text{-}2^d$), because inbred athymic nude and SCID mice have a BALB/c genetic background, and C57BL/6 mice, representing a different *H-2* haplotype ($H\text{-}2^b$) and a non-major histocompatibility complex-linked genetic background.

The inoculum size was selected from preliminary studies with strain 613D, the most virulent of the four strains studied, at i.t. inocula of 10^2, 10^3, 10^4, and 10^5 organisms (data not shown). At doses of 10^2 and 10^3 organisms, dissemination to the brain occurred in only 3 of 10 animals. At doses of 10^4 and 10^5 organisms, dissemination to the brain occurred in 10 of 10 animals. Since the objective was not to overwhelm the developing immune response but, at the same time, to choose an inoculum large enough to facilitate detecting the yeast cells in tissue, an intermediate dose of $10^{3.5}$ organisms was selected.

Two sets of time points were initially selected: 7 to 9 days (before immunity was likely to be expressed, based on previous studies with an intranasal model [27]) and 35 to 49 days (after the development of immunity [27]). Because preliminary studies indicated that the spleen is an early site for extrapulmonary infection and that brain infection is a frequent cause of death in cryptococcus-infected mice, spleen and brain CFU were evaluated in addition to lung CFU.

In C57BL/6 mice, each cryptococcal strain exhibited a characteristic pattern of infection following i.t. inoculation (Fig. 1A-C). Unencapsulated strain 305 was nearly cleared from the lungs by day 7 but remained at low levels through day 35. Measurable extrapulmonary dissemination did not occur. Slowly growing strain 68A remained essentially static in the lungs, and there was no detectable dissemination to the brain or spleen. Strain 613D continued to replicate in the lungs through day 35, and CFU were detected in both the brain and the spleen in all animals studied at day 35. Strain 52D initially grew rapidly in the lungs and was already present, although in low numbers, in the spleen by day 7. By day 35, however, pulmonary clearance was occurring, although organisms were still detected in the spleen and in low numbers in the brain in most animals.

In general, the growth and dissemination patterns of the four strains of *C. neoformans* in BALB/c mice were similar to those in C57BL/6 mice (Fig. 2A-C). There was a trend toward more lung CFU and dissemination in C57BL/6 mice than in BALB/c mice, but more detailed studies will be required to determine whether there are murine strain differences in resistance. Both strains 305 and 68A were successfully cleared from the lungs, and no dissemination was detected. Strain 613D grew progressively and disseminated to the brain and spleen. By day 49, all animals had large numbers of CFU in the brain and one had died. Previous studies demonstrated that all mice receiving 10^3 CFU of this strain i.t. survived less than 60 days (data not shown). Strain 52D grew rapidly in the lungs from days 0 to 9 but was progressively cleared through days 35 and 49. Small numbers of CFU were detectable in the spleen in nearly half of the mice at 9 and 35 days but in none of the mice by day 49. Additional kinetic analyses indicated that the numbers of strain 52D organisms in the spleen peaked 21 to 28 days after i.t. inoculation and then decreased (data not shown; see also Fig. 4C). Brain CFU were detected at low levels in a small number of mice throughout the study, but no animals died of infection with this strain.

Development of DTH during infection. Mice were tested for a response to CneF Ag as a measure of systemic CMI and to determine whether the kinetics of a DTH response would correlate with the development of pulmonary resistance. In BALB/c mice, DTH was detected on day 7 and persisted after infection with strain 52D but was not detected at any time after infection with the other three strains (Fig. 3A). C57BL/6 mice failed to develop DTH following infection, even when infected with strain 52D (Fig. 3B), despite their capacity to develop pulmonary resistance to this strain. Strain 52D-infected C57BL/6 mice were able to generate DTH to sRBC which was abrogated when $CD4^+$ cells were depleted with injections of anti-CD4 MAb (Fig. 3C). This

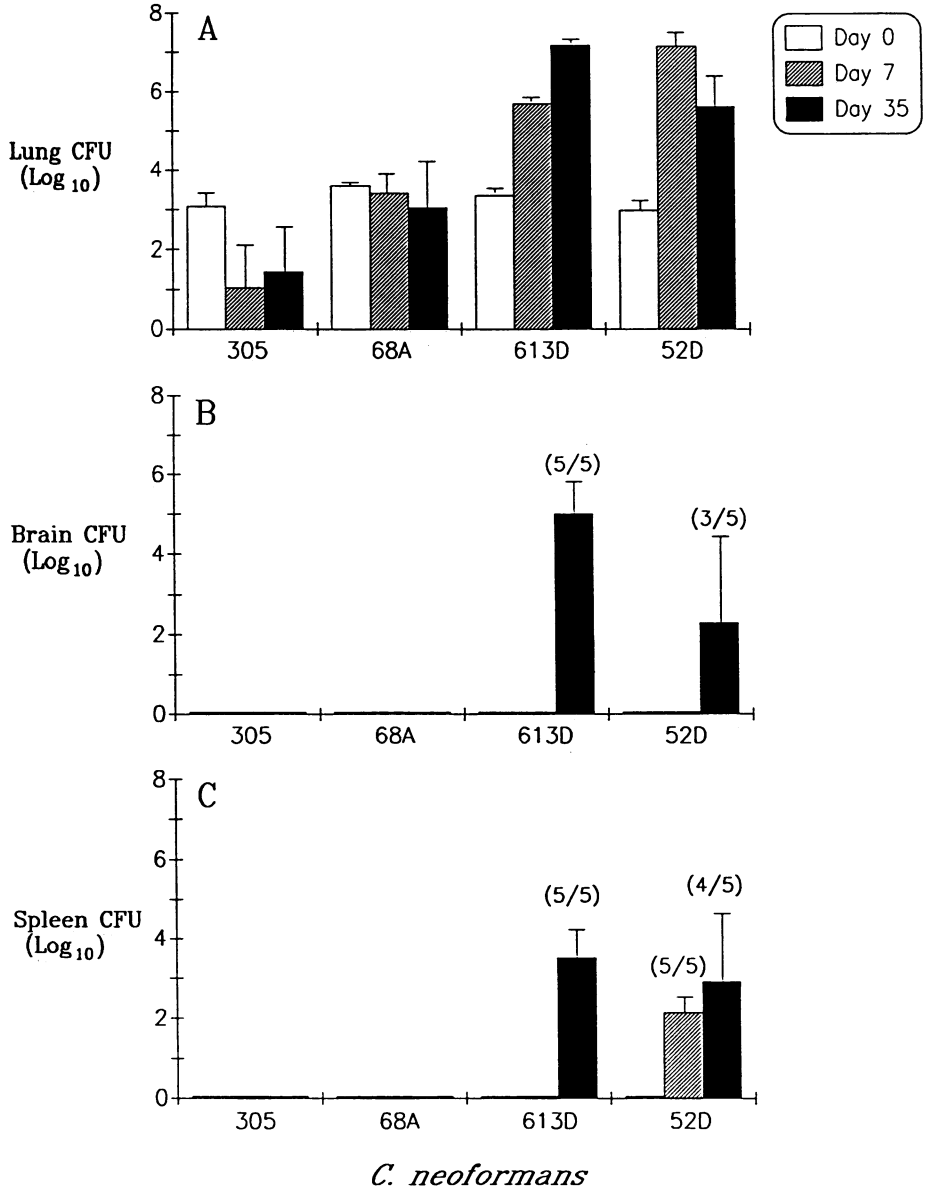


FIG. 1. Clearance and dissemination of the four strains of *C. neoformans* in C57BL/6 mice. Bars indicate the SD. (A) Pulmonary clearance. (B) Brain dissemination. (C) Splenic dissemination. There were five mice per time point. In panels B and C, the numbers in parentheses indicate the number of mice with dissemination to that organ/the total number of mice examined at that time point. Organs without detectable CFU were included in the analysis as $\log_{10}$ CFU = 0.

result suggested that the lack of DTH to CneF Ag in C57BL/6 mice was not due to a generalized anergy but, rather, was likely an antigen-specific defect developing in the context of an intrapulmonary infection.

Role of T cells in acquired pulmonary resistance. The kinetics of the resolution of *C. neoformans* 52D infection in BALB/c mice indicated that pulmonary resistance was acquired, i.e., an immune response had developed. The concomitant appearance of DTH in BALB/c mice was further evidence for the development of CMI and suggested that T cells could be the mediators of pulmonary resistance. To more directly demonstrate that pulmonary resistance to strain 52D was mediated by T cells, we infected T cell-deficient nude, T and B cell-deficient SCID, and control C.B-17 mice via i.t. inoculation with strain 52D. As previously reported (16, 33, 39), nude mice were markedly susceptible to cryptococcal infection. So, too, were SCID mice, and both strains began to die by day 35. Thus, CFU were not analyzed after this time in these mice. The clearance and dissemination kinetics of strain 52D were nearly identical after i.t. inoculation of either the BALB/c mouse strain or the congenic mouse strain, C.B-17 (see Fig. 2 and 4; data not shown).

There were no differences in the numbers of lung CFU in C.B-17, nude, or SCID mice at day 7 (Fig. 4A). However, between days 8 and 20 pulmonary clearance occurred in immunocompetent C.B-17 mice, and clearance continued through the end of the experiment. In both nude and SCID

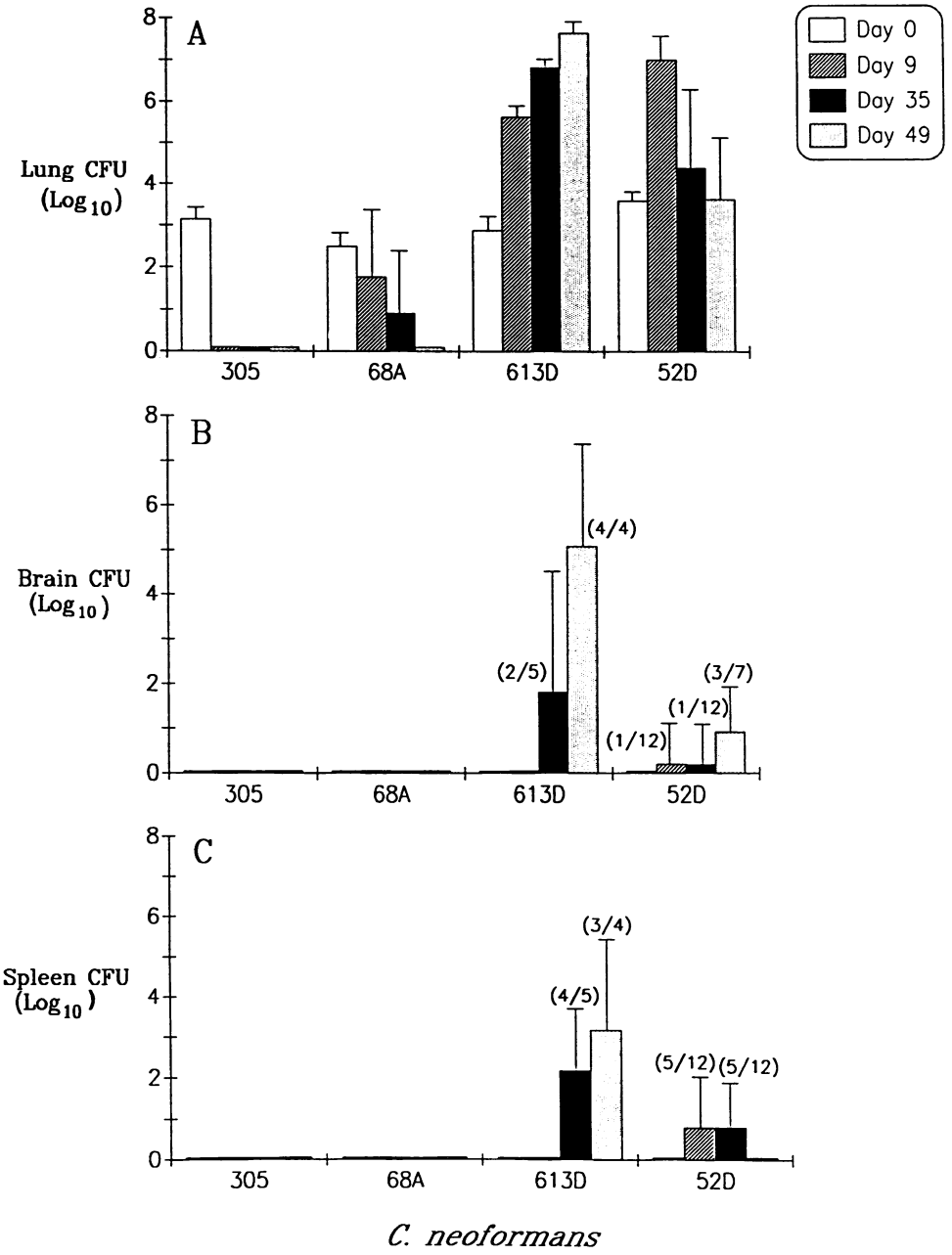


FIG. 2. Clearance and dissemination of the four strains of *C. neoformans* in BALB/c mice. Bars indicate the SD. (A) Pulmonary clearance. (B) Brain dissemination. (C) Splenic dissemination. There were five mice per time point for strains 305, 68A, and 613D (unless otherwise noted) and 7 or 12 mice per time point for strain 52D. In panels B and C, the numbers in parentheses indicate the number of mice with dissemination to that organ/the total number of mice examined at that time point. Organs without detectable CFU were included in the analysis as $\log_{10}$ CFU = 0.

mice, pulmonary resistance failed to develop. There were also differences in the recovery of organisms from extrapulmonary organs. Limited dissemination to the brain occurred by day 7 in all three mouse strains, but C.B-17 mice had no further increase in brain CFU (Fig. 4B). In contrast, nude mice had large numbers of yeast cells in the brain at days 21 and 35. SCID mice had only a moderate amount of dissemination to the brain, suggesting that some resistance mechanism(s) was present. Dissemination to the spleen occurred between days 7 and 21 in all three strains of mice. The splenic infection was cleared by day 35 in immunocompetent C.B-17 mice (Fig. 4C). In nude mice, the numbers of splenic CFU continued to increase. Surprisingly, in SCID mice, the numbers of splenic CFU were relatively static after day 21, again suggesting that some resistance mechanism(s) was present.

In these and previous experiments, it was noted that mice dying of pulmonary disease (no CNS symptoms) had 10^7 to 10^8 CFU in the lungs and that animals dying of meningitis (lethargy and a staggering gait) had 10^6 to 10^7 CFU in the

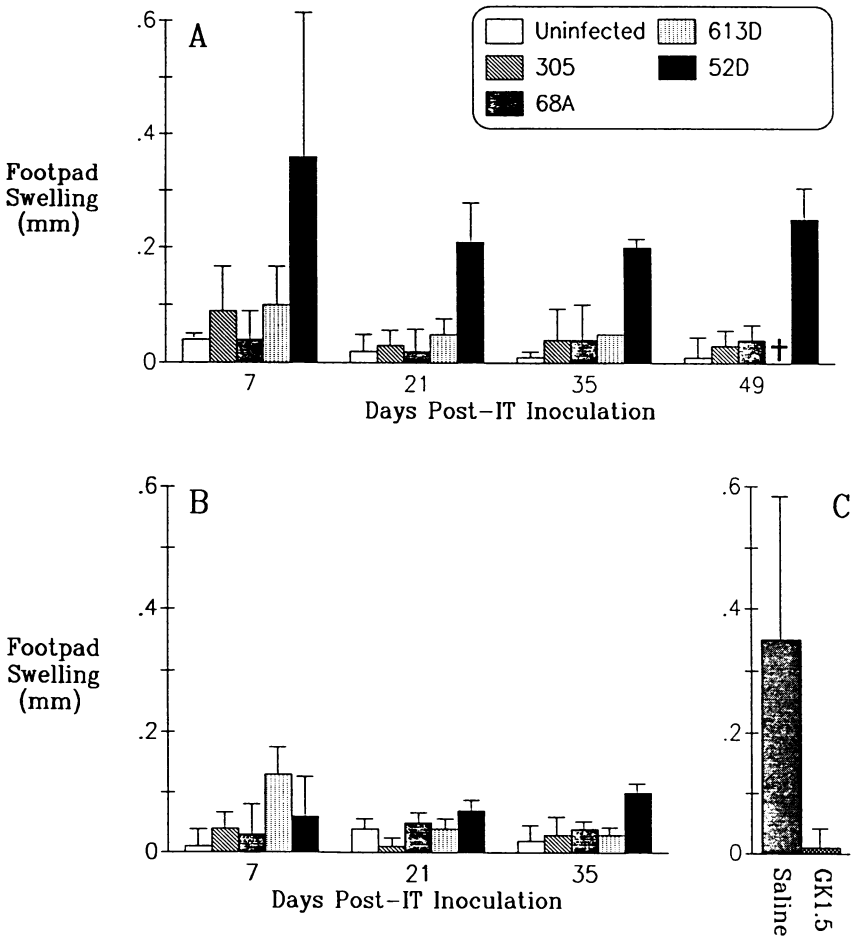


FIG. 3. DTH response to CneF Ag after i.t. inoculation of one of four strains of *C. neoformans* in BALB/c (A) or C57BL/6 (B) mice. Bars indicate the SD. There were five mice per time point, except for strain 613D-infected mice on day 35, when there was only one surviving mouse, and day 49, when there were no surviving mice (†). (C) DTH response to sRBC of strain 52D-infected, sRBC-sensitized C57BL/6 mice. Mice were treated with either saline or MAb GK1.5 (anti-CD4) before sRBC sensitization.

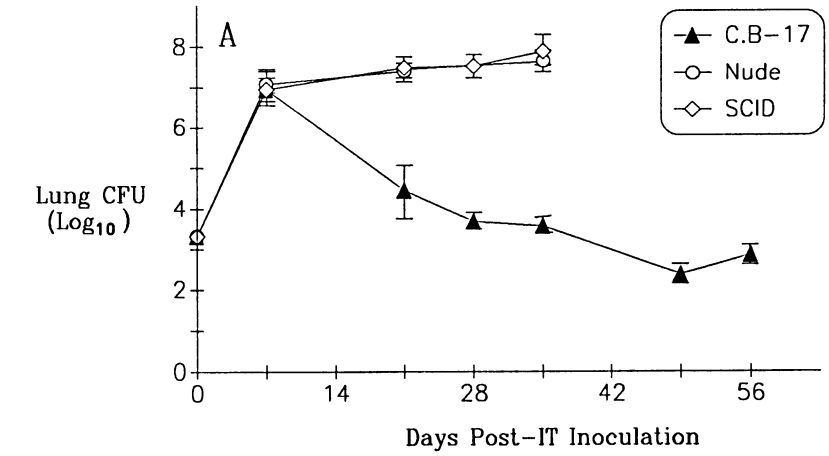
brain. Nude mice died with large numbers of yeast cells in both the lungs and CNS, while SCID mice died with large numbers of yeast cells in the lungs but relatively few in the CNS.

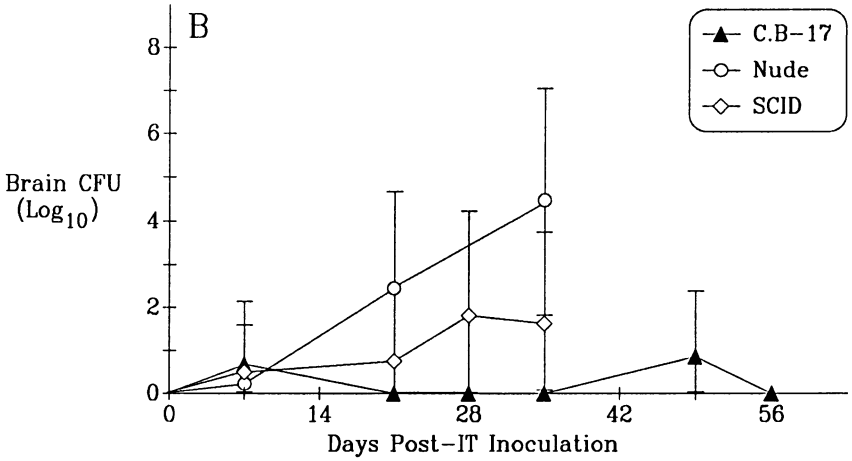
T cells isolated from infected, but recovering, C.B-17 donors were inoculated into SCID recipients infected 2 days previously to determine whether pulmonary protection could be adoptively transferred by immune T cells (Fig. 5). Additionally, the relative efficacies of splenic versus lung and hilar lymph node T cells for adoptive transfer were studied.

Mice receiving either immune spleen T cells or immune lung and hilar lymph node T cells displayed good DTH 14 days following infection, and this was abolished by depletion of the $CD4^+$ and $CD8^+$ cells from the cell populations with MAb and complement before transfer (Fig. 5A). Transfer of nonimmune T cells failed to induce significant DTH in most animals at the time studied, demonstrating that the ability to mount a DTH response was not primarily the result of T cell reconstitution but rather depended on the adoptive transfer of immune cells.

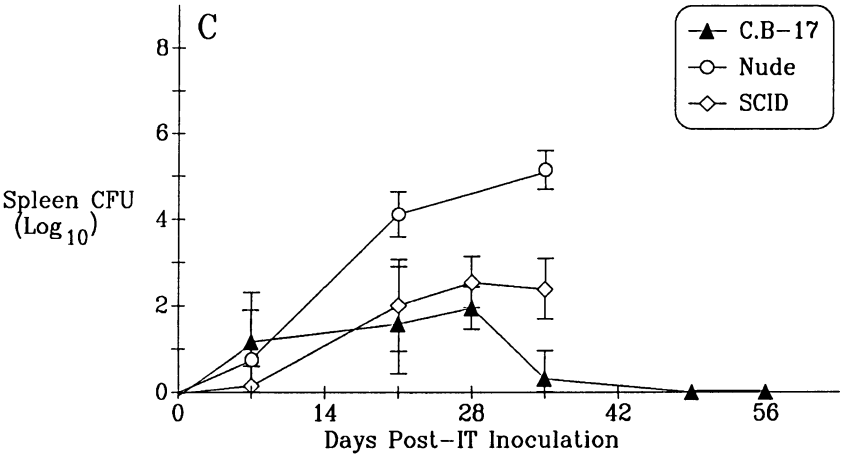
Although infected SCID mice receiving either the immune splenic or the immune lung and hilar lymph node T cells displayed similar levels of DTH, those receiving lung and hilar lymph node T cells had a greater ability to control the pulmonary cryptococcal infection (Fig. 5B). Immune splenic T cells conferred some pulmonary protection to infected mice ($P < 0.005$), but immune lung and hilar lymph node T cells were nearly 100-fold more effective than were immune splenic T cells ($P < 0.005$). Groups of mice receiving either immune spleen or immune lung cells that were depleted of $CD4^+$ and $CD8^+$ cells failed to display pulmonary resistance. A small degree of protection may have developed after adoptive transfer of nonimmune splenic T cells, since

FIG. 4. Clearance and dissemination of *C. neoformans* 52D in T cell-deficient nude mice and T and B cell-deficient SCID mice as compared with control C.B-17 mice. Bars indicate the SD. (A) Pulmonary clearance. (B) Brain dissemination. (C) Splenic dissemination. In panels B and C, the numbers in the box below the graph represent the number of mice with dissemination to that organ/the total number of mice examined at that time point in the experiments shown in panels A to C. Organs without detectable CFU were included in the analysis as $\log_{10}$ CFU = 0. Survival of nude and SCID mice beyond day 35 was limited, so the experiments with these mice were terminated at day 35. d, Day.





	d7	d21	d28	d35	d49	d56
SCID	2/10	2/11	3/6	2/5		
Nude	1/9	7/10		4/5		
C.B-17	2/9	0/9	0/5	0/5	1/3	0/4



	d7	d21	d28	d35	d49	d56
SCID	1/10	9/11	6/6	5/5		
Nude	3/9	10/10		5/5		
C.B-17	5/9	6/9	5/5	1/5	0/3	0/4

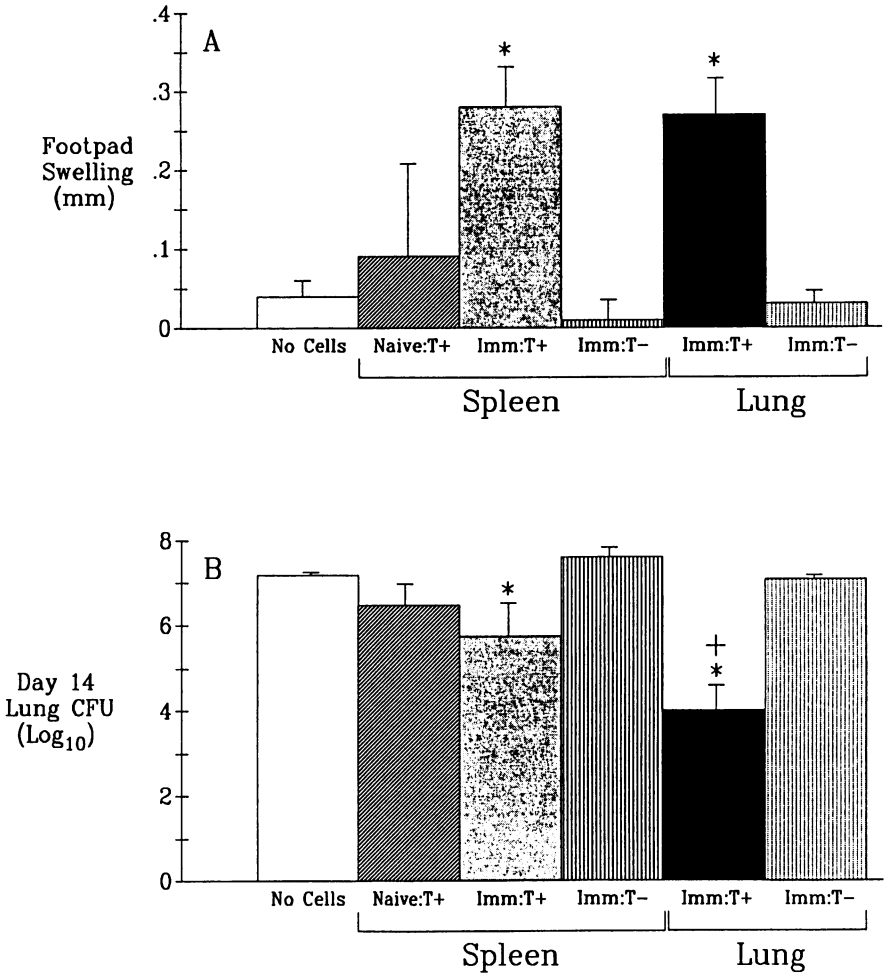


FIG. 5. DTH response (A) and pulmonary CFU (B) of *C. neoformans* 52D-infected SCID mice 14 days after i.t. inoculation (12 days after adoptive transfer). Bars indicate the SD. Infected SCID mice received one mouse equivalent (as described in the text) of T cells at day 2 postinfection. At day 12 postinfection, they were challenged for DTH to CneF Ag; 48 h later, footpad swelling was assessed and lung CFU were assayed. Abbreviations for donor cells: Spleen, splenic T cells; Lung, T cells from the lungs and hilar lymph nodes; Naive:T+, T cells from uninfected BALB/c mice, Imm:T+, T cells from day-21 52D-infected BALB/c mice; Imm:T−, cells from day-21 52D-infected BALB/c mice depleted of CD4 and CD8 cells by antibody and complement treatment before transfer. (A) *, $P < 0.01$ as compared with "No Cells" control. (B) *, $P < 0.005$ as compared with No Cells control; +, $P < 0.005$ as compared with "Spleen Imm:T+." In panel A, for the "Spleen Naive:T+" group, footpad swelling was >0.20 nm for two of five mice and <0.05 mm for three of five mice.

mice receiving these cells demonstrated a level of pulmonary protection (a one-half log decrease in CFU in the lung as compared with controls) intermediate between those of control and immune splenic T cell-reconstituted SCID mice. This difference was not statistically significant but, as a trend, was not unexpected, since naive BALB/c and C.B-17 mice develop DTH by day 7 after infection.

Possible role of $CD8^+$ cells in suppressing innate cryptococcal resistance in nude mice. It has been reported that $CD8^+$ T cells can develop from an extrathymic site in nude mice (20, 29). It was possible that the increased dissemination of strain 52D of *C. neoformans* seen in nude mice as compared with SCID mice was the result of the systemic development of $CD8^+$ suppressor T cells during the infection (36, 37). Therefore, nude mice were injected i.p. with anti-CD8 MAb to deplete $CD8^+$ T cells prior to the initiation of infection (Fig. 6). Twenty-eight days after infection, there were no significant differences in lung, spleen, or brain CFU between the control and antibody-treated nude mice.

DISCUSSION

In the current experiments, a murine model was established to pursue studies of the mechanisms of the acquisition and expression of pulmonary CMI against *C. neoformans*. A cryptococcal strain (52D), inoculating dose, and mouse strain (BALB/c) were identified such that after i.t. inoculation, progressive growth of the organism occurred until local resistance developed. A pivotal role for T cells was demonstrated in the acquisition of pulmonary immunity, because (i) T cell-deficient athymic (nude) and SCID mice were unable to clear the infection from their lungs and (ii) pulmonary resistance could be reconstituted in infected SCID mice by the adoptive transfer of anticryptococcal immune T cells. Furthermore, there was a dichotomy between the development of lung resistance and DTH, demonstrated by comparing these parameters in infected BALB/c and C57BL/6 mice and in SCID mice receiving immune spleen versus lung and hilar lymph node T cells. Lastly, an innate extrapulmonary

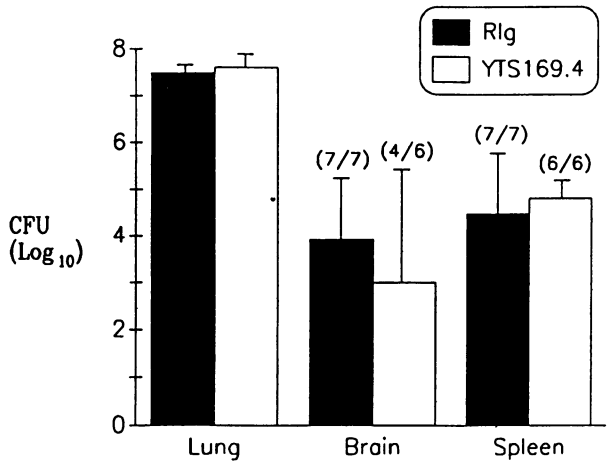


FIG. 6. Growth and dissemination of strain 52D at day 28 in nude mice treated with MAb YTS-169.4 (anti-CD8) or an irrelevant isotype-matched rat immunoglobulin (RIg). The numbers in parentheses represent the number of mice with dissemination to that organ/the total number of mice examined. Treatment of strain 52D-infected immunocompetent C.B-17 mice with YTS-169.4 resulted in a decrease in positively staining cells in the spleen from 14 to 3% and the complete elimination of the CD8 "brightly staining" peak (19a). A comparable analysis was not possible for treated nude mice, since CD8 cells are not normally detectable in the spleen.

resistance mechanism that was absent in nude mice was observed in SCID mice, suggesting that a nonimmune-mediated resistance mechanism may play an important role in cryptococcal resistance in mice.

The critical role of T cells in experimental cryptococcosis is well documented (12, 36, 37). Experiments involving immunization of mice before infection, infection of nude mice, and adoptive transfer of immune T cells all demonstrated this point (12, 36, 37). However, the mechanism(s) by which T cells effect intraalveolar clearance and protection has not been addressed.

i.t. infection of mice is a useful technique to examine the manifestation of pulmonary anticryptococcal immunity. Graybill et al. reported on i.t. infection of rats with *C. neoformans* (15). They observed a decrease in the survival of athymic animals as compared with controls and noted a significant pulmonary tissue burden in athymic rats at the time of death. However, growth of the organisms in the lungs and other organs was not monitored, and tissue burdens in athymic and matched control rats were not compared. Previous reports from this laboratory described i.t. inoculation of mice with an oral-i.t. cannula (31–33). However, the strains of *C. neoformans* selected for those studies grew progressively within the lungs, with no evidence for the acquisition of pulmonary immunity. Although both nude and $CD4^+$ T cell-depleted mice had more aggressive extrapulmonary disease than had control mice, there were no differences in the growth of the organisms in the lungs at any time studied (31, 33).

Our goal was to select a strain of *C. neoformans* which, after i.t. inoculation, would establish a progressive pulmonary infection in naive, normal mice until immune defenses developed and cleared the infection. There are significant variations in virulence between strains of *C. neoformans*, and many strains are extremely virulent in mice (18, 19, 22). Four strains were examined in this study. Infections with strains 68A and 305 were readily controlled in both BALB/c and C57BL/6 mice, probably because of their slow growth rate and lack of capsule, respectively (22). Rapid, early clearance may have accounted for the lack of development of DTH in animals infected with these strains of *C. neoformans*. Strain 613D, however, was not controlled by host pulmonary defenses. This strain grew progressively in the lungs and disseminated to the spleen and brain in both C57BL/6 and BALB/c mice. The failure to develop DTH to our antigen preparation during this infection may have resulted from suppression induced by the release of excess cryptococcal antigen (35). Strain 52D grew very rapidly in the lungs from the initial infection until some time between days 7 and 21, when clearance began. The clearance kinetics were indicative of an immune response against the organism, and infection of SCID and nude mice with strain 52D confirmed that the clearance mechanism(s) was T cell dependent.

Consistent with the development of CMI, strain 52D-infected BALB/c mice developed DTH as clearance occurred. Anticryptococcal DTH developed earlier (by 7 days) than was reported following intranasal infection (21 days [27]), possibly because of a larger antigen burden reaching draining lymph nodes earlier in the i.t. model than in the intranasal model. In strain 52D-infected C57BL/6 mice, however, DTH failed to develop, although the organisms were cleared from the lungs as efficiently as in BALB/c mice. Blackstock and Hall (5) observed that C57BL/6 mice injected intravenously with a lethal dose of *C. neoformans* NU-2 developed generalized immunosuppression and lost the ability to generate DTH against sRBC. Our results indicated that after i.t. inoculation, infected C57BL/6 mice were capable of mounting a DTH response to sRBC. Therefore, the inability to generate DTH was likely antigen specific and may indicate a dichotomy between DTH and CMI, similar to the results reported by Lim et al. (27). However, even though the kinetics of pulmonary clearance of 52D were similar between C57BL/6 and BALB/c mice and were immune mediated in BALB/c mice, it was not demonstrated in this report that pulmonary clearance of strain 52D in C57BL/6 mice was the result of CMI.

Consistent with these findings, Murphy first noted (34a) that C57BL/6 mice did not develop DTH to CneF Ag. However, Hall et al. reported the development of DTH in immunized C75BL/6 mice after challenge with three types of cryptococcal antigen preparations (17). The reasons for these discrepancies are unclear but may result from different antigen preparations and/or routes of immunization.

The failure to detect DTH in a mouse that appears to demonstrate pulmonary CMI suggests that at least two different subsets of cells are involved in the two phenomena. In a somewhat analogous infection model, mice infected with the facultative intracellular bacterium, *Listeria monocytogenes*, DTH and CMI are mediated by different T cell subsets, $CD4^+$ and $CD8^+$, respectively (2, 4, 10, 30). $CD4^+$ T cells are largely responsible for DTH reactions to many different nonviral antigens (7, 34). $CD8^+$ T cells play a major role in clearing listerial infections, possibly by lysing bacterium-laden unactivated macrophages (24). It was possible that in C57BL/6 mice and perhaps even BALB/c mice, CMI in the lungs and DTH to *C. neoformans* were mediated by distinct T cell subsets (even within the $CD4^+$ T cell population) and the DTH subset failed to develop or was suppressed in C57BL/6 mice. Alternatively, the antigen-specific cells that would have mediated DTH might have been sequestered at the sites of infection.

Reconstitution of infected SCID mice with immune T cells

from either the spleen or the lungs and hilar lymph nodes also demonstrated a dichotomy between the relative degrees of DTH and pulmonary resistance conferred. Lung and hilar lymph node T cells were markedly better at providing pulmonary protection after adoptive transfer than were splenic T cells, although both sets of cells were equivalent in transferring DTH. There are two explanations which are not mutually exclusive. First, lung and hilar lymph node cells may have a higher ratio of T cells that mediate CMI to T cells that mediate DTH than has the spleen. Second, the lung cells may contain a population of cryptococcus-specific T cells that home to the lung better than do splenic cryptococcus-specific T cells. Whatever the explanation, the ability to reconstitute infected SCID mice with immune T cells from the lungs and hilar lymph nodes allows one to dissect the mechanism(s) of T cell-mediated pulmonary clearance of yeast cells.

As compared with their heterozygous littermates, nude mice have increased early resistance to some bacterial, viral, and fungal infections, including a cryptococcal infection initiated following i.p. inoculation (39). This resistance has been attributed to activated phagocytes and/or natural killer cells (39). In the current experiments, extrapulmonary cryptococcal dissemination was greater in nude mice than in SCID mice. Treatment of athymic nude mice with anti-CD8 antibody did not affect the degree of spleen and brain infections present in these animals, although this $CD8^+$ cell depletion regime was able to deplete splenic $CD8^+$ T cells in *C. neoformans*-infected BALB/c controls. A possible explanation for the greater sensitivity of athymic nude mice than of SCID mice is that a nonopsonizing immunoglobulin M antibody which "masks" the yeast cells from phagocytic effector cells may be secreted by B cells in nude mice. However, in other studies from our laboratory, antibody-mediated in vivo double depletion of both $CD4^+$ and $CD8^+$ T cells in BALB/c or C.B-17 mice (which, like nude mice, have normal B cells) caused dissemination patterns similar to those seen in SCID mice (19a). This observation makes less likely a facilitating role for antibody in the increased extrapulmonary CFU in nude mice. Alternatively, the cause of the difference between nude and SCID mice in controlling extrapulmonary dissemination could reside in extrapulmonary phagocytic effector cells, or the rate of escape of the organism from the lungs might be different in the two strains of mice.

Further studies with this fungal infection model, in which CMI appears to play a pivotal role in controlling pulmonary infection, should provide important insights into the mechanisms for the profound susceptibility of AIDS patients and others with T cell defects to developing opportunistic pulmonary infections.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI-21951 from the National Institutes of Health, and additional support (to G.B.H.) was provided by National Cancer Institute grant CA09082 and a grant from the Texas Department, Ladies Auxiliary of the Veterans of Foreign Wars.

We thank Juneann Murphy, Vinay Kumar, and Robert Tigelaar for helpful discussions and suggestions during the course of this work. We also thank Patricia Pipes and Donna Todd for help in the preparation of this manuscript.

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