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Local Immunity in Lung-Associated Lymph Nodes in a Murine Model of Pulmonary Histoplasmosis

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Local immunity against acute pulmonary histoplasmosis was studied in the lung-associated lymph nodes of normal nonimmune mice infected intratracheally with live *Histoplasma capsulatum* yeasts. The phenotypes and distribution of cells in lung-associated lymph nodes and spleens were determined by flow cytometry. In addition, the immune responsiveness of these cells was evaluated by in vitro blastogenesis. Anti-*H. capsulatum* antibodies in serum and *H. capsulatum* antigen in tissue were measured by immunoassays. Cellular immune responses were greater in the lymph nodes than in the spleens. In lymph nodes 7 days after infection, a marked increase in the number of B lymphocytes caused the percentage to rise to 43%, compared with 26% in controls, and it remained elevated throughout the course of infection. A $CD3^+$ cell that did not express CD4 or CD8 increased in number until it constituted 21% of lymph node cells, compared with 5% in controls, by day 14. The numbers of $CD4^+$ and $CD8^+$ T lymphocytes were modestly increased from days 7 to 35, but their percentages dropped because of the greater numbers of B lymphocytes and $CD3^+4^-8^-$ cells. Macrophages consistently constituted 2 to 3% of lymph node cells during the study. In spleens 7 days after infection, the percentage of macrophages in infected mice rose to 21%, compared with 9% in controls, but the total spleen cell number did not increase until day 14, when all cell subsets were nearly double in number. The in vitro blastogenic response of lymph node cells to *H. capsulatum* peaked at day 7, but spleen cell response was minimal during the course of infection. *Histoplasma*-specific serum immunoglobulin G antibodies reached peak levels by day 21 and remained high to the end of the study. In contrast, levels of antigen-specific immunoglobulin M antibodies were very low. These data suggest that antigen-specific immune responses occur in lung-associated lymph nodes and that this draining lymph node response may be an important component in host defense against *Histoplasma* lung infection.

Histoplasmosis is a systemic infection caused by the dimorphic fungus *Histoplasma capsulatum* var. *capsulatum*. Primary infection, which is acquired by inhalation of mycelial spores into the lungs, is usually self limited, but it sometimes disseminates to other organs (15). Cell-mediated immunity is the principal host defense against *Histoplasma* infection (35). Immune responses against primary *H. capsulatum* lung infection have been described mostly for the lung (4, 11, 25, 29) and sometimes the spleen (22, 25). Little is known about the immune responses of lung-associated lymph nodes (LALN) against pulmonary histoplasmosis. Two abstract reports have described the distribution of B and T lymphocytes in LALN and the in vitro blastogenic response of these lymph node cells to *H. capsulatum* (14, 29). There are a few studies of cell-mediated immune responses in extrathoracic lymph nodes of animals infected with *H. capsulatum* via extrapulmonary routes. In those investigations, lymph nodes have been shown to become infiltrated with macrophages (1) and to have no change in the percentage or number of $Thy\text{-}1.2^+$, $Lyt\text{-}1^+$, or $Lyt\text{-}2^+$ lymphocytes (31). A more detailed study of immune responses in LALN would help us understand more clearly the pathophysiology of pulmonary histoplasmosis and the factors which influence the outcome of this infection.

We used a mouse model of acute *H. capsulatum* lung infection to study how cell-mediated immune responses develop in the LALN and spleens of normal nonimmune hosts during the course of acute pulmonary histoplasmosis. In this report, we describe the phenotypes and distribution of immune cells in these organs during the course of infection, the in vitro immune responsiveness of these cells, the *H. capsulatum*-specific antibody response in blood, and the clearance of *H. capsulatum* antigen from the lungs, spleen, and blood.

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MATERIALS AND METHODS

Experimental animals. Male $B6C3F_1$ (C3H/HeJ × C57BL/6) mice, 6 to 8 weeks old, were obtained from Harlan Sprague Dawley, Inc., Indianapolis, Ind. Experimental *Histoplasma* infections have been demonstrated previously in each of the parental strains (10), as well as in the F_1 hybrid (34). Use of the F_1 hybrid for this study investigates the genetic immune responsiveness contributed by both the $H\text{-}2^b$ and $H\text{-}2^k$ haplotypes. After intratracheal inoculation, mice were kept in microisolator cages and given food and water ad libitum.

***H. capsulatum*.** The yeast phase of a stock *H. capsulatum* strain isolated from a patient was used for infecting mice and for subsequent in vitro analyses. The yeasts were grown in brain heart infusion broth at 37°C for 48 to 72 h in a gyratory shaker (New Brunswick Scientific Co., Inc., New Bruns-

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TABLE 1. Monoclonal antibodies against mouse cell surface antigens

Tube no.	Cell surface determinant[a]	Antibody[a]	Predominant reactivity
1	Ly5, CLA	CD45-FITC[b]	All leukocytes
1	CD11b/CD18	Mac-1-PE[c]	Macrophages and granulocytes
2	CD3-ε	CD3-FITC[b]	T lymphocytes
2	B220	CD45R-PE[b]	B lymphocytes
3	TCR γδ	TCR γδ-FITC[c]	T lymphocyte receptor γδ
3	Ly2	CD8a-PE[b]	T cytotoxic or suppressor lymphocytes
4	TCR αβ	TCR αβ-FITC[c]	T lymphocyte receptor αβ
4	L3T4	CD4-PE[b]	T helper or inducer lymphocytes

[a] Abbreviation: TCR, T-cell receptor.
[b] Antibody obtained from GIBCO-BRL, Grand Island, N.Y.
[c] Antibody obtained from Boehringer Mannheim.

wick, N.J.). Organisms in an aliquot of the broth culture were pelleted by centrifugation at 230 × *g* at 4°C for 10 min, washed three times by resuspension in a sterile 0.9% NaCl solution, and then centrifuged as described before. After the last wash, the yeasts were adjusted with 0.9% NaCl solution to a concentration of 2×10^7 yeasts per ml for infecting mice. The remainder of the broth culture was autoclaved at 115°C for 30 min. The dead yeast cells were washed three times as described before, resuspended in 0.9% NaCl solution, and stored at 4°C for later use.

Inoculation of mice. Experimental and control mice were inoculated intratracheally as described previously (28). Briefly, mice were anesthetized in an induction jar that was supplied with 5.0% halothane and oxygen delivered at a rate of 1 liter/min by an anesthesia machine (Foregger Co., Inc., Smithtown, N.Y.) until deep, gasping respirations were observed. After a mouse was anesthetized, a wire speculum was inserted behind the incisor teeth, the tongue was retracted, and the epiglottis was viewed. A 20-gauge, 1.25-in. (ca. 3.18-cm)-long intravenous catheter was passed into the trachea. Correct placement of the catheter was verified by observing symmetrical expansion of the thorax when the mouse was ventilated with air from a glass syringe attached to the catheter. A 50-μl syringe (Hamilton, Reno, Nev.), fitted with a stylet that passed into the tip of the catheter, was used to deliver intratracheally either 5×10^5 *H. capsulatum* yeasts in 25 μl of sterile 0.9% NaCl solution into experimental mice or 25 μl of sterile 0.9% NaCl solution alone into control mice. Subsequently, groups of infected and control mice were studied 4, 7, 14, 21, 28, and 35 days after inoculation. There were five to six mice in the infected and control groups at each time point.

Cultures. Mice were anesthetized with halothane (as described for inoculation) and then exsanguinated by cutting a brachial artery. The blood was collected, and the serum was stored at −20°C for *H. capsulatum* antibody and antigen quantitation. Spleens, tracheobronchial lymph nodes, and lungs were removed aseptically. The spleens and LALN were placed in petri dishes containing RPMI 1640 medium supplemented with penicillin and streptomycin (Sigma Chemical Co., St. Louis, Mo.) and teased apart with fine-toothed tweezers. Sterile glass tissue grinders were used to homogenize portions of the lungs. Aliquots of spleen and lung cell suspensions were inoculated onto tube slants of brain heart infusion agar and cultured at 37°C in 7% CO_2 for 4 weeks. The cultures were examined three times a week for growth. Identification of *H. capsulatum* was made on the basis of colony morphology and microscopic appearance.

Flow cytometry. Lymph node and spleen cells were analyzed by direct two-color immunofluorescence. For each lymph node or spleen cell sample, aliquots of 5×10^5 to 1×10^6 cells were placed in quadruplicate into polystyrene tubes (12 by 75 mm) and washed with diluent, which consisted of calcium- and magnesium-free phosphate-buffered saline (pH 7.4), 1% bovine serum albumin, and 0.1% sodium azide. The sample tubes were centrifuged at 400 × *g* for 6 min and decanted, and the appropriate amount of fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated antibody (Table 1) was added. The tubes were gently mixed, and after 30 min at room temperature, 4 ml of diluent was added to each tube and the samples were centrifuged. The supernatant was decanted, and the cells were fixed in cacodylate buffer (pH 7.2) containing 1% paraformaldehyde and stored at 4°C in the dark until analysis on a Becton Dickinson FACScan flow cytometer.

In a separate experiment, lymph node cells were analyzed by two-color immunofluorescence for the presence of natural killer (NK) cells. For these experiments, cells were stained indirectly with antibodies against the NK1.1 and 4D11 determinants (received as a gift from P. L. Dunn, Trudeau Institute, Saranac Lake, N.Y.). NK1.1 binding was detected by using an anti-mouse immunoglobulin G (IgG)-PE antibody, while 4D11 was detected by using an anti-rat IgG-PE antibody. Each NK cell determinant was analyzed in conjunction with anti-CD3-FITC antibodies. Thus, whether a cell that stained positive for an NK cell determinant might also express CD3 cell surface molecules could be determined.

Lymphocyte blastogenesis. LALN and spleen cells were centrifuged at 230 × *g* at 4°C for 10 min and resuspended in complete medium, which consisted of RPMI 1640 medium supplemented with penicillin and streptomycin (Sigma) and 2% heat-inactivated fetal calf serum (GIBCO-BRL, Gaithersburg, Md.). Spleen cells were resuspended in warm (37°C) lysing buffer (0.17 M NH_4Cl, 0.01 M $KHCO_3$, 0.1 M EDTA [pH 7.3]) for 5 min to remove erythrocytes, centrifuged, and washed once again. LALN and spleen cells were adjusted to a final concentration of 5×10^6 cells per ml. Aliquots of 100 μl were placed in the wells of 96-well flat-bottom microtiter plates (Costar, Cambridge, Mass.). For each cell sample, 10^5 heat-killed *H. capsulatum* yeasts in 100 μl of complete medium was added to one set of triplicate wells (antigen-containing wells), and 100 μl of complete medium alone was added to another set of triplicate wells (control wells). The plates were incubated for 96 h in 5% CO_2 at 37°C. After 72 h of incubation, 1 μCi of [^{3}H]thymidine with a specific activity of 2.0 Ci/mmol (Amersham Corporation, Arlington Heights, Ill.) was added to each well. Cells

were harvested (PHD model 290 cell harvester; Cambridge Technology, Inc., Watertown, Mass.), and the radioactivity of incorporated [^{3}H]thymidine was measured (LS 8000 scintillation counter; Beckman Instruments, Inc., Irvine, Calif.). The results were expressed as counts per minute.

Anti-*H. capsulatum* antibody assay. Serum IgM and IgG antibodies against *H. capsulatum* were measured with an enzyme immunoassay kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Each sample was tested in triplicate for both immunoglobulin isotypes. In the initial step, 50 μl of 0.9% NaCl solution containing 10^8 heat-killed *H. capsulatum* yeasts were added to each microtiter plate well, and the plates were incubated for 1 h at 37°C. The wells were washed three times with a solution of 0.9% NaCl and 0.1% Tween 20 and then blocked for 15 min with the postcoating buffer supplied with the kit. The wells were washed three times before 50 μl of test serum was added to each one. After a 30-min incubation at 37°C, the wells were washed three times. Goat anti-mouse IgG-horseradish peroxidase conjugate solution (50 μl) was added to one set of triplicate wells, and goat anti-mouse IgM-horseradish peroxidase conjugate solution (50 μl) was added to the second set of triplicate wells. The microtiter plates were incubated for 30 min at 37°C and then washed three times. Horseradish peroxidase substrate (2,2′-azino-di-[3-thylbenzthiazoline sulfonate]) (6) supplied with the kit was added to the wells, and the plates were incubated for another 30 min at 37°C. The plates were read at a wavelength of 450 nm by an automatic plate reader (Molecular Devices, Menlo Park, Calif.).

***H. capsulatum* antigen assay.** Serum, lung, and spleen tissues were tested for *H. capsulatum* antigen by a solid-phase radioimmunoassay as described previously (33). Briefly, rabbit anti-*H. capsulatum* IgG antibodies were used as both the capture and ^{125}I-radiolabeled detector antibodies. For the assay, sera were diluted 1/5, spleen homogenates were diluted 1/10, and lung homogenates were diluted 1/50 as done in a previous experiment (34). Results were expressed as radioimmunoassay units, which were calculated as the ratio of the counts per minute for tissues from infected mice to 1.5 times the counts per minute for the corresponding tissues from uninfected control mice. A radioimmunoassay unit of at least 1.0 was considered to be positive.

Statistical analysis. Student's *t* test was used to compare data between infected and control mice. Analysis of variance was used to compare data within the infected and control groups of mice. A P value of <0.05 was considered significant.

RESULTS

Immunocompetent mice clear acute *H. capsulatum* lung infection after 2 weeks, but some develop disseminated infection. Of the infected mice examined 4 days after infection, 50% had lung cultures positive for *H. capsulatum*. Lung cultures for 100% of the mice studied 7 and 14 days after infection grew *H. capsulatum*, but none were positive for any infected mice at subsequent times. Spleen cultures were positive for 17% of the mice studied at day 7, 67% at day 14, and 17% at day 35. None of the mice at days 4, 21, and 28 had positive spleen cultures. Infected mice appeared ill (ruffled fur and lethargy) only during the period 7 to 14 days after infection.

Acute pulmonary *H. capsulatum* infection produces different cell distribution patterns in LALN and spleens. The cellular composition of LALN and spleens was determined by flow cytometry. LALN and spleens differed in the distribution of lymphocytes and macrophages during the course of acute pulmonary histoplasmosis. On day 4 after infection, the percentages and total numbers of LALN lymphocytes and macrophages from infected mice were similar to those of uninfected control mice. After day 4, the number of lymphocytes increased and peaked at day 14 (Fig. 1). The decrease in cell numbers after day 14 coincided with the clearance of lung infection as shown by negative lung cultures. Although all subpopulations of lymphocytes showed some increase in cell number, the increase in total LALN cell number was largely due to B lymphocytes and $CD3^+4^-8^-$ cells. At day 14, the percentage of B cells was 43%, compared with 26% in controls, and the percentage of $CD3^+4^-8^-$ cells was 21%, compared with 5% in controls. On days 7 and 14, $CD4^+$ and $CD8^+$ T lymphocytes were also increased in number, but their percentages fell because of the greater B lymphocyte and $CD3^+4^-8^-$ cell numbers. The percentage of macrophages in LALN remained 2 to 3% throughout the course of infection.

In comparison with LALN, changes in the distribution of spleen cells were less pronounced. As in the LALN, lymphocyte and macrophage percentages and numbers in spleens were similar for both infected and control mice at day 4 after infection. At day 7, macrophages constituted 21% of spleen cells, compared with 9% in controls, but decreases in T lymphocytes kept the total spleen cell number unchanged (Fig. 2). On day 14, the total spleen cell number was doubled, but the percentages of the spleen cell subsets were unchanged from day 7 because all cell subsets had uniformly doubled in number. The $CD3^+4^-8^-$ cells seen in the LALN were also seen in the spleens. The percentage of these cells ranged from 6 to 14% and did not vary much during the course of pulmonary infection as they did in the LALN.

LALN and spleen $CD3^+4^-8^-$ cells lack NK cell surface markers. Flow cytometry revealed that all of the LALN and spleen cells which possessed either αβ or γδ T-cell receptors were $CD4^+$ and $CD8^+$ T lymphocytes. However, a substantial portion of the $CD3^+$ cell population lacked CD4 and CD8 cell surface markers. $CD3^+$ cells are usually considered to be T lymphocytes, but some $CD3^+4^-8^-$ cells have been shown to express NK cell markers (2, 3, 17, 27). To determine whether the $CD3^+4^-8^-$ cells we observed were NK cells, we infected another group of mice and identified the phenotypes of their LALN cells 14 days after infection. Flow cytometric analysis performed with two antibodies against NK cell surface markers showed that the $CD3^+4^-8^-$ cells were not NK cells (data not shown).

In acute pulmonary histoplasmosis, LALN cells have a strong in vitro blastogenic response to *H. capsulatum*, but spleen cells do not. Lymphocyte transformation was used to determine the immune responsiveness of LALN and spleen cells to *H. capsulatum* in vitro. LALN cell blastogenesis was seen only with cells obtained on day 7 (Fig. 3). There were not enough LALN cells from control mice to test for comparison. However, in preliminary experiments, heat-killed *H. capsulatum* yeast did not have a significant mitogenic effect on LALN or spleen cells (data not shown).

Spleen cells had a weaker in vitro blastogenic response to *H. capsulatum*. Blastogenesis of spleen cells obtained from infected mice at day 14 was mildly suppressed in the presence of *H. capsulatum* (Fig. 4). When the cells were exposed to *H. capsulatum* in vitro, the mean ± standard error of the mean for [^{3}H]thymidine incorporation was $2.4 \times 10^3 \pm 0.3 \times 10^3$ cpm compared with $4.5 \times 10^3 \pm 0.4 \times 10^3$ cpm when they were not exposed to antigen ($P < 0.001$,

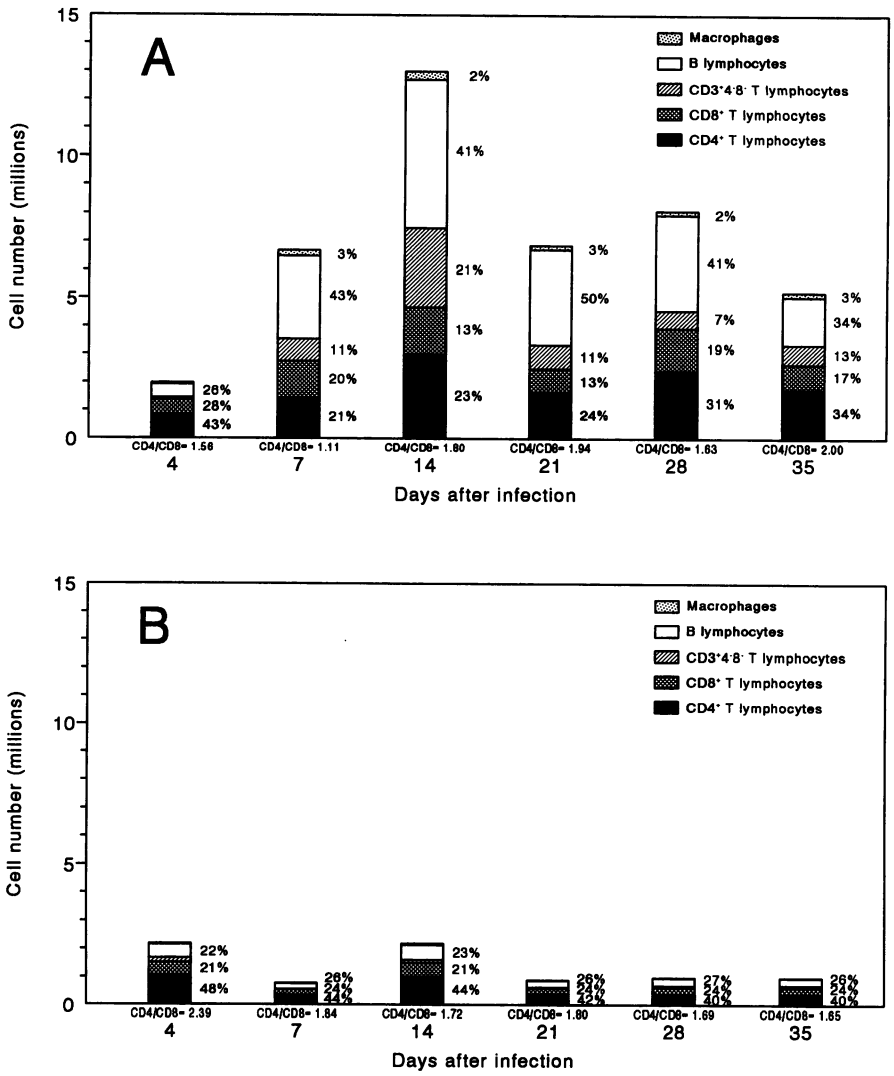

FIG. 1. Distribution of cell subsets in LALN during the course of pulmonary histoplasmosis. (A) Infected mice; (B) control mice. Bar heights correspond to the mean total cell number for six infected and five control mice. The percentage of all lymph node cells that each cell subset constitutes is shown to the right of the bars. The mean CD4/CD8 T-lymphocyte ratio is shown below the bars.

Student's *t* test). At day 28, the blastogenic response of cells from infected mice was slightly enhanced, i.e., $5.3 \times 10^3 \pm 1.9 \times 10^3$ cpm with *H. capsulatum*, compared with $1.4 \times 10^3 \pm 0.3 \times 10^3$ cpm without antigen ($P = 0.027$). Suppression of spleen cell blastogenesis coincided with the increase in macrophages. To determine whether the macrophages were the cause of the suppression, lymphocyte transformation was repeated with spleen cells from infected and control mice 14 days after infection. For each mouse, an aliquot of spleen cell suspension was placed in a plastic six-well plate for 1 h to remove macrophages by adherence. The blastogenesis assay was then performed with both adherence-treated and untreated cells. In this experiment, *H. capsulatum* had no in vitro effect on adherence-treated spleen cells of infected mice ($2.3 \times 10^3 \pm 0.2 \times 10^3$ cpm with *H. capsulatum* compared with $2.4 \times 10^3 \pm 0.2 \times 10^3$ cpm without antigen; $P = 0.492$). In contrast to the prior experiment, untreated spleen cells of infected mice were slightly stimulated by *H. capsulatum* ($2.3 \times 10^3 \pm 0.2 \times 10^3$ cpm with antigen compared with $1.5 \times 10^3 \pm 0.1 \times 10^3$ cpm without antigen; $P < 0.001$).

Acute pulmonary histoplasmosis in mice produces a significant rise in serum anti-*H. capsulatum* IgG antibodies but not IgM. In our study, a large increase in the levels of serum IgG antibodies against *H. capsulatum* was seen at day 14, and the levels peaked at day 21 and remained high at day 35 (Fig. 5). In contrast, there was no significant rise in IgM levels during the course of the lung infection.

High levels of *H. capsulatum* antigen can be detected in lungs, but not blood or spleens, of normal mice with pulmonary histoplasmosis. High levels of *H. capsulatum* antigen were detected in lungs beginning with the earliest time point (day 4) of the experiment, and maximum levels were measured at day 14 (Fig. 6). Antigen continued to be detected in the lungs afterwards, although *H. capsulatum* did not grow in any cultures after day 14. Very low levels of antigen could be detected in the blood on days 14 and 21 but not in spleens

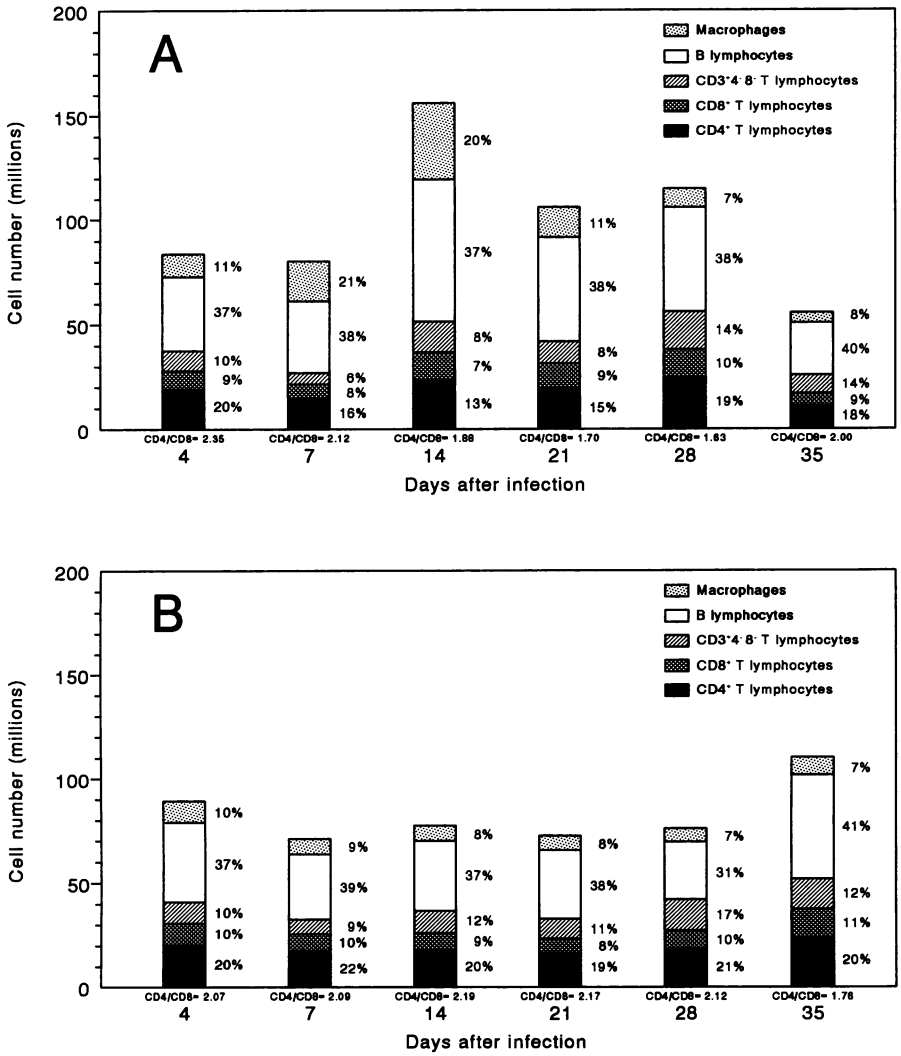

FIG. 2. Distribution of cell subsets in spleens during the course of pulmonary histoplasmosis. (A) Infected mice; (B) control mice. Bar heights correspond to the mean total cell number for six infected and five control mice. The percentage of all spleen cells that each cell subset constitutes is shown to the right of the bars. The mean CD4/CD8 T-lymphocyte ratio is shown below the bars.

at any time during the course of the experiment, even though some spleen cultures grew *H. capsulatum*.

DISCUSSION

Our study shows that strong immune responses develop in LALN in normal nonimmune mice with acute pulmonary histoplasmosis. Both cell distribution and proliferation varied considerably between LALN and spleens. This variation may be due partly to higher concentrations of *Histoplasma* antigen in LALN, which are closer to the primary site of infection. Even though some spleens grew *H. capsulatum* during the course of the experiment, they contained no appreciable amounts of antigen, and the splenic immune cell distribution changes and in vitro blastogenic response were diminished compared with those of LALN. In a previous experiment, we found that spleens of intravenously infected mice showed responses that were similar to those of LALN of intratracheally infected mice (14). In intravenously infected mice, changes in cell distribution and in vitro blastogenic responses to *H. capsulatum* were greater in spleens

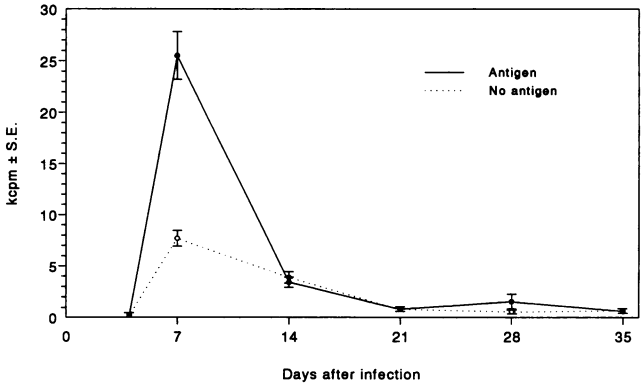

FIG. 3. Incorporation of [^{3}H]thymidine by lymph node cells exposed to antigen (*H. capsulatum* yeasts) in vitro. Results are shown as the mean counts per minute in thousands ± standard error (S.E.). Lymphocyte transformation was performed only with cells of infected mice because there were insufficient numbers of cells from controls.

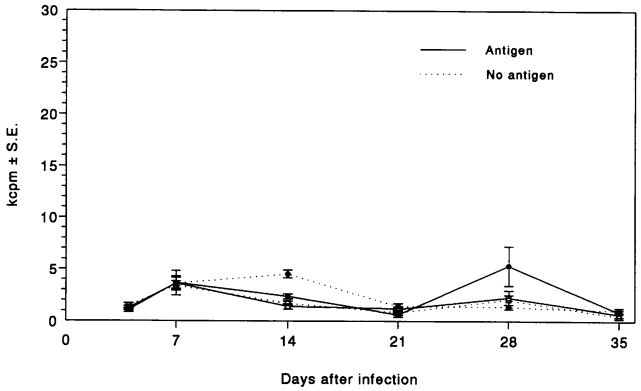

FIG. 4. Incorporation of [^{3}H]thymidine by spleen cells exposed to antigen (*H. capsulatum* yeasts) in vitro. Results are shown as the mean counts per minute in thousands ± standard error (S.E.) for infected (●) and control (□) mice.

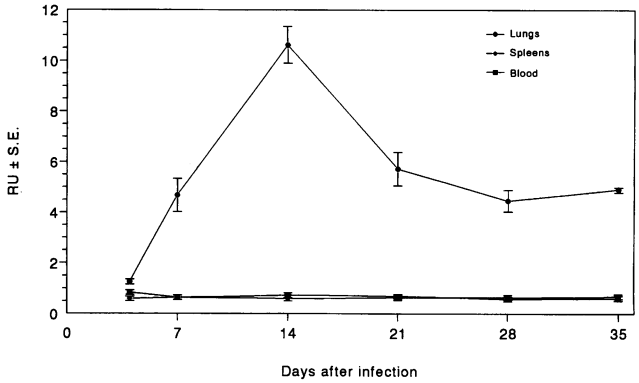

FIG. 6. Levels of *Histoplasma* antigen in lungs, spleens, and blood measured by radioimmunoassay. Results are expressed as the mean radioimmunoassay unit (RU) ± standard error (S.E.). A radioimmunoassay unit of at least 1.0 is a positive result.

than in LALN. However, in intratracheally infected mice, these immune responses were greater in LALN than in spleens. This suggests that immune response patterns are related to the route of inoculation, perhaps as a function of local antigen concentration.

Of the cell populations that we identified (B lymphocytes, $CD4^+$ and $CD8^+$ T lymphocytes, $CD3^+4^-8^-$ cells, and macrophages), the B-lymphocyte subset was the largest in both LALN and spleens of infected mice. Since cell-mediated immunity is considered to be the principal host defense against *Histoplasma* infection, the question arises as to why B lymphocytes proliferated more than the other cells. Although B lymphocytes can be stimulated directly by antigen, there is no information suggesting that this is important for *H. capsulatum* immunity (35). It is more likely that the B lymphocytes are stimulated by $CD4^+$ T lymphocytes. Mice have two $CD4^+$ T helper (T_H) lymphocyte subsets which have different cytokine secretion patterns: T_H1, which secretes interleukin-2 (IL-2) and gamma interferon, and T_H2, which secretes IL-4 and IL-5 (21). Since IL-4 is a potent activator of B lymphocytes, T_H2 cells are thought to be the primary source of helper activity for B lymphocytes. However, in the presence of various cytokines in vitro, both types of T_H cells can stimulate B-lymphocyte proliferation (9). This suggests that the predominance of one T_H cell type over another might be determined by the concentration of cytokines in the immediate environment of the cells prior to infection and by the concentration of cytokines secreted by activated macrophages. Quantifying the cytokines may provide clues as to which T_H cell predominates and as to how cell-mediated and humoral immunity against *H. capsulatum* develops.

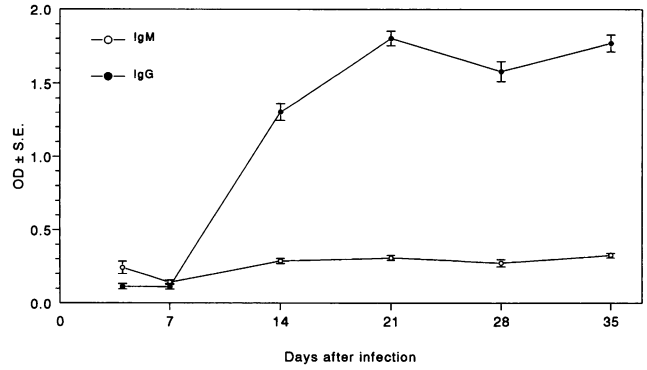

FIG. 5. Levels of serum *Histoplasma*-specific IgG and IgM antibodies measured by enzyme immunoassay. Results are expressed as the mean optical density (OD) ± standard error (S.E.).

In both the LALN and spleens, we identified a cell with a $CD3^+4^-8^-$ phenotype. This double-negative $CD3^+$ cell had the greatest rate of increase in number and percentage of T lymphocytes in the LALN. Other investigators have reported a similar cell population in animals and humans and identified the cells as T lymphocytes because of the presence of T-cell receptors (6, 7, 18, 19, 26). Recently, mouse $CD8^+$ lymphocytes that have been activated in vitro with phorbol mitogen, ionomycin, and IL-2 in the presence of IL-4 lost their surface expression of CD8 to become $CD3^+4^-8^-$ lymphocytes (12). These double-negative lymphocytes lost their cytolytic activity but gained the capacity to produce IL-4, IL-5, and IL-10. This pattern of cytokine release is consistent with that produced by the T_H2 subset of lymphocytes which provide the necessary signals for the activation and differentiation of resting B cells into antibody-producing cells (23). Therefore, it is possible that the increase in the $CD3^+4^-8^-$ subset may have represented a loss of CD8 from $CD3^+8^+$ lymphocytes which would have resulted in an increased $CD4^+/CD8^+$ ratio (which was observed) and may have supplied the necessary signals for the large B-cell response noted in the LALN. Some NK cells also have a $CD3^+4^-8^-$ phenotype (2, 3, 17, 27). However, the $CD3^+4^-8^-$ cell we saw in our experiments lacked NK cell surface markers.

LALN cells had a strong in vitro blastogenic response to *H. capsulatum*, but spleen cells responded minimally. Flow cytometry demonstrated that in the LALN, B lymphocytes and $CD3^+4^-8^-$ cells responded most vigorously. A blastogenic response was not seen beyond day 7 after infection, even though substantial amounts of *H. capsulatum* antigen were present in the lung. The reason for the transience of the response is unknown. Possible explanations might be the induction of B-lymphocyte tolerance to antigen or the activation of $CD8^+$ T suppressor lymphocytes.

As mentioned earlier, the weaker blastogenic response of spleen cells might be due to lower concentrations of *Histoplasma* antigen. Another possibility is that macrophages, which made up a larger proportion of the spleen than of the

LALN, inhibited the lymphocytes in vitro (13, 16). Indeed, the suppressed blastogenesis of spleen cells in our experiments was seen at the time when macrophages were increasing in number faster than other spleen cells. We tested this hypothesis by repeating the in vitro blastogenesis experiment with spleen cells depleted of macrophages and found that the proliferation of spleen cells from infected mice was not enhanced in response to *Histoplasma* antigen regardless of the presence or absence of macrophages. Thus, the presence of macrophages is not needed to inhibit spleen cell blastogenic responses, although there might be other ways in which macrophages suppress lymphocyte proliferation.

Infected mice in our experiments had high levels of serum anti-*Histoplasma* IgG antibodies and very low levels of anti-*Histoplasma* IgM antibodies. These findings are similar to results of another experiment in which mice were intraperitoneally infected with *H. capsulatum* yeasts (30). Patients with histoplasmosis have also been found to have high quantities of anti-*Histoplasma* IgG in blood either alone (8, 24) or with elevated levels of IgM (5, 32). The type of *Histoplasma* antigen might determine the pattern of the antibody response. Mycelial-phase antigens may be more likely than yeast-phase antigens to induce an IgM response. Indeed, we have observed in our laboratory that mice immunized with mycelial antigens often have IgM levels at least as high as the IgG levels. Although anti-*Histoplasma* antibodies have no significant role in host immunity to *H. capsulatum* infection, they are useful in diagnosis (32).

The mice in our study were inoculated with the yeast form of *H. capsulatum*. Although pulmonary histoplasmosis develops by inhalation of mycelial spores, it is the yeast form that causes infection and progression of disease. When mice are inoculated with *Histoplasma* mycelia that are irreversibly inhibited from converting to yeasts, infection fails to occur, and neither fungi nor granulomas are found at autopsy (20). Use of the yeast form also had the advantage of permitting greater control of standardization of the inoculum given to mice.

In summary, acute pulmonary *H. capsulatum* infection in mice produces stronger immune responses in LALN than it does in spleens. In primary histoplasmosis, LALN may help regulate the progression of pulmonary histoplasmosis. However, another important site of host defense that requires further study is the lung. The results of the present experiments are largely descriptive, and it remains to be shown how LALN and spleens contribute to immunity against pulmonary *H. capsulatum* infection.

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