Age-Related Resistance of C57BL/6 Mice to *Cryptococcus neoformans* Is Dependent on Maturation of NKT Cells

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Conflicting results have been reported regarding the ability of C57BL/6 mice to clear infections due to *Cryptococcus neoformans*. Examination of the various experimental protocols used suggested that C57BL/6 mice might develop the ability to resist infection as they mature. We analyzed the ability of C57BL/6 mice of different ages to respond to immunization with cryptococcal antigen or to clear a cryptococcal infection. Mice were immunized with a soluble cryptococcal culture filtrate antigen (CneF) emulsified in complete Freund’s adjuvant (CneF-CFA). Delayed-type hypersensitivity (DTH) reactions elicited by the immunization were significantly stronger in 15-week-old C57BL/6 mice than in 7-week-old mice. Analysis of cryptococcal CFU 8 weeks following intratracheal infection of 7-week-old mice or 15-week-old mice revealed a relative inability of the younger animals to control the infection. Six-week-old immunized and infected mice cleared cryptococci from brain, spleen, and liver in a manner similar to that of immunized and infected 15-week-old mice. However, the older mice cleared cryptococci much more efficiently from the lungs. The possible role for NKT cells was determined by passive transfer of thymocytes from 10-week-old mice (containing mature NKT cells) or 2-week-old mice (containing immature NKT cells) to 6-week-old mice. The 10-week-old thymocytes significantly enhanced the ability of the mice to develop a DTH response after immunization with CneF-CFA, while animals treated with 2-week-old thymocytes did not improve their DTH response after immunization. The cells in the 10-week-old thymocyte population responsible for improvement of DTH responses were identified as being NK1.1 positive.

C57BL/6 (B6) mice have been reported to be susceptible to infection with the yeast-like organism *Cryptococcus neoformans* (12, 14, 15). Our early experience with *C. neoformans* infections in B6 mice indicated that B6 mice were relatively resistant to this organism compared to highly susceptible BALB/c mice or more resistant CBA/J mice (J. W. Murphy, unpublished results). Upon careful examination of the experimental designs of cryptococcosis experiments in B6 mice, we found that investigators reporting B6 mice as susceptible to cryptococcal infection were using mice that were 6 to 8 weeks of age (15), whereas we had been infecting much older animals, generally from 15 to 24 weeks of age. Another observation that we and others have made is that B6 mice in the 6-to-8-week age range do not develop a strong cell-mediated immune (CMI) response to either immunization with cryptococcal antigen or infection with *C. neoformans* (15; Murphy, unpublished).

B6 mice have been characterized as being resistant to certain infectious agents, such as *Leishmania major*, that are eliminated from the host or held in check by T helper 1 (Th1) lymphocytes (10). Mouse strains such as BALB/c that are susceptible to this same group of organisms develop Th2 responses preferentially (13). Th1 lymphocytes, which are essential to a CMI response, produce gamma interferon (IFN-γ) upon restimulation with the immunogen, whereas Th2 cells, which are essential to the humoral immune response, produce interleukin-4 (IL-4) (23). Protection against the group of infectious agents eliminated by the CMI response is mediated by IFN-γ-activated macrophages, which then make greater quantities of toxic substances such as nitric oxide (NO) and oxygen intermediates that kill the organisms (4, 20). Macrophages from the Th1 mouse strains, such as B6 mice, are more readily activated by IFN-γ to produce NO than are macrophages from Th2 mouse strains, such as BALB/c (22). *C. neoformans* is an organism that is cleared from the host primarily by CMI, and IFN-γ is essential for protection (24). Consequently, it would be anticipated that B6 mice, which tend to respond to antigens with a Th1 response, should be resistant to *C. neoformans* and should develop a strong anticytotoxic cell-mediated CMI response. The reports (15) that B6 mice are almost as susceptible as BALB/c mice to *C. neoformans* infection do not fit this logic. Furthermore, the conflicting results that we have obtained with regard to resistance of B6 mice to *C. neoformans* infection need to be explained.

Considering that the CMI response is an important protective mechanism against *C. neoformans* (24), one would expect that mice lacking significant CMI responses to cryptococcal antigen would be more susceptible to cryptococcosis than mice that develop a strong anticytotoxic cell-mediated CMI response during the infection. Thus, the objective of this investigation was to gain an understanding of the reasons for the conflicting results of susceptibility of B6 mice to a cryptococcal infection and to define a parameter(s) that is important in development of the anticytotoxic CMI response. We hypothesized that 6- to 7-week-old B6 mice would respond differently to *C. neoformans* or cryptococcal antigens than mice that were 10 or more

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weeks of age. B6 mice are used extensively to study infectious diseases, such as cryptococcosis, because so many gene knock-outs are on the B6 background. Consequently, to interpret results from B6 mouse studies it is necessary to understand the effects of age on the protective immune response in B6 mice.

MATERIALS AND METHODS

Mice. B6 female mice were purchased from the Jackson Laboratories (Bar Harbor, Maine). Animals were put into experiments at 6, 7, or 15 weeks of age. Mice used as donors of thymocytes in passive transfer experiments were either 2 or 10 weeks of age at the time of thymocyte removal. Mice were housed in the University of Oklahoma Health Sciences Center animal facility. The facility is accredited by the American Association for the Accreditation of Laboratory Animal Care. All animal protocols were reviewed and approved by the University of Oklahoma Health Sciences Center Institutional Animal Care and Use Committee.

Maintenance of endotoxin-free conditions. All experiments were performed under conditions that would minimize endotoxin contamination. Endotoxin-free plasticware was used whenever possible. Glassware was heated for 3 h at 180°C. Reagents were tested for endotoxin contamination by the chromogenic Limulus amebocyte lysate assay (Whittaker Bioproducts, Inc., Walkersville, Md.) and contained less than the minimal detectable level of endotoxin (0.1 endotoxin unit/ml).

Cryptococcal isolate and infection of mice. *C. neoformans* isolate 184A, which is a weakly encapsulated isolate, was used throughout this investigation. This serotype A isolate was originally obtained from L. Friedman (Tulane University School of Medicine). The organism was maintained in the laboratory by serial passage on Sabouraud’s dextrose agar. A new stock was established from frozen stocks every 2 months. Prior to infecting mice, *C. neoformans* isolate 184A was grown on Sabouraud’s dextrose agar for 24 h at room temperature. Mice were infected with 184A by injecting 10⁵ cryptococci intravenously or by intratracheal instillation of 10⁶ cryptococci. The method for surgical intratracheal infection has been published previously (3). CFU of cryptococci in lungs, liver, spleen, and brain were determined 3 days following intravenous infection or 8 weeks after intratracheal infection.

Preparation of soluble cryptococcal antigen. Cryptococcal culture filtrate antigen (CneF) was prepared from *C. neoformans* isolate 184A by the method of Buchanan and Murphy (5). The preparation used in this investigation had a protein content of 0.281 mg per ml as determined by the bicinchoninic assay (Pierce Chemical Co., Rockford, Ill.) and a carbohydrate content of 5.36 mg per ml as determined by the phenol-sulfuric assay (7). Based on the Limulus assay, this lot of CneF had a reaction that was less than 0.1 endotoxin unit per ml.

Immunization with cryptococcal CneF antigen. Mice were immunized by subcutaneous injection of 0.1 ml of an emulsion of equal volumes of CneF and complete Freund’s adjuvant (CneF-CFA) in two separate sites at the base of the tail. Control animals were similarly injected with sterile physiological saline (SPSS) or saline-CFA. Mice were evaluated for their delayed-type hypersensitivity (DTH) response to CneF 7 days following immunization. In some experiments immunized mice were challenged with 10⁵ viable cryptococci intravenously followed by analysis of cryptococcal CFU in tissues 7 days following infection.

Passive transfer of thymocytes. Thymocytes were harvested from 2-week-old B6 mice or from 10-week-old B6 mice. After passage through a 60-gauge wire screen to obtain a single-cell suspension, the cells were pelleted by centrifugation and treated with Tris-NH₄Cl (pH 7.2 to 0.83% NH₄Cl) to remove any contaminating red blood cells. Following two additional washes in SPSS, the cells were counted and suspended to a concentration of 2 × 10⁶ per ml. A 0.5-ml aliquot of the cell suspension (10⁵ cells per mouse) was administered to 6-week-old recipient B6 mice by intravenous injection.

Depletion of NK1.1⁺ T cells from thymocyte suspensions by MACS. Single-cell suspensions of 5 × 10⁶ thymocytes from 10-week-old B6 mice were treated with 0.5 mg of biotinylated immunoglobulin G2a (IgG2a; BD Biosciences, San Diego, Calif.) or 0.5 mg of biotinylated anti-NK1.1 IgG2a (BD Biosciences). The cells were incubated in the refrigerator for 15 min. NK1.1⁺ cells were removed from the cell suspension by passage over a Vario magnetic-activated cell sorter column (MACS) column (Miltenyi Biotec, Inc., Auburn, Calif.) according to the manufacturer’s instructions. Control cells (treated with biotinylated IgG2a) were passed over the MACS column in a similar fashion. Analysis of fractions by flow cytometry showed that the MACS depletion lowered the number of NK1.1⁺ T cells in the thymocyte population from 6.1 to 0.0%. Cells that passed through the column (NK1.1⁻ or isotype-depleted fraction) were transferred intravenously (3 × 10⁷ cells/mouse) to naive recipients immediately prior to immunization with CneF-CFA or saline-CFA. A control group was included that received 0.5 ml of SPSS intravenously in place of the thymocyte suspension.

Elicitation of the anticyryptococcal DTH response. The hind footpads of mice were measured with a gauge micrometer. Immediately thereafter, 30 μl of SPSS was injected into the left footpad of the mice and 30 μl of CneF was injected into the right footpad. The footpads were measured a second time 24 h later. The increase in footpad swelling was calculated by subtracting the thickness of the footpad prior to injection from the thickness at 24 h after injection. The CneF-specific response was calculated by subtraction of the swelling of the SPSS-injected footpad from the swelling of the CneF-injected footpad.

Analysis of cryptococcal CFU in tissues. Groups of five experimental mice were euthanized 8 weeks after intratracheal infection or 7 days after intravenous infection with 10⁴ *C. neoformans* 184A cryptococci. The brain, liver, spleen, and lungs of the mice were removed to evaluate numbers of cryptococcal CFU in the tissues. Each organ was homogenized in 5 ml of SPSS, and dilutions of the homogenate were plated in duplicate on Sabouraud’s dextrose agar plates. CFU were enumerated after 3 days of incubation at room temperature.

Statistical analysis. When two groups were compared, Student’s t test was used for statistical analysis, and when more than two groups were compared, statistical comparisons were made with an analysis of variance (ANOVA) and the Bonferroni post-test. Data with a P value of 0.05 or less were considered to be significantly different. Each experiment was repeated a minimum of two times.

RESULTS

Fifteen-week-old mice developed stronger DTH responses than 7-week-old mice after immunization with CneF-CFA. B6 mice of two different ages were evaluated for their ability to respond to immunization with CneF-CFA, which is known to induce a strong protective CMI response to *C. neoformans* (5). The animals were immunized with CneF-CFA or saline-CFA (negative controls). Seven days after immunization, the mice were footpad tested with CneF to determine their DTH response. Footpad swelling reactions obtained in this analysis are shown in Fig. 1. Mice that were 7 weeks of age at the time of immunization with CneF-CFA developed a minimal anticyryptococcal DTH response, whereas 15-week-old mice developed a strong anticyryptococcal DTH response. The DTH response

![FIG. 1. DTH responses of 7-week-old and 15-week-old B6 mice immunized with saline-CFA or CneF-CFA 7 days prior to footpad testing. Data represent the mean increase (expressed in inches) ± the standard error of the mean of footpad swelling from five animals per group. P values were determined using Student’s t test. Footpad swelling in CneF-injected footpads of mice that were immunized with CneF-CFA was significantly greater than in the CneF-injected footpads of mice immunized with saline-CFA (P < 0.001).](http://iai.asm.org/)
in the 15-week-old B6 mice was higher \((P < 0.0001)\) than the DTH response in the 7-week-old mice.

Six- or 7-week-old B6 mice did not clear \(C.\) neoformans from tissues as effectively as 15-week-old B6 mice. B6 mice were also evaluated for their ability to clear an infection with cryptococcal isolate 184A following intratracheal infection. Mice (five per group) that were 7 or 15 weeks old were infected with \(10^5\) \(C.\) neoformans cryptococci by the intratracheal route. Eight weeks following infection, the mice were euthanized and their lungs, liver, spleen, and brain were removed, homogenized, and cultured to determine the number of cryptococcal CFU in each organ. Results from this analysis can be seen in Table 1. It was evident that 7-week-old mice were far less able to clear a cryptococcal infection than 15-week-old mice, because significantly more viable cryptococci were cultured from every organ examined from the 7-week-old mice than from the 15-week-old mice.

We evaluated the ability of immunization to enhance protective immunity in B6 mice that were 6 or 15 weeks old. Groups of five mice each were immunized with CneF-CFA. Seven days after immunization, the mice were challenged intravenously with \(10^5\) \(C.\) neoformans 184A. In this experiment the intravenous route of infection was used, since infection with the 184A isolate of \(C.\) neoformans cannot be established in immunized mice using the intratracheal route. On the 14th day following immunization (7 days after infection), the brain, spleen, liver, and lungs of each mouse were evaluated for the number of viable cryptococci present. The results are shown in Fig. 2. Significantly \((P = 0.02)\) fewer cryptococcal CFU were cultured from the lungs of 15-week-old immunized mice than from 6-week-old immunized animals that were challenged with viable cryptococci. Numbers of CFU in other organs were not significantly different between the two groups.

**Transfer of NK1.1\(^+\) T cells from 10-week-old B6 mice to 6-week-old B6 mice enhanced the anticytrococcal CMI response.** Considering (i) the importance of NKT cells for development of Th1 responses and immunity in cryptococcosis (18) and (ii) that NKT cells are not fully mature in B6 mice until after the 10th week of age (8), we hypothesized that the age-related differences in the anticytrococcal CMI response we were detecting in our experiments could be due to lack of a sufficient mature NKT-cell response during the induction phase of the CMI response to cryptococcal immunization. To

<table>
<thead>
<tr>
<th>Age (wk)</th>
<th>No. of (C.) neoformans [((\text{CFU} \pm \text{SEM}) \times 10^3)]/organ in:</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>Lungs: 5.8 ± 2.0, Liver: 5.0 ± 4.9, Spleen: 0.58 ± 0.19, Brain: 1.5 ± 2.0</td>
</tr>
<tr>
<td>15</td>
<td>Lungs: 0.01 ± 0.091(^b), Liver: 0.01 ± 0.0068, Spleen: 0.025 ± 0.01(^b), Brain: 0.07 ± 0.029(^d)</td>
</tr>
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\(^a\) Groups of five B6 mice were infected intratracheally with \(10^5\) \(C.\) neoformans 184A cryptococci. Organs were analyzed for numbers of CFU 8 weeks after infection.

\(^b\) \(P < 0.03\) by Student’s \(t\) test, compared to 7-week-old mice.

\(^c\) \(P = 0.0002\) by Student’s \(t\) test, compared to 7-week-old mice.

\(^d\) \(P < 0.0001\) by Student’s \(t\) test, compared to 7-week-old mice.

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**FIG. 2.** Cryptococcal CFU from brain, spleen, liver, and lungs of 6-week-old and 15-week-old B6 mice infected with \(10^5\) \(C.\) neoformans cryptococci intravenously. Mice were infected 7 days after immunization, and CFU were determined 7 days following infection. Numbers of CFU represent the mean ± standard error of the mean of total organ CFU obtained from five individual mice per group. \(P\) values were determined using Student’s \(t\) test.
test this hypothesis, an initial experiment was performed to provide increased numbers of mature NKT cells to younger B6 mice to determine if this would enhance development of their anticyptococcal CMI response after immunization with cryptococcal antigen. Six-week-old B6 mice were treated with thymocytes harvested from 10-week-old donors, which are known to have fully mature NKT cells (8). Control animals were treated with an equal number of thymocytes harvested from 2-week-old B6 mice, which are known to contain immature NKT cells (8). After the cell transfer the mice were immunized with CneF-CFA or saline-CFA, and 7 days after immunization the anticyptococcal DTH response was evaluated. The data from a typical experiment are shown in Fig. 3. Footpad swelling in the antigen-injected footpads of CneF-CFA-immunized mice was significantly increased in all experimental groups (P < 0.001 for all groups) compared to saline-CFA-immunized groups. However, significant enhancement of DTH responses to CneF immunization occurred in 6-week-old mice that received thymocytes from 10-week-old mice that had received unseparated thymocytes or thymocytes treated with isotype control antibody prior to MACS separation, compared to responses in 6-week-old mice that received saline. Mice that received NK1.1-depleted thymocytes from 10-week-old B6 mice developed significantly weaker DTH responses than 6-week-old mice that received thymocytes treated with isotype control antibody. In fact, the DTH response of mice given NK1.1-depleted thymocytes was similar to the DTH response of the 6-week-old mice that did not receive a thymocyte transfer.

**DISCUSSION**

B6 mice have been reported to be more susceptible to infection with *C. neoformans* than BALB/c or C.B-17 (BALB/c congenic for the B6 Ig heavy chain gene segment) mice (11, 12, 15). This susceptibility has been attributed to development of an eosinophilic infiltrate into infected lungs and production of high levels of IL-5 (14). On the other hand, we (Murphy, unpublished) and others (6) have found that B6 mice at 15 to 24 weeks of age are resistant to infection with *C. neoformans*. It has also been observed by us (Murphy, unpublished) and others (15) that younger (6- to 8-week-old) B6 mice do not develop as strong a DTH response to *C. neoformans* antigen as do more mature B6 mice. In general, B6 mice have been noted.
for their ability to develop Th1-mediated immune responses, whereas BALB/c mice have been considered the best mouse strain for development of Th2-type immune responses (10). Consequently, the previous observations with the murine model of cryptococcosis did not fit with the B6 mouse characteristic of a strong Th1 response. Thus, this investigation was initiated to determine the possible cause of these conflicting results. One possibility included our anecdotal observation that B6 mice over the age of 12 weeks seemed to be more resistant to cryptococcal infection and developed strong anticryptococcal CMI responses as measured by DTH reactions. However, these findings had never been proven by direct comparison of cryptococcal infections or the anticryptococcal CMI responses in B6 mice of different ages.

In the present investigation direct comparison of cryptococcal CFU from C. neoformans-infected B6 mice showed that 6- or 7-week-old B6 mice did not clear the organism from tissues as well as did 15-week-old B6 mice. The difference in clearance of organisms was reflected in the relative abilities of mice of these two ages to respond with a strong anticryptococcal CMI response following immunization with CneF-CFA. On the other hand, following immunization with CneF-CFA, infected 6-week-old mice cleared cryptococci from most organs in a manner similar to CneF-CFA-immunized 15-week-old B6 mice with the exception of the lung. In the lung, significantly more organisms were found in immunized 6-week-old mice that were challenged with a cryptococcal infection than were found in immunized and infected 15-week-old animals. The enhanced clearance in lungs of immunized 15-week-old B6 mice compared to immunized 6-week-old mice may be sufficient to have an overall long-term impact on progression of cryptococcosis if the organism enters the body via the respiratory route. In fact, our data from the CFU analysis of 7- and 15-week-old mice infected by the respiratory route support this concept.

Recently, a link between the innate and adaptive immune responses has been established, with NK cells and NKT cells playing a role in directing the differentiation pathway (Th1 versus Th2) followed by CD4+ T cells after antigenic stimulation. Originally it was thought that NK cells secreted IFN-γ and contributed to Th1 development, while NKT cells secreted IL-4 and contributed to Th2 development (21). However, later reports showed that NKT cells can also secrete large amounts of IFN-γ upon TcR engagement (21). Thus, NKT cells can secrete both IL-4 and IFN-γ and contribute to the development of the CMI response. NKT cells have a very limited T-cell receptor repertoire, with a Vα14-Jα281 Vα chain and Vβ chains skewed to use of Vβ8.2, Vβ7, and Vβ2 (9, 29). The activation of NKT cells is restricted to the major histocompatibility complex-like molecule CD1d. The natural ligand for the receptor is not known, but the receptor can be detected by the binding of CD1d tetramers loaded with a synthetic glycolipid, α-galactosylceramide (α-Gal-Cer) (19). After engagement of the TcR with α-Gal-Cer, the cells rapidly secrete large amounts of IL-4 and IFN-γ, suggesting that these cells play an important role in regulating immune responses (28). The contribution of NKT cells to some microbial infections that are controlled by CMI has been reported. These analyses demonstrated that NKT cells do not contribute to immunity in Mycobacterium tuberculosis (2) or Salmonella enterica serotype Choleraesuis (16) infections but are important for immunity to Listeria monocytogenes (27), Toxoplasma gondii (26), and C. neoformans (17).

Studies of the maturation of NKT cells in B6 mice revealed that the cell type does not fully mature until the 10th week of life (8). During early maturation, NKT cells secrete large amounts of IL-4 and very little IFN-γ. After the 10th week of life, NKT cells from B6 mice are characterized by secretion of relatively more IFN-γ than IL-4 (8). For this reason, we hypothesized that the level of maturation of NKT cells may be responsible for the age-related differences in CMI that were detected in our studies of B6 mice of different ages, since IFN-γ is essential to drive the development of the CMI response (1). Mature NKT cells can be recognized by their expression of the restricted TcR detected with the α-Gal-Cer-CD1d tetramer and having the phenotype NK1.1+ DX58 (8). Based upon this expression profile, Gadue and Stein (8) found that only 4% of the NKT cells in the thymus of 2-week-old B6 mice are mature. This number increased gradually to 16% of the NKT cells being mature at 4 weeks of life and 49% being mature by the 10th week of life. Our initial experiments determined the ability of thymocytes harvested from 10-week-old B6 mice to improve DTH responses of 6-week-old mice immunized with CneF-CFA. Negative controls included mice that received thymocytes from 2-week-old mice as a source of immature NKT thymocytes. These experiments showed that transfer of 10-week-old thymocytes to 6-week-old B6 mice just prior to immunization significantly improved the ability of the mice to develop an anticryptococcal DTH response induced by CneF-CFA immunization. Further experiments showed that removal of NK1.1+ cells from the thymocyte suspension of 10-week-old mice eliminated the ability of the thymocyte population to augment the anticryptococcal DTH response. Kawakami and coworkers (17) reported that NKT cells play an important role in anticryptococcal immunity and that these cells accumulate rapidly in the lungs of B6 mice following intratracheal infection. We speculate that accumulation of more-immature NKT cells (secreting IL-4) in the lungs of 7-week-old mice, compared to mature NKT cells (secreting IFN-γ) that would accumulate in the lungs of mice over the age of 10 weeks, sets up local conditions in the lung that favor the persistence of the infection in the lungs. This agrees with the data reported here, revealing the selective inability of CneF-CFA-immunized 7-week-old mice to clear a cryptococcal lung infection compared to immunized 15-week-old B6 mice.

Previous investigations have shown that mice that respond to cryptococcal immunization with a classical DTH reaction induced by CneF-CFA are highly resistant to infection with C. neoformans (25). In this investigation we report that relatively young B6 mice do not develop a strong DTH response following immunization with the cryptococcal vaccine CneF-CFA, while mice that are 14 to 15 weeks old respond with a very strong DTH response. This observation, coupled with those in this paper revealing the importance of NK1.1+ T cells for the development of the DTH response, helps explain the conflicting results obtained by various investigators who have studied the susceptibility of B6 mice to infection with C. neoformans.
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