Acquired Resistance to *Cryptococcus neoformans* in Adult Mice Vaccinated as Newborns

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Although *Cryptococcus neoformans* causes serious infections in AIDS patients, cryptococcosis in immunologically immature infants, as in immunocompetent adults, is rare. To investigate the resistance of neonates to *C. neoformans* and to determine whether they could be efficiently vaccinated as neonates against challenge with the yeast as adults, the course of infection was monitored in the lungs of mice infected intranasally with yeast cells. Neonates were less able than adults to reduce yeast burdens less than 24 h postinoculation and less able to control the progressive growth of yeast over several weeks. However, in both neonates and adults, yeasts were substantially eradicated by 6 to 8 weeks after infection. Numbers of all classes of leukocytes recovered from lung lavages of infected neonates and adults were similar. Significant differences appeared only on day 14, when neonates had more neutrophils and adults had significantly more CD4⁺ CD45RB cells with low fluorescence intensity. When vaccinated neonates were rechallenged after reaching adulthood, they expressed resistance to *C. neoformans* as effectively as did mice immunized as adults and survived an intravenous challenge that was lethal for unimmunized controls. Thus, exposure of neonatal mice to viable *C. neoformans* yeast, which persists in the lungs for many weeks, does not result in immunological tolerance to a yeast challenge in adult mice, as predicted by immunological dogma, but instead immunizes them. Therefore, even in immunologically immature individuals, the immune system serves to protect against pathogens rather than simply to distinguish self from nonself.

The immunological capability of neonates has long been of interest both for practical reasons, related to immunizing neonates against infections, and for theoretical reasons, dealing with how the immune system learns to distinguish pathogens from innocuous self. Immunological thinking in this area has been dominated by the paradigm that if infants are inoculated with significant amounts of a persistent antigen very early in their immunological development, they will exhibit unresponsiveness, rather than immunity, when challenged with the same antigen as adults (2). Previously published studies from this laboratory cast doubt on that paradigm. Many mice exposed in utero to the protozoan parasite *Toxoplasma gondii* by inoculation of their mothers or inoculated directly with *T. gondii* as neonates exhibited resistance to the parasite as adults (16). This resistance was manifest in the mice infected in utero as control of parasites that chronically infected the central nervous system and more directly in the neonatally inoculated mice as extended survival after rechallenge with highly virulent *T. gondii*. However, acquired immunity in neonatally vaccinated mice was clearly inferior to that of controls immunized as adults.

The present study was undertaken to investigate the resistance of neonatal mice to another opportunistic pathogen of immunocompromised individuals, the yeast *Cryptococcus neoformans*. It is believed that *C. neoformans* infection is initiated by the inhalation of the ubiquitous organism (17) and that pulmonary clearance is dependent upon CD4⁺ and CD8⁺ T lymphocytes (8, 12, 15, 20). We were specifically interested in whether pathogens in addition to *T. gondii* could induce acquired resistance when introduced to neonates by a physiologically relevant route. Although *C. neoformans* is a significant

cause of morbidity and mortality in AIDS patients, cases of cryptococcosis in neonates are very rare (18). This is perhaps unexpected given the ubiquity of *C. neoformans* in the environment (17, 18) and the immaturity of neonatal host defense mechanisms (26). Because *C. neoformans* is a pathogen which takes weeks to be cleared from the lungs of immunocompetent adult mice (8, 15), conventional immunological wisdom predicts that neonates exposed to *C. neoformans* should become immunologically tolerant of yeast antigens upon exposure to the yeast later in life. Most importantly, the foregoing suggests that unlike mice immunized as adults, mice inoculated with yeast as neonates might be highly susceptible as adults to a rechallenge (1, 9).

The following experiments were performed to determine (i) the level of susceptibility of neonatal mice to intranasally inoculated yeast and (ii) the capability of neonatally inoculated mice to resist pulmonary or systemic challenge with yeast as adults.

MATERIALS AND METHODS

Mice. Mice were purchased from the Animal Breeding Facility of the Trudeau Institute. Female BALB/cByJ (BALB/c) mice were estrus synchronized by crowding for 7 to 10 days prior to mating to C57BL/6J (B6) males in order to obtain several litters of mice born within a single 24- to 48-h period. Simultaneously born litters were pooled for experimental manipulation, after which the neonates were randomized and returned to nursing mothers. Adult (BALB/cByJ × C57BL/6J)F₁ (CB6F₁) control mice were also purchased from the Animal Breeding Facility and were 2 months of age at the time of inoculation. The mice received commercially prepared chow and acidified water ad libitum.

***C. neoformans*.** The moderately virulent serotype A strain 184 (22) was the gift of J. Murphy and was maintained as previously described (1). The avirulent serotype D *ura5* mutant was a gift from K. J. Kwon-Chung (4). *ura5* mutants require exogenous uracil and grow on Sabouraud dextrose (Sab-dex) agar but are unable to grow on yeast synthetic minimal medium. The *ura5* strain can therefore be distinguished from strain 184 by its growth requirements and is used as the challenge strain in experiments examining acquired resistance as a result of vaccination with strain 184.

Inocula were prepared by seeding yeast cells onto Sab-dex agar slants for 24 h at 26°C and then washing yeast cells off the slants and suspending the cells in Sab-dex broth. Cultures were incubated with shaking for 24 h at 37°C. Log-phase

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suspensions were centrifuged to pellet yeast cells and were then resuspended in phosphate-buffered saline (PBS) and counted. Suspensions were adjusted to the desired concentration for inoculation. Inocula were always plated to determine the number of viable yeast cells delivered per mouse.

To determine the numbers of yeast cells in the lungs or brains of mice at various times after infection, the mice were killed and their brains and lungs were homogenized in sterile PBS. Organ suspensions were then serially diluted and plated on appropriate media to detect the *ura5* strain or strain 184 yeast cells, as described previously (9).

Inoculation of mice. Neonates (<48 h old) and adult controls were given intranasal inoculations as described previously (6). Anesthetized (halothane-O₂) neonates received 10³, 10⁴, 10⁵, or 10⁶ yeast cells delivered in 10 μ l of sterile PBS which was placed in two successive 5- μ l drops over the nares of each mouse. Neonates were returned to nursing mothers chosen at random. Adult control mice were handled analogously and received 10⁴, 10⁵, 10⁶, or 10⁷ yeast cells in 50- μ l volumes. Intragroup uniformity of seeding by the intranasal route was checked by enumerating viable yeast cells recovered from lung homogenates obtained 1 to 2 h after inoculation, diluting the homogenates in sterile PBS, and plating the diluted samples on Sab-dex agar as previously described (1). The number of yeast cells recovered from each animal was within 10% of the mean for its group.

Cell recovery. At various times, infiltrating leukocytes in lung lavage fluids were sampled as described elsewhere (6). Briefly, mice were killed by halothane asphyxiation and exsanguinated by severance of the hepatic portal vein. The trachea was exposed, and narrow-gauge tubing attached to lavage apparatus was inserted through a small incision. The lungs of the neonates were lavaged with five 0.1-ml aliquots of sterile PBS, and the adult lungs were lavaged with three 1-ml aliquots of sterile PBS. Aliquots were cytocentrifuged and stained (Diff-Quik; Baxter, Hialeah, Fla.), and 100 cells/slide were scored to distinguish polymorphonuclear leukocytes (PMNs), macrophages, and lymphocytes by their morphological characteristics. The remainder of the lavage was used for plating to enumerate yeast cells and for flow-cytometric analyses. Tracheobronchial lymph nodes were harvested, placed in sterile PBS, crushed through stainless-steel screens and 100-mesh nylon gauze funnels, washed, and resuspended in PBS-1% bovine serum albumin-0.02% Na₂S₂O₃ for flow cytometric analyses.

Flow cytometry. Three-color analyses were performed on cells recovered by lung lavage or from single-cell suspensions prepared from the lung-draining lymph nodes as described above. Cells were suspended to approximately 10⁸/ml in PBS-1% bovine serum albumin-0.02% Na₂S₂O₃, and 0.01-ml aliquots were stained with fluorescein isothiocyanate-conjugated anti-CD4 (GK1.5; American Type Culture Collection), phycoerythrin-conjugated anti-CD8 (clone 53-6.7; PharMingen, San Diego, Calif.), and biotin-conjugated CD45RB (clone 16A; PharMingen), followed by an additional incubation with streptavidin-CyChrome (PharMingen). Fluorescence-labelled cells were analyzed with a FACScan and LYSIS II software (Becton Dickinson, Sunnyvale, Calif.), and 3,000 to 4,000 events were routinely acquired.

Statistics. Numbers (log₁₀) of viable *C. neoformans* cells are expressed as the means $\pm$ standard deviations (SD), as are numbers of leukocytes in Fig. 4. These data were statistically analyzed by Student's *t* test, with differences deemed significant when *P* was <0.05. As multiple comparisons were made in the experiment whose results are shown in Fig. 6, a more stringent criterion of significance (*P* < 0.025) was employed. Morbidity data were analyzed by the Mann-Whitney method.

RESULTS

Neonates are deficient in limiting yeast proliferation in the lungs at early time points but eventually control infection. Adult CB6F₁ mice inoculated intratracheally or intranasally with 10⁶ cells of *C. neoformans* 184 typically survive the infection, clearing the yeast from the lungs between 6 and 10 weeks after infection (data not shown). When seven 24-h-old neonates were infected intranasally with 10⁶ yeast cells, all were dead by 20 days postinfection. Five of five neonates given 10⁵ yeast cells survived for 52 days, and seven of eight neonates given 10⁴ yeast cells survived for 52 days, at which point the experiment was terminated. Therefore, neonates were not able to control yeast proliferation as well as adults that were given identical numbers of yeast cells. However, at lower doses, the neonates were equally capable of surviving cryptococcal infection. (In subsequent experiments, there were occasional early deaths among mice given 10⁵ yeast cells within 48 h of birth, but a large majority survived.)

In order to examine the kinetics of yeast clearance in neonates and adults, 20 neonates and 20 adult mice were inoculated with 10⁵ yeast cells (Fig. 1). Approximately five times as

FIG. 1. Yeast burdens in lungs of neonatal and adult mice early after intranasal inoculation. Groups of four or five neonates and adults inoculated with 10⁵ strain 184 yeast cells were killed at various times, and the number of lung CFU of *C. neoformans* was determined. Data are expressed as mean log₁₀ CFU $\pm$ SD. The differences between the means in neonates and adults were statistically significant (*P* < 0.05) at each time point examined.

many viable yeast cells were recovered from the lungs of neonates as from those of the adults at 2 h after inoculation. In each of several experiments, adult mice were able to clear up to 90% of the original inoculum during the first 24 h. In contrast, neonates consistently failed to reduce numbers of yeast cells during the first day of infection. Subsequently, adult mice continued to limit the proliferation of yeast in their lungs, but the numbers of yeast cells in lungs of mice inoculated as neonates increased more than 100-fold by 14 days of infection. Very few yeast cells were found in the brains of the neonates or the adult mice at any point.

To examine later times after inoculation and to monitor yeast clearance, 25 additional neonates and 25 adult mice were inoculated with 10⁵ yeast cells, and groups of 5 were killed 1, 4, 18, and 35 days later (Fig. 2). (It should be noted that the experiments whose results are shown in Fig. 1 and 2 were performed separately due to the difficulty of obtaining larger numbers of neonates born within a 48-h period.) In this experiment, in contrast to the results described above, yeast burdens increased somewhat in adult mice during the first 18 days of infection, but not as rapidly as in mice infected as neonates. Eighteen days after infection, mice inoculated as neonates had approximately 100-fold-more yeast cells in their lungs than did mice inoculated as adults. Between 18 and 35 days after infection, neonates reduced their lung burdens 99.9% (approximately 10⁶ viable yeast cells were recovered at day 18 versus approximately 10³ at day 35), and adults reduced their lung burdens >99.0% (approximately 10⁴ viable yeast cells were recovered at day 18 versus <10² at day 35).

These data suggest that neonates are profoundly deficient in the very early mechanisms that are deployed by adult mice to efficiently limit seeding and proliferation of *C. neoformans* in the respiratory tract and that they continue to be deficient in limiting growth of lung burdens for at least 2 weeks. Eventually, however, mice inoculated as neonates are able to handle the lung infection effectively.

FIG. 2. Yeast burdens later in infection in lungs of mice inoculated intranasally as neonates or as adults. Groups of four or five neonates and adults were inoculated intranasally with 10^5 strain 184 yeast cells, and the number of lung CFU was determined at later time points in infection to examine the kinetics of clearance of infection. Data are expressed as mean $\log_{10}$ CFU $\pm$ SD. Differences between the means for neonates and adults were significant ($P < 0.05$) at each time point examined.

We wondered whether 10^5 yeast cells was simply too many organisms for a presumably limited number of effector cells in the neonates to handle effectively during the first weeks of infection. If this was the case, the neonate might be able to demonstrate kinetics similar to those of adults (such as 90% killing during the first 24 h of infection) if the dose was decreased to 10^4 or 10^3 yeast cells.

To investigate dose-dependent resistance in neonates, 48

mice were infected within 48 h of birth with 10^5 , 10^4 , or 10^3 yeast cells. Adults were inoculated with 10^7 , 10^6 , 10^5 , or 10^4 yeast cells (Fig. 3). In the experiment whose results are shown, neonates were able to effect a small reduction in yeast numbers between the first 2 and 24 h at all three doses examined; however, differences in the number of CFU recovered from neonates at 2 and at 24 h after infection were not significant ($P > 0.05$) at any dose. (In four other experiments in this study, where neonates were inoculated with 10^6 or 10^5 yeast cells, there was no reduction in numbers of yeast cells at 24 h after infection.) After 24 h, yeast cells grew progressively in the lungs of neonates. By day 10, lung burdens were 10- to 100-fold greater than the initial instillation. This difference between lung burdens of yeast at 1 and 10 days was significant ($P < 0.05$) for each of the three doses examined.

A very different pattern of yeast proliferation was seen in adult mice. During the first 24 h, the adult mice were able to control yeast growth and, at the two lowest doses, to significantly reduce numbers of yeast cells. Furthermore, adult mice were capable of holding lung burdens more or less in check, or even diminishing them, by day 10. Thus, there was no significant difference between the numbers of CFU per lung recovered at 1 and 10 days from adult mice that had received inocula of 10^7 or 10^4 yeast cells, and there was even a reduction in the number of CFU per lung recovered from mice at 1 and 10 days from mice receiving the two intermediate doses. Together, these data suggest that the resistance capabilities of the neonates were not simply overwhelmed with too many organisms. Instead, the deficiency of neonatal resistance mechanisms appears to be qualitative.

The cellular response of neonates is similar to that of mice inoculated as adults. Controlling yeast proliferation in the lungs and containing yeast in the lungs after primary pulmonary infection are functions that are dependent upon $CD8^+$ and $CD4^+$ cells (8, 12, 15, 20) and upon tumor necrosis factor (11, 14, 19) and other cytokines (3, 10, 13, 21, 23). To examine the composition of the cellular infiltrate in the infected lungs, 25 neonates and 25 adult controls were infected intranasally

FIG. 3. Yeast burdens in lungs of neonates and adult mice given various doses of yeast. Groups of five neonatal (A) and five adult (B) mice received intranasal inoculations of different numbers of yeast cells. CFU of yeast in the lungs were enumerated at the indicated time points. The statistical significance of differences between groups is discussed in the text.

with 10^5 yeast cells, and the leukocyte composition of bronchoalveolar lavage fluids was assessed at various time points. The yeast proliferated to higher numbers in the lungs in this experiment than in the experiments whose results are shown in Fig. 1 and 2, and clearance of yeast was delayed in both groups. However, the relative growth pattern of the yeast and the limitation of proliferation by the host were similar to the results of the earlier experiments, with both groups of mice reducing numbers of yeast cells substantially by 41 days of infection. At various times, mice were killed, and leukocytes were washed from the lungs of the animals by lavage, identified by morphological characteristics, and counted (Fig. 4). Although neonates had an average of two- to fourfold fewer macrophages in lavage fluids than did adults at 1 and 4 days after infection, these differences were not statistically significant. By day 14, however, the number of macrophages in the lungs of neonates varied considerably but the mean number was comparable to the number of macrophages in adults. Also at day 14, neonates had 10 times as many PMNs as did adults. The PMNs were largely neutrophils; fewer than 1.0% were eosinophils. On average, the adult mice had six times as many lymphocytes as did neonates in their lung lavage fluid by day 14; however, due to animal-to-animal variability, this difference was not statistically significant. By day 41, the numbers of macrophages, PMNs, and lymphocytes in both groups of mice were approximately equal. Therefore, the relatively deficient resistance capabilities that we observed in neonates at early points after infection do not appear to be due to gross differences in the number of macrophages or PMNs in the bronchoalveolar infiltrates but rather suggest that the cells are not functionally equivalent effectors of yeast elimination.

Lung lavage lymphocyte populations were analyzed by flow cytometry as well (Fig. 5). Very few lymphocytes were observed on day 1 or 4 after infection. By day 14, adults had significantly more CD4⁺ T cells than did neonates, and of the adult CD4⁺ cells, a larger number and proportion were CD45RB^{lo} and therefore presumably activated. The difference in the numbers of CD8⁺ T cells was not significant. By day 41, mice infected as neonates and those infected as adults had roughly equivalent numbers of both CD45RB^{hi} and CD45RB^{lo} CD4⁺ T cells, but the number of CD45RB^{lo} CD4⁺ T cells in mice infected as neonates had increased between days 14 and 41, while those in the mice infected as adults had decreased. Similarly, the number of CD8⁺ cells was diminishing in the adult mice but remained elevated in the mice infected as neonates. These data suggest that the protracted period of yeast clearance observed in the neonatal mice may have been due to a lag in the recruitment of T cells to the site of infection and a lag in their activation. On the other hand, a different situation was observed in one experiment in which tracheobronchial lymph nodes were harvested 14 and 35 days after infection. (These nodes could not be reliably detected in and removed from the neonatal mice at day 1 or 4.) When tracheobronchial lymph node-derived lymphocytes were analyzed at day 14, there were more lymphocytes of both the CD4 and the CD8 classes in the lung-draining lymph nodes of mice infected as neonates ($[6.43 \pm 2.18] \times 10^6$ CD4⁺ cells and $[2.58 \pm 0.93] \times 10^6$ CD8⁺ cells) than in those of the mice infected as adults ($[1.60 \pm 0.81] \times 10^6$ CD4⁺ cells and $[0.73 \pm 0.35] \times 10^6$ CD8⁺ cells). Similarly, at day 35 there were more T cells in the nodes taken from mice infected as neonates ($[10.6 \pm 1.47] \times 10^6$ CD4⁺ cells and $[4.00 \pm 0.50] \times 10^6$ CD8⁺ cells) than in those taken from mice infected as adults ($[4.03 \pm 0.05] \times 10^6$ CD4⁺ cells and $[1.71 \pm 0.82] \times 10^6$ CD8⁺ cells). This finding could reflect the higher numbers of yeast cells in the lungs of mice infected as neonates, which

FIG. 4. Cellular composition of lung lavages of neonates and adult mice at various times after intranasal inoculation with *C. neoformans*. Mice received 10^5 strain 184 yeast cells. Groups of four mice were killed, and their lungs were lavaged as described in the text. Aliquots of leukocytes recovered were cytocentrifuged, stained, and identified on the basis of morphology as described in Materials and Methods. Data are expressed as means $\pm$ SD. Differences in the number of macrophages between neonates and adults were not significant ($P > 0.05$) at day 1 or 4. The number of PMNs differed significantly between neonates and adults only at day 14. Differences between neonates and adults in total lymphocyte numbers were not significant at any time point shown.

would result in more extensive stimulation of lymphocytes in the draining nodes.

Neonates can be effectively vaccinated to express efficacious immunity to *C. neoformans* upon rechallenge as adults. Previous work from this laboratory has demonstrated that the intratracheal vaccination of adult mice with 10^6 strain 184 yeast cells protects mice against subsequent systemic cryptococcal challenge (1, 9). A large majority of vaccinated mice survive a rechallenge and eventually sterilize their brains of yeast, while naive mice die in 28 to 35 days with $>10^8$ yeast cells in their brains. We have also shown that such vaccination results in

FIG. 5. Lymphocyte subset composition of lung lavages of neonates and adults at various times after infection. The remainder of the cells harvested from lavages as described in the legend to Fig. 4 were stained and analyzed by flow cytometry as described in Materials and Methods. Data are expressed as the means $\pm$ SD. Adults had significantly more ($P < 0.05$) total CD4⁺ T cells as well as CD4⁺ CD45RB^{lo} cells only at day 14. There was no significant difference between neonates and adults in the number of CD8⁺ T cells.

significantly lower brain burdens of yeast at 10 days after intravenous challenge with the avirulent *ura5* mutant strain. The growth requirements of the *ura5* strain allow us to distinguish between yeast cells derived from the vaccinating inoculum (strain 184) and those derived from the challenge (the *ura5* strain).

To determine whether mice can be induced to express efficacious immunity to *C. neoformans* by vaccination as neonates, groups of five mice were inoculated with 10^5 strain 184 yeast cells within 24 h of birth, along with adult controls. Six weeks later, the neonates, along with the naive controls, were infected intravenously with 2×10^4 *ura5* yeast cells. Mice were killed at 10 days, and the viable *ura5* cells in their brains were enumerated (Fig. 6). Mice vaccinated as neonates were, if anything, more efficient in limiting yeast growth in their brains than mice vaccinated as adults. There were 10- to 100-fold fewer yeast cells in the brains of neonatally vaccinated mice than in those of the naive controls.

To determine the long-term efficacy of neonatal vaccination, the experiment was repeated with 2×10^4 yeast cells of the

potentially virulent strain 184 to challenge mice immunized 6 weeks previously. All (five of five) naive mice given the virulent strain 184 yeast died between 18 and 25 days after infection (times to death, 18, 20, 23, 23, and 25 days). All mice vaccinated as neonates (five of five) or adults (five of five) and given the virulent strain survived for 56 days, when the experiment was terminated.

To determine whether mice vaccinated as neonates could express immunity to intratracheal challenge with a large inoculum of yeast, they, along with the mice vaccinated as adults and the naive controls, were challenged with 10^6 *ura5* yeast cells. Both groups of vaccinated mice expressed good resistance to pulmonary rechallenge (Fig. 7). Seven days after infection, both groups of immunized mice had 100-fold fewer viable *ura5* cells in their lungs than did naive controls. The difference between the numbers of 184 yeast cells remaining from the immunizing infection in the two groups of vaccinated mice was not significant. Twelve days after infection, no viable yeast cells of either strain were detected in the lungs of the vaccinated mice; however, a significant number of *ura5* cells ($\log_{10}$ CFU, 4.21 ± 0.29) remained in the unvaccinated mice. No organisms of either strain were detected in the brains of any immunized mice after this intratracheal instillation of yeast. In contrast, small numbers of *ura5* cells were recovered from the brains of three of five naive mice at day 7 (data not shown). The limit of detection for this experiment was 30 yeast cells per brain or pair of lungs.

These results indicate that exposure of mice to a sublethal dose of *C. neoformans* within 24 h of birth effectively vaccinates mice, protecting them against subsequent challenge as adults.

DISCUSSION

In this study, the resistance of neonatal mice to intranasally inoculated yeast along with the capability of mice vaccinated as neonates to resist challenge with yeast as adults was investi-

FIG. 6. Yeast burdens in brains of mice vaccinated as neonates or as adults 10 days after intravenous challenge. Groups of five mice were vaccinated intranasally as neonates or as adults with 10^5 strain 184 yeast cells and received an intravenous challenge with the *ura5* mutant strain 6 weeks later. Mice were killed, and brain yeast burdens were determined 10 days later. Data are expressed as means $\pm$ SD. The mean numbers of brain CFU for both groups of vaccinated mice were significantly different ($P < 0.02$) from that of the unvaccinated controls in each of two experiments.

FIG. 7. Yeast burdens in lungs of mice vaccinated as neonates and adults after intratracheal challenge. Six weeks after vaccination, groups of 10 mice vaccinated intranasally as neonates or as adults with 10^5 strain 184 yeast cells received an intratracheal challenge of 10^6 *ura5* yeast cells. The mice were killed, and lung yeast burdens were determined 7 days later. Data are expressed as means $\pm$ SD. The mean lung CFU for both groups of vaccinated mice was significantly different ($P < 0.05$) from that for the unvaccinated controls.

gated. Neonates were found to be less capable than adults of reducing lung burdens of yeast at very early time points after intranasal infection. The deficiency in neonates of resistance mechanisms that allow adult mice to limit yeast proliferation continued to be evident for the first 2 to 3 weeks after infection. Yeast growth curves for adult mice were flat during this period, in contrast to the progressive growth of yeast in neonates. Even when neonates were inoculated with 10- to 100-fold fewer yeast cells, they controlled yeast growth very poorly, if at all, implying that resistance capabilities operating during this period were not simply overwhelmed by the number of infecting organisms. Rather, the inefficiency of the resistance mechanisms in neonates relative to those in adult mice appears to be qualitative.

Studies with adult mice deficient in CD4⁺ and CD8⁺ T cells clearly indicate that these cells are critical for the ultimate clearance of the yeast from the lungs (12, 15, 18, 20). However, this T-cell-dependent clearance does not begin until several weeks into infection (8, 15). Prior to that time, however, adult mice hold the yeast in check. This is evidently managed by innate resistance mechanisms independent of CD4⁺ and CD8⁺ T cells, because the mice deficient in these T cells had lung burdens equivalent to those of T-cell-sufficient mice over this interval (8, 20). Thus, it is likely that the larger lung burdens of yeast attained in neonates during the first few weeks of infection reflects a deficiency in their innate resistance mechanism rather than a deficiency in CD4⁺ and CD8⁺ T-cell-dependent acquired immunity. Indeed, beginning at about 3 weeks of infection, the clearance of yeast from the lungs of neonates and adults proceeded at similar relative rates (Fig. 2), presumably via T-cell-dependent mechanisms, although this was not investigated in the present study. Since lung burdens were higher at the onset of clearance in neonates, clearance took longer to achieve. The delay in lung clearance may also have been a result of neonates' having fewer activated (i.e., CD45RB^{lo}) CD4⁺ T cells at 2 weeks, when significant numbers of these cells are first evident in adults (Fig. 5). It is noteworthy that the numbers and types of leukocytes recovered from in-

fected lungs did not differ greatly between adults and neonates until about 2 weeks after infection. Yet it was during this interval that neonates were clearly less able to control yeast than were adults (Fig. 1). This observation supports the conclusion that the innate resistance mechanisms of neonates during this period are functionally inferior to those of adults rather than simply numerically inferior.

At 2 weeks, the number of lung lavage PMNs in neonates was markedly greater than that in adults (Fig. 4). A similar finding has been reported (7) for neonatal mice with pulmonary *Pneumocystis carinii* infection. In neonatal mice infected with cryptococcus, it is likely that the large numbers of PMNs resulted from the heavy burdens of yeast present in the lungs at 2 weeks of infection. Whether this influx of PMNs is functionally important in yeast clearance remains to be determined, however.

Defects present in neonates are ultimately inconsequential since they affect neither the outcome of infection nor the ability of 24- to 48-h-old mice to be effectively vaccinated against rechallenge as adults. According to long-standing immunological dogma, the exposure of the immature immune system to an antigen that persists for several weeks ought to induce tolerance in mice. On the contrary, we observed that very young mice could be vaccinated to express robust and efficacious immunity to systemic or pulmonary challenge, demonstrating directly in a physiologically relevant model that exposure of immature animals to replicating antigen may result in immunity rather than in tolerance or anergy.

Three recent studies also support the conclusion that protective immune responses, rather than immunological tolerance, can be induced in neonatal mice (5, 24, 25). In each of those studies, the induction of immune responses required special manipulation of the conditions of immunization. Specifically, the induction of H-Y antigen-specific cytotoxic T lymphocytes in neonatal females depended on the coadministration of enriched dendritic cells (24), the induction of cytotoxic T lymphocyte protection against a murine retrovirus depended critically on immunization with small amounts of virus (25), and the induction of Th1- or Th2-like responses to a protein antigen required the coadministration of adjuvants (5). In contrast, in both the present study and a previous study from this laboratory (16), protective immune responses were elicited against naturally occurring pathogens administered by normal routes of infection. These results lend further credence to the notion that even in immunologically immature individuals, the immune system, is keyed to respond protectively against pathogens rather than to distinguish self from nonself (24).

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