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A Role for B Cells in Resistance to *Cryptococcus neoformans* in Mice

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The role of B cells in immunity to *Cryptococcus neoformans* was investigated. Genetically targeted, B-cell-deficient mice (μMt) examined at various times after intravenous infection with *C. neoformans* 184 had lung and brain yeast burdens that were equivalent to tissue burdens in control B-cell-sufficient mice. Both B-cell-deficient and B-cell-sufficient control mice were effectively vaccinated by a sublethal intratracheal instillation of strain 184 yeast against a systemic infection with the *C. neoformans* strain carrying *ura5*; vaccinated control and vaccinated B-cell-deficient mice had equivalent brain and lung burdens of the *ura5* strain 10 days after intravenous rechallenge. Additionally, B-cell-deficient and B-cell-sufficient vaccinated mice survived an intravenous rechallenge with a dose of yeast cells which is normally lethal for unimmunized mice. In further studies of the role of B cells in murine cryptococcosis, SCID mice were reconstituted with lymphocytes from B-cell-deficient and B-cell-sufficient mice. SCID mice reconstituted with lymphocytes from vaccinated B-cell-deficient animals failed to express effective adoptive immunity to *C. neoformans* brain infection. In contrast, SCID mice reconstituted with lymphocytes from vaccinated B-cell-sufficient mice had 10-fold fewer yeast cells in their brains than did uninfused SCID controls. However, SCID mice given lymphocytes from B-cell-deficient immune donors had fewer yeast cells in their lungs than did uninfused controls. Fewer $CD4^+$ lymphocytes were recovered at 7 and 11 days after infection from the peripheral blood and spleens of SCID mice reconstituted with lymphocyte suspensions from B-cell-deficient animals than from the peripheral blood and spleens of SCID mice reconstituted with suspensions from B-cell-sufficient control donors. These data suggest that B cells can play an important role in host defense against *Cryptococcus* in the brain under conditions in which T-cell-mediated immunity is impaired.

A role for $CD8^+$ and $CD4^+$ T cells in resistance to the opportunistic fungal pathogen *Cryptococcus neoformans* is widely accepted (12, 14, 20); however, the role of B lymphocytes remains controversial (for a recent review, see reference 5). Administration of polyclonal immune sera to mice has been reported to be either protective (9, 10) or ineffective (19) against experimental infection with *C. neoformans*. Individuals with deficiencies in humoral immunity are not characteristically at risk for cryptococcal disease (17). However, cryptococcosis has been reported in a few individuals with hyperimmunoglobulin M (hyper-IgM) syndrome (15) and hypogammaglobulinemia (29). Administration of monoclonal antibodies (MAbs) specific for the polysaccharide capsule component glucuronoxylomannan prolonged survival and reduced yeast burdens in mice in some organs (7, 22, 24, 25, 28). However, the beneficial effects were dependent upon route and timing of administration of antibody relative to delivery of infecting organisms. Supplementation of antifungal chemotherapy with MAbs to glucuronoxylomannan can help limit yeast proliferation (23).

Perhaps the strongest evidence against a role for B cells in cryptococcal infection is the demonstration that mice rendered B cell deficient by administration of antimouse μ Ig as newborns were indistinguishable from controls with respect to organ burdens of yeast, development of delayed-type hypersensitivity, and mortality after experimental intravenous yeast infection (21). However, intravenously inoculated yeast cells seeded the brain and other organs, with rapid proliferation within the central nervous system leading to a lethal meningoencephalitis. As a consequence, the intravenous infection model most likely did not mimic the infection dose and route under which humans are believed to contract systemic cryptococcal disease. In humans, cryptococcosis is believed to be initiated as a mild pulmonary infection (17). Although yeast cells are often controlled within the lungs of individuals with defects in cell-mediated immunity, there is frequent dissemination to the brain and other organs (12, 14).

Experimental studies at this laboratory have attempted to model more closely the immunologic events attending a primary pulmonary exposure to *C. neoformans* that is followed by dissemination of yeast to the brain. Mice are vaccinated with a sublethal pulmonary infection and allowed to develop specific anticryptococcal resistance. Six to eight weeks after vaccination, they are challenged intravenously with yeast. Vaccinated mice express an acquired resistance to *C. neoformans*, surviving an infection which is lethal for naive mice and, in most instances, eradicating yeast from the central nervous system by 12 to 16 weeks after challenge (1, 13). Moreover, transfer of T cells from vaccinated donors into lymphodeficient SCID mice protects the latter against intravenous infection with *C. neoformans*. Recipients of the cells from vaccinated donors survive 2 to 3 times longer than untreated control SCID mice (or SCID mice given an infusion of cells from naive immunocompetent donors), provided that $CD4^+$ T cells are included in the suspension (1).

Although no role for B cells was found in the primary intravenous infection model cited previously (21), the model did not rule out the possibility that B cells may be an important component of acquired resistance in vaccinated mice. Furthermore, the finding that T cells are necessary for acquired resistance in vaccinated mice does not imply that they are sufficient in the absence of B cells. Therefore, we wished to determine

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whether B cells are, in fact, an important component of acquired resistance to *C. neoformans.*

MATERIALS AND METHODS

Mice. μMt mice (16), which are B cell deficient due to the disruption of a membrane exon of the IgM heavy-chain gene, were provided by A. G. Harmsen (Trudeau Institute). This stock was obtained originally from B & K Ltd., Grimston, Aldbrough Hull, United Kingdom. Mice homozygous for the disrupted gene were mated to heterozygous progeny of C57BL/6J (B6) × μMt crosses to produce homozygous and heterozygous littermate controls having comparable genetic backgrounds. Offspring were typed for the presence or absence of B220+ and/or Ig+ cells by flow cytometric analyses of peripheral blood cells (see below). C57BL/6 *scid*/*scid* (B6 SCID) lymphodeficient mice were used as recipients in adoptive immunity experiments. Immunodeficient mice, along with controls, were housed in microisolator cages and were supplied with sterilized food, HEPA-filtered air, and water ad libitum. Male mice between 10 and 20 weeks old were used in all experiments.

C. neoformans. Strain 184 is a mildly virulent serotype A strain that was obtained from J. W. Murphy (26). Strain 184 was maintained on Sabouraud dextrose agar and cultured in Sabouraud dextrose broth for inoculum preparation as previously described (12). The *ura5* strain, an avirulent mutant derived from the serotype D *C. neoformans* B3501, was a gift from K. J. Kwon-Chung (31). The *ura5* strain was maintained on yeast essential peptide derivative (YEPD) agar and cultured in YEPD broth as previously described (1). *ura5* strain yeast cells do not grow on yeast synthetic minimal medium (YSM) plates because the *ura5* strain requires an exogenous source of uracil. Strain 184 grows on YSM. Therefore, it is possible to distinguish between the two strains by differential growth requirements.

Inoculations were delivered via two routes in this study. Intravenous inocula of 2×10^4 organisms in 0.2-ml volumes were delivered via the retro-orbital sinus (2); this resulted in 0.1 to 1.0% of the initial inoculum being deposited in the brain. Intratracheal instillation of 10^6 yeast cells was used to vaccinate mice (12). Inocula were prepared as previously described (12).

Recovery of yeast from mice. Mice were killed by CO_2 asphyxiation. Organs were harvested and homogenized in ice-cold phosphate-buffered saline (PBS), and the homogenate was serially diluted and plated on appropriate media to detect *ura5* strain or strain 184 yeast as described previously (12).

Reconstitution of SCID mice with cells from vaccinated donors. Donor mice were killed 6 to 8 weeks after immunization. Spleens and lung-draining lymph nodes were harvested from several donors and pooled, and single-cell suspensions were prepared by mincing and crushing spleens and nodes through sterile stainless steel screens and 100-mesh nylon gauze funnels. The cells were collected by centrifugation and washed with sterile PBS, and the erythrocytes were lysed hypotonically. The unlysed cells were washed in PBS, filtered again through nylon gauze, and resuspended in sterile PBS at approximately 5×10^7 lymphocytes per ml. Mice received a number of cells from the pool that was equivalent to the yield from a single donor. Cells were delivered intravenously via the retro-orbital sinus. Suspensions were analyzed by flow cytometry (see below) to determine numbers of $CD4^+$, $CD8^+$, Thy-1^+, and Ig^+ cells. Eighteen hours after transfer of lymphocytes, mice were given an inoculum of the yeast via the retro-orbital sinus.

Flow cytometry. Mice were examined before use in experiments to verify the absence of Ig^+ lymphocytes in mice putatively homozygous for the disrupted IgM heavy-chain gene. Approximately 30 μl of peripheral blood was collected from the tails of mice and placed in microcentrifuge tubes containing PBS and 20 U of heparin per ml. Cells were pelleted by centrifugation for 1 min at $9,500 \times g$, and the supernatant was discarded. After hypotonic lysis of erythrocytes, nucleated cells were suspended to approximately 10^7 cells/ml in RPMI 1640 containing 1% bovine serum albumin and 10 mM sodium azide, then incubated with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated monoclonal antibodies to detect Ig^+ cells (goat anti-mouse IgG plus IgM [Tago, Burlingame, Calif.]) and $B220^+$ cells (PE-CD45R-B220; Pharmingen, San Diego, Calif.).

The composition of lymphocyte subsets in recipient mice during the course of the adoptive immunization was analyzed by collecting peripheral blood lymphocytes (PBL) as described above and incubating them with FITC- or PE-conjugated MAbs to detect $CD4^+$ (GK1.5, ATCC TIB 207), $CD8^+$ (2.43, TIB 210), Thy-1.2^+ (30H12, ATCC TIB 107), and Ig^+ cells (goat anti-mouse IgG plus IgM). GK1.5, TIB 210, and 30H12 antibodies were prepared at this institute as FITC-conjugated $F(ab')_2$ fragments. Labelled cells, gated for lymphocytes by forward scatter and side scatter characteristics, were analyzed with a FACScan flow cytometer and LYSIS II software (Becton Dickinson, Sunnyvale, Calif.). One thousand to five thousand events were routinely collected from peripheral blood samples. Lymphocyte suspensions used in adoptive immunity experiments were also analyzed by flow cytometry, as were splenocytes prepared from adoptively immunized SCID mice that were killed 12 days after infection. Splenocyte suspensions were analyzed in the same manner as PBL; however, the number of events collected was increased to 5,000 to 10,000.

MAb treatments. Twenty-four hours prior to intravenous infection with *C. neoformans*, mice received intraperitoneal injections of 1.0 mg of anti-CD4 MAb or 1.0 mg of irrelevant rat antibody of the identical isotype. The anti-CD4 MAb was partially purified by DEAE fractionation of ascites fluid from mice injected with hybridoma GK1.5. Flow cytometric analysis of splenocytes of the GK1.5-treated mice showed that <1.03% of the lymphocytes were $CD4^+$, whereas 41.6% of the lymphocytes from mice treated with the control MAb were $CD4^+$.

Statistics. Differences in numbers of yeast cells per organ were analyzed by Student's *t* test. Values shown are means ± standard deviations. The Mann-Whitney test was used to analyze data in survival studies and to analyze lymphocyte populations in the experiments shown in Fig. 3. Differences were deemed significant when *P* was less than 0.05.

RESULTS

Acquired resistance to *C. neoformans* is expressed in the absence of B cells. To test whether B-cell-deficient mice were capable of being vaccinated to express acquired immunity to *C. neoformans*, groups of five male μMt (−/−) mice and five (+/−) littermates were given an intratracheal instillation of 10^6 cells of strain 184. Six weeks after vaccination, vaccinated mice and naive controls were challenged intravenously with 2×10^4 mutant *ura5* strain yeast cells. The use of the *ura5* strain allows yeast cells derived from the challenge infection to be distinguished from residual yeast cells from the vaccinating inoculum. Ten days after rechallenge, mice were killed and yeasts were enumerated in brains and lungs (Fig. 1A). Vaccinated B-cell-deficient mice and B-cell-sufficient controls each had 10- to 100-fold fewer *ura5* strain yeast cells in their brains than did naive controls. None of the mice had appreciable numbers of *ura5* strain cells in their lungs. Figure 1B shows that there was little difference in the brain burdens of vaccination strain 184 yeast between B-cell-deficient and B-cell-sufficient mice. However, some individuals (two of four in the B-cell-deficient group and 1 of 4 in the B-cell-sufficient group) continued to harbor large numbers of strain 184 yeast in their lungs. Nonetheless, mice that had large burdens of strain 184 yeast in their lungs did not have defects in acquired immunity because the same mice had brain burdens of *ura5* strain yeast that were 10- to 100-fold lower than those of naive mice of the identical genotype (Fig. 1A).

A similar experiment was performed, using strain 184 yeast cells for both vaccinating and challenge inocula, to determine whether B-cell-deficient mice were capable of resisting a challenge with virulent organisms. A longer period (12 weeks) was incorporated between vaccination and challenge to allow for clearance of the primary pulmonary infection. Mice were killed 11 days after intravenous challenge, and yeast cells were enumerated in brains and lungs. Again, vaccinated B-cell-deficient and B-cell-sufficient mice were similarly resistant, with 100- to 1,000-fold fewer yeast cells in their brains than unvaccinated controls (data not shown). Yeast cells were not detectable (detection limit, 30 yeast cells/organ) in the lungs of either set of vaccinated animals, but a small number of organisms (<1,000) were harvested from naive +/− mice. Because μMt mice were available in limited numbers, only heterozygous mice were included as naive controls in this experiment.

The capacity of vaccinated B-cell-deficient mice to survive long term after an intravenous challenge that is lethal for nonimmune mice was tested. Five B-cell-deficient mice and five B-cell-sufficient mice were intratracheally inoculated, along with naive controls, and challenged 12 weeks later with strain 184 yeast. The median time to death of naive B-cell-deficient mice (24 days) did not differ significantly from that of B-cell-sufficient controls (25 days). Eighty days after the intravenous challenge, four vaccinated mice remained in each group. The single death in the B-cell-deficient group was not due to cryptococcal infection; the cause of the single death in the B-cell-sufficient group was not determined. Yeast cells were enumerated in the brains of the remaining eight mice. Viable yeast cells were not detected in two of the four B-cell-

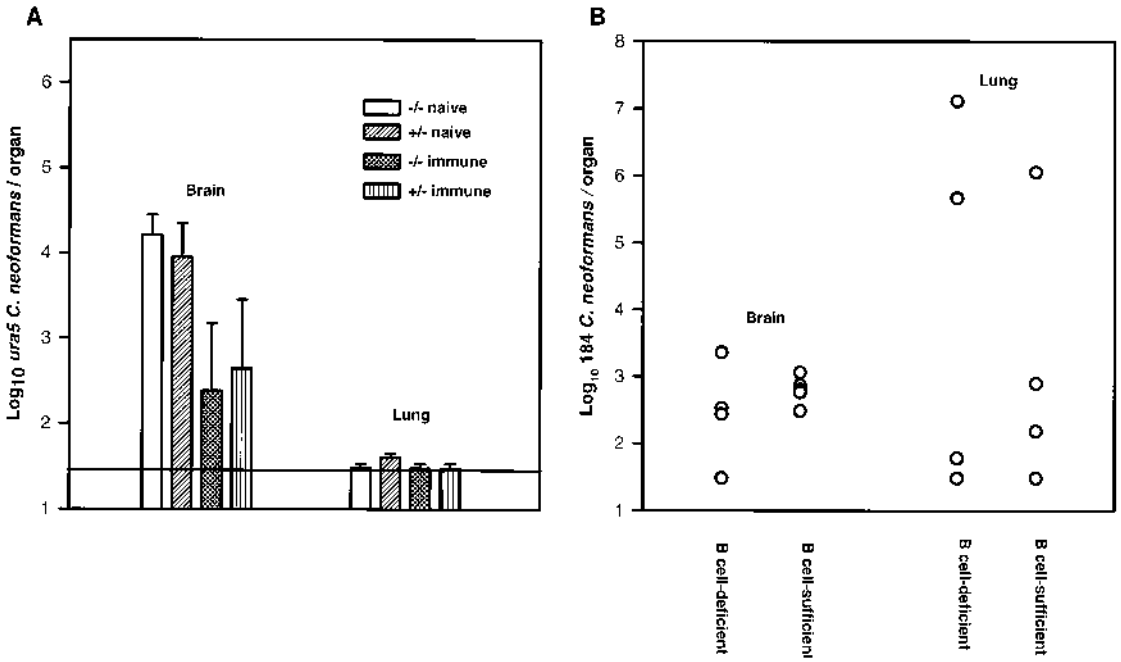

FIG. 1. Resistance to *C. neoformans* infection in the brains of vaccinated B-cell-deficient mice. Male B-cell-deficient and B-cell-sufficient mice were infected intratracheally with 10^6 cells of strain 184. Eight weeks after immunization, vaccinated mice, along with control unvaccinated mice, received 2×10^4 *ura5* strain yeast cells intravenously. (A) *ura5* strain brain and lung burdens 10 days after intravenous infection. The horizontal line represents the limit of detection of yeast cells per organ for these experiments. The difference between brain yeast burdens of naive and immune mice was significant ($P < 0.05$) for both B-cell-deficient and B-cell-sufficient mice. Differences in lung burdens were not significant. Data are the means plus standard deviations for four or five mice per group. (B) Strain 184 brain and lung burdens of yeast cells in individual mice whose burdens are depicted in panel A.

deficient mice; the remaining two had 100 to 1,000 yeast cells. Viable yeast cells were not detected in the brains of any of the four B-cell-sufficient mice.

These data suggest that B cells play little if any role in the generation or expression of specific acquired immunity to *C. neoformans*. To identify a lymphocyte population that is responsible for resistance in B-cell-deficient mice, intratracheally vaccinated B-cell-deficient mice were given 1.0 mg of MAb against $CD4^+$ T cells 12 weeks after the intratracheal infection was initiated and were killed 9 days later. The burdens of yeast in the brains of these mice were greater than those of vaccinated mice given isotype-matched control antibody (Fig. 2). Therefore, the acquired immunity expressed by B-cell-deficient mice, like that of B-cell-sufficient mice (13), depends on $CD4^+$ T cells.

Transfer of lymphocytes from vaccinated donors into SCID mice does not protect recipients against systemic cryptococcal challenge. Previously, this laboratory has shown that adoptive immunization of C.B-17 SCID mice with lymphocyte suspensions containing $CD4^+$ T cells from vaccinated immunocompetent C.B-17 mice offers a measure of protection against subsequent intravenous challenge. C.B-17 SCID mice given lymphocytes from immune congenic donors have significantly (6- to 10-fold) fewer yeast cells in their brains 7 to 10 days after infection and survive 2 to 3 times longer than controls given no lymphocytes (1, 13).

To test whether T cells from vaccinated B-cell-deficient mice can adoptively immunize SCID mice, we prepared suspensions of lymphocytes from spleens and lung-draining lymph nodes of vaccinated B-cell-deficient and B-cell-sufficient mice and transferred the cells into B6 SCID mice. Flow cytometric analyses revealed that the suspension prepared from B-cell-deficient mice contained 2.8×10^7 total cells and 4.2×10^6 $CD4^+$ cells. The suspension prepared from B-cell-sufficient mice contained 6.8×10^7 total cells and 5.0×10^6 $CD4^+$ cells. Thus, the numbers of $CD4^+$ T cells were similar in the two suspensions, and the difference in total numbers was almost entirely due to Ig^+ cells, as determined by flow cytometry. Suspensions were also plated on Sabdex agar to determine the numbers of yeast

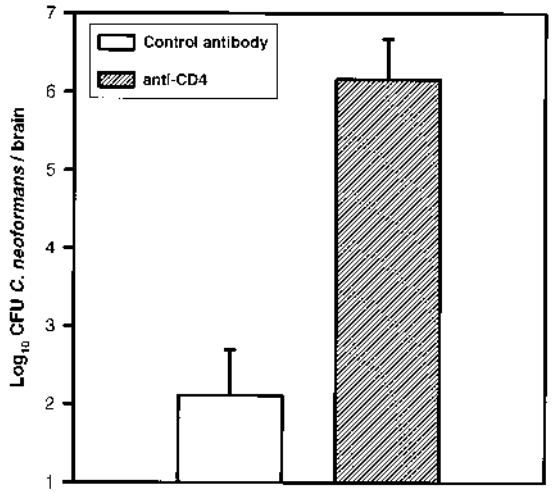

FIG. 2. Effect of depletion of $CD4^+$ T cells on *C. neoformans* brain infection in B-cell-deficient mice. Twelve weeks after intratracheal infection with 10^6 cells of strain 184, groups of B-cell-deficient male mice received a single intraperitoneal injection (1 mg) of anti-CD4 MAb or an isotype-matched control antibody and were killed 9 days later. The difference in brain yeast burdens between the two treatment groups is significant ($P < 0.05$). Data shown are the means plus standard deviations for four mice per group.

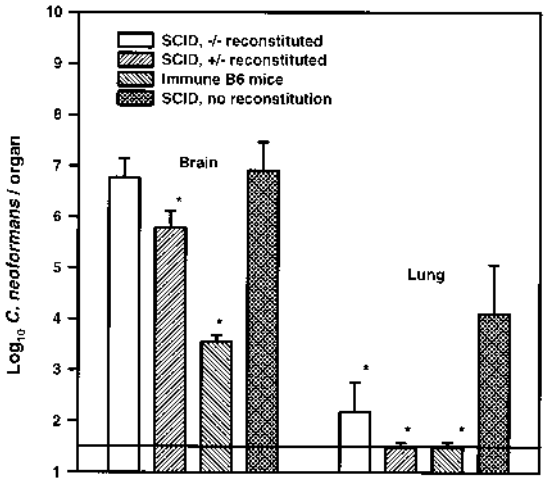

FIG. 3. SCID mice given lymphocytes from vaccinated B-cell-deficient mice express adoptive immunity in their lungs but fail to express adoptive immunity to *C. neoformans* brain infection. Male B6 SCID mice received intravenous infusions of splenocytes and lymphocytes from lung-draining lymph nodes of B-cell-deficient or B-cell-sufficient mice that had been vaccinated 8 weeks earlier. Eighteen hours after transfer, the mice, along with uninfused SCID controls and vaccinated B-cell-sufficient controls, were intravenously infected with 2×10^4 cells of strain 184. Mice were killed 10 days after infection. Data are the means plus standard deviations of five mice per group. *, significantly different from uninfused control mice ($P < 0.05$). The horizontal line represents the limit of detection of yeast cells for these experiments.

cells contained in the lymphocyte suspensions. It was calculated that 246 yeast cells were delivered to each mouse receiving a B-cell-deficient suspension and 80 yeast cells were delivered to those receiving B-cell-sufficient suspensions. Thus, the numbers of yeast cells in the lymphocyte suspensions were negligible compared to the 2×10^4 organisms delivered 18 h later. Eleven days after challenge, SCID mice given lymphocytes from the vaccinated B-cell-deficient donors appeared sick, evincing impaired movement, hydrocephaly, and piloerection. In contrast, the SCID mice given lymphocytes from vaccinated B-cell-sufficient mice appeared relatively healthy. At this point, mice were killed and the yeast cells in brains and lungs were enumerated (Fig. 3). Yeast burdens in the brains of mice given lymphocyte suspensions from B-cell-deficient mice were equivalent to those in the brains of uninfused SCID controls. In contrast, mice given lymphocyte suspensions from B-cell-sufficient donors had approximately 10-fold fewer yeast cells in their brains than the control mice. These data suggest that B cells can have a protective effect in systemic cryptococcal infection. However, mice given suspensions from B-cell-deficient mice had fewer yeast cells in their lungs than did uninfused SCID controls, showing that the T lymphocytes from the B-cell-deficient mice were efficacious against the typically less rapid yeast proliferation in the lungs.

Kinetics of lymphocyte reconstitution of *C. neoformans*-infected SCID mice. In a pilot experiment performed previous to the experiment represented in Fig. 3, splenocyte suspensions from B-cell-deficient mice failed to protect SCID recipients, whereas suspensions from B-cell-sufficient mice were protective (data not shown). In that pilot experiment, splenocytes of recipient mice were analyzed by flow cytometry at the time mice were killed, and a trend toward higher numbers of $CD4^+$ T cells was observed in SCID mice given lymphocytes from B-cell-sufficient immune donors. We hypothesized that B cells might be somehow involved in sustaining an ongoing lymphocyte-mediated response to *C. neoformans* in these mice. To test this idea, lymphocytes from the reconstituted mice were analyzed during the course of the infection whose outcome is shown in Fig. 3. Peripheral blood was sampled, and lymphocytes were analyzed for expression of CD4, CD8, and Ig by flow cytometry 1, 4, 7, and 11 days after infection, along with splenocyte suspensions on day 11 (Fig. 4). Lymphocytes were not uniformly detected in the peripheral blood of SCID mice reconstituted by suspensions from either B-cell-deficient or B-cell-sufficient mice until day 7 of infection. At day 7, there were approximately twofold more $CD4^+$ T cells in the peripheral blood of mice given lymphocytes from B-cell-sufficient mice than in that of mice given lymphocytes from the B-cell-deficient mice, despite the near equivalence of $CD4^+$ T-cell numbers in the reconstituting suspension (see above). Similarly, by day 11, there were threefold more $CD4^+$ T cells in the peripheral blood of mice given lymphocytes from B-cell-sufficient mice. There was no significant difference in the numbers of $CD8^+$ T cells in mice given lymphocytes from B-cell-deficient versus B-cell-sufficient donors at any time when PBL or splenocytes were sampled. An analysis of splenocytes from mice killed at day 11 showed approximately twofold more $CD4^+$ T cells in samples from SCID mice reconstituted by lymphocytes from B-cell-sufficient mice than in samples from SCID mice reconstituted by lymphocytes from B-cell-deficient mice. Thus, B cells may be involved directly or indirectly in stimulating the proliferation of $CD4^+$ T cells and sustaining their response to cryptococcal infection.

B-cell-deficient mice do not show superior innate resistance to *C. neoformans* in the lungs. An alternative explanation for the disparate results from intact vaccinated B-cell-deficient mice and SCID mice reconstituted with lymphocytes from vaccinated animals could be that μMt −/− mice develop other mechanisms which compensate for their B-cell deficiency. To determine the resistance capabilities of B-cell-deficient and B-cell-sufficient littermates in the very early stages of systemic infection, before specific T-cell-mediated immunity is functioning in this system (13), groups of four mice were inoculated with 2×10^4 strain 184 yeast cells intravenously and killed 1 or 4 days later. One day after infection, the $\log_{10}$ CFU of yeast in the lungs or in the brains of B-cell-deficient mice and B-cell-sufficient mice were not significantly different (1.77 ± 0.40 in −/− mouse lung versus 2.36 ± 0.27 in +/− mouse lung; 2.05 ± 0.20 in −/− mouse brain versus 2.32 ± 0.28 in +/− mouse brain). Neither were significant differences in $\log_{10}$ CFU per organ observed at 4 days (2.18 ± 0.34 in −/− mouse lung versus 2.65 ± 0.32 in +/− mouse lung; 5.07 ± 0.33 in −/− mouse brain versus 5.05 ± 0.52 in +/− mouse brain). These results argue that the innate resistance mechanisms B-cell-deficient mice deploy against *C. neoformans* are not superior to those of B-cell-sufficient mice.

DISCUSSION

We have shown that B-cell-deficient mice are capable of mounting a long-lived immune response that protects them against a challenge infection that is lethal for nonimmune mice. However, SCID recipients of T lymphocytes from immune B-cell-deficient mice fail to express the adoptive immunity seen in SCID recipients of T lymphocytes from immune B-cell-sufficient mice.

One explanation for the apparently paradoxical results posed by the adoptive immunization experiments could be that μMt −/− mice resist yeast infections by mechanisms distinct from those of B-cell-sufficient mice. For example, B-cell-deficient mice have more peripheral blood natural killer cells than uninfected B-cell-sufficient littermates (29a), which could con-

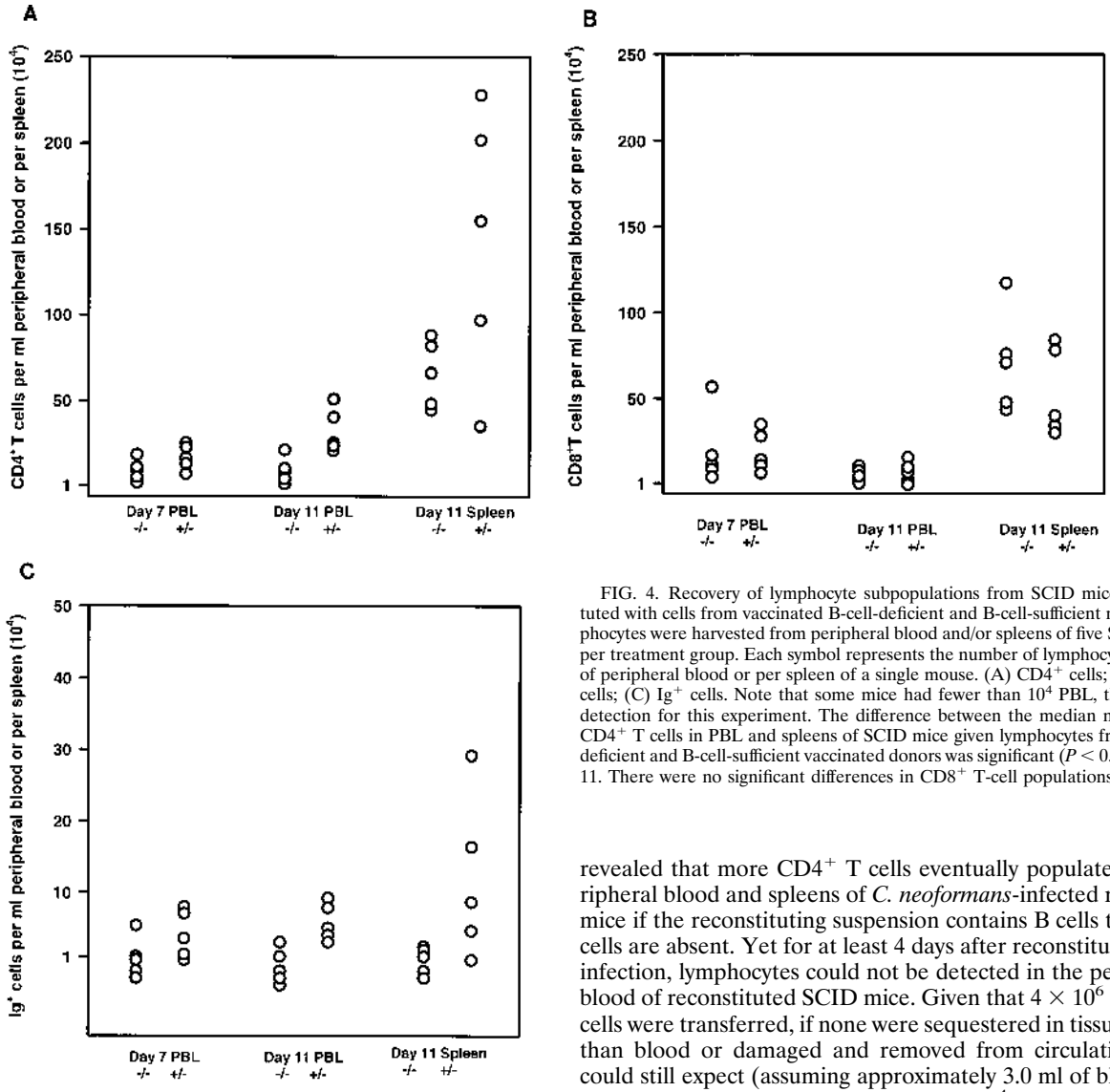

FIG. 4. Recovery of lymphocyte subpopulations from SCID mice reconstituted with cells from vaccinated B-cell-deficient and B-cell-sufficient mice. Lymphocytes were harvested from peripheral blood and/or spleens of five SCID mice per treatment group. Each symbol represents the number of lymphocytes per ml of peripheral blood or per spleen of a single mouse. (A) $CD4^+$ cells; (B) $CD8^+$ cells; (C) Ig^+ cells. Note that some mice had fewer than 10^4 PBL, the limit of detection for this experiment. The difference between the median numbers of $CD4^+$ T cells in PBL and spleens of SCID mice given lymphocytes from B-cell-deficient and B-cell-sufficient vaccinated donors was significant ($P < 0.05$) on day 11. There were no significant differences in $CD8^+$ T-cell populations.

ceivably be less efficiently transferred to SCID recipients. Although antigen-specific T-cell expansion in the spleens of B-cell-deficient mice has been reported to be impaired (32), T-cell priming and memory functions are apparently normal in μMt −/− mice (4, 8), as is allograft rejection (15a). These observations, together with the dependence of vaccinated B-cell-deficient mice on $CD4^+$ T cells for resistance to *C. neoformans* and their apparent lack of functionally enhanced innate immunity, argue that the mechanisms of resistance used by these mice are the same as those of B-cell-sufficient mice. Therefore a differentially efficient transfer to SCID mice of different resistance mechanisms possessed by B-cell-sufficient and B-cell-deficient mice seems unlikely to be a cause of the differing requirement for B cells in donor μMt mice and in SCID recipients of their cells.

An alternative explanation for the disparity of immune capability between vaccinated B-cell-deficient mice and SCID mice infused with lymphocytes from immune B-cell-deficient mice can be found in the examination of lymphocyte subsets in the recipients of immune cells. Analysis of lymphocyte populations in the reconstituted mice during the course of infection revealed that more $CD4^+$ T cells eventually populate the peripheral blood and spleens of *C. neoformans*-infected recipient mice if the reconstituting suspension contains B cells than if B cells are absent. Yet for at least 4 days after reconstitution and infection, lymphocytes could not be detected in the peripheral blood of reconstituted SCID mice. Given that 4×10^6 $CD4^+$ T cells were transferred, if none were sequestered in tissues other than blood or damaged and removed from circulation, one could still expect (assuming approximately 3.0 ml of blood per mouse) to recover approximately 4×10^4 cells in the small (0.03-ml) volume analyzed. It is more likely that only a fraction of the infused cells successfully engrafted, as has been reported previously (11, 27, 30). Also, some lymphocytes may have been recruited to foci of infection in the mice and so were removed from the circulation. The fact that lymphocytes were detected at days 7 and 11 suggests that some lymphocyte proliferation occurred after day 4 in both groups of reconstituted SCID mice. The two- to threefold higher numbers of $CD4^+$ T cells in mice that received suspensions containing B cells, while modest, suggests a B-cell involvement in stimulation of T-cell proliferation under these conditions. The lack of B cells may be especially important in the reconstituted SCID mouse, which has only a limited number of transferred T cells available to it.

Investigators working with B-cell-deficient JHD mice have shown that the mice are severely deficient in $CD4^+$ T-cell clonal expansion (18). A possible mechanism of B-cell involvement in T-cell proliferation is through the engagement of CD40L (expressed on activated T cells) by CD40, which is expressed on B cells, dendritic cells, and monocytes. It has been reported that this interaction results in the proliferation of T cells (3), and especially of $CD4^+$ T cells (6). Alternatively, B cells might affect T-cell proliferation less directly, by secret-

ing antibodies that opsonize the yeast, increasing yeast phagocytosis by macrophages (33) and ultimately potentiating antigen presentation to T cells.

Limited attempts to quantitate antibodies in immune B-cell-sufficient mice were inconclusive in this study, possibly because anticryptococcal antibody titers were low in these mice. Further experiments are planned to investigate how B cells and/or antibodies are involved in adoptive immunity to *C. neoformans* in SCID mice.

Finally, it is possible that T-cell-mediated immunity to *C. neoformans* is so effective in vaccinated mice that it is difficult to detect the B-cell contribution to resistance which, although present, is not ordinarily needed. It should be noted that the adoptive immunity to *C. neoformans* seen when lymphocytes from immunized congenic mice are given to SCID mice is not as effective as the immunity of the vaccinated congenic mice themselves. Immune C.B-17 mice (and immune B6 mice) have 10- to 1,000-fold fewer yeast cells in their brains at day 10 of infection than naive controls and survive the challenge infection indefinitely, eventually sterilizing their brains in most cases. Reconstituted C.B-17 *scid*/*scid* mice and B6 *scid*/*scid* mice, however, typically have 5- to 10-fold fewer yeast cells than do control SCID mice in their brains at day 10 of infection, and reconstituted C.B-17 *scid*/*scid* mice survive only 2 to 4 times as long as the controls. (Survival data are not yet available for B6 *scid*/*scid* mice.) Thus, in this situation of limited T-cell-mediated immunity, a B-cell component to resistance may become apparent.

We conclude that B cells can play an important role in host defense against *Cryptococcus* infection under certain conditions. This confirms the results of investigators working with antibody-based therapies for cryptococcosis (5, 23) and should encourage the investigation of this therapeutic approach. Also, our results underscore the need to exercise great care in interpreting negative findings from genetically engineered knockout mice.

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REFERENCES

1. **Aguirre, K., E. A. Havell, G. W. Gibson, and L. L. Johnson.** 1995. Role of tumor necrosis factor and gamma interferon in acquired resistance to *Cryptococcus neoformans* in the central nervous system of mice. Infect. Immun. **63:**1725–1731.
2. **Aguirre, K. M., P. C. Sayles, G. W. Gibson, and L. L. Johnson.** 1996. Resistance to *Cryptococcus neoformans* is associated with an inflammatory response to *Toxoplasma gondii* in the central nervous system of mice. Infect. Immun. **64:**77–82.
3. **Armitage, R. J., T. W. Tough, B. M. Macduff, W. C. Fanslow, M. K. Spriggs, F. Ramdell, and M. R. Alderson.** 1993. CD40 ligand is a T cell growth factor. Eur. J. Immunol. **23:**2326–2331.
4. **Asano, M. S., and R. Ahmed.** 1996. CD8 T cell memory in B cell-deficient mice. J. Exp. Med. **183:**2165–2174.
5. **Casadevall, A.** 1995. Antibody immunity and invasive fungal infections. Infect. Immun. **63:**4211–4218.
6. **Cayabyab, M., J. H. Phillips, and L. L. Lanier.** 1994. CD40 preferentially costimulates activation of $CD4^+$ T lymphocytes. J. Immunol. **152:**1523–1531.
7. **Dromer, F., J. Charreire, A. Contrepois, C. Carbon, and P. Yeni.** 1987. Protection of mice against experimental cryptococcosis by anti-*Cryptococcus neoformans* monoclonal antibody. Infect. Immun. **55:**749–752.
8. **Epstein, M. M., F. DiRosa, D. Jankovic, A. Sher, and P. Matzinger.** 1995. Successful T cell priming in B cell-deficient mice. J. Exp. Med. **182:**915–922.
9. **Gadebusch, H. H.** 1981. Passive immunization against *Cryptococcus neoformans*. Proc. Soc. Exp. Biol. **98:**611–614.
10. **Graybill, J. R., M. Hague, and D. J. Drutz.** 1981. Passive immunization in murine cryptococcosis. Sabouraudia **19:**237–244.
11. **Hilbert, D. M., A. O. Anderson, K. L. Holmes, and S. Rudikoff.** 1994. Long-term lymphoid reconstitution of SCID mice suggests self-renewing B and T cell populations in peripheral and mucosal tissue. Transplantation **58:**466–475.
12. **Hill, J. O., and A. G. Harmsen.** 1991. Intrapulmonary growth and dissemination of an avirulent strain of *Cryptococcus neoformans* in mice depleted of $CD4^+$ or $CD8^+$ T cells. J. Exp. Med. **173:**755–758.
13. **Hill, J. O., and K. M. Aguirre.** 1994. $CD4^+$ T cell-dependent acquired state of immunity that protects the brain against *Cryptococcus neoformans*. J. Immunol. **152:**2344–2350.
14. **Huffnagle, G. B., J. L. Yates, and M. Lipscomb.** 1991. Immunity to a pulmonary *Cryptococcus neoformans* infection requires both $CD4^+$ and $CD8^+$ T cells. J. Exp. Med. **173:**793–798.
15. **Iseki, M., M. Anzo, N. Yamashita, and N. Matsuo.** 1994. Hyper-IgM immunodeficiency with disseminated cryptococcosis. Acta Paediatr. **83:**780–782.

15a. **Johnson, L. L.** Unpublished data.

16. **Kitamura, D., J. Roes, R. Kuhn, and K. Rajewsky.** 1991. A B cell-deficient mouse by targeted disruption of a membrane exon of the immunoglobulin μ chain gene. Nature **350:**423–426.
17. **Kozel, T. R.** 1993. Cryptococcosis, p. 277–302. *In* J. W. Murphy (ed.), Fungal infection and immune responses. Plenum Press, New York.
18. **Liu, Y., Y. Wu, L. Ramarathinam, Y. Guo, D. Huszar, M. Trounstein, and M. Zhzo.** 1995. Gene-targeted B-deficient mice reveal a critical role for B cells in the CD4 T cell response. Int. Immunol. **7:**1353–1362.
19. **Louria, D. B., and T. Kaminski.** 1965. Passively-acquired immunity to experimental cryptococcosis. Sabouraudia **4:**80–84.
20. **Mody, C. H., G.-H. Chen, C. Jackson, J. L. Curtis, and G. B. Toews.** 1993. Depletion of $CD8^+$ T cells *in vivo* decreases pulmonary clearance of a moderately virulent strain of *Cryptococcus neoformans*. J. Lab. Clin. Med. **121:**765–769.
21. **Monga, D. P., R. Kumar, L. N. Mohapatra, and A. N. Malaviya.** 1979. Experimental cryptococcosis in normal and B-cell-deficient mice. Infect. Immun. **26:**1–3.
22. **Mukherjee, J., A. Casadevall, and M. D. Scharff.** 1993. Molecular characterization of the antibody responses to *Cryptococcus neoformans* infection and glucuronoxylomannan-tetanus toxoid conjugate immunization. J. Exp. Med. **177:**1105–1106.
23. **Mukherjee, J., L. Zuckier, M. D. Scharff, and A. Casadevall.** 1994. Therapeutic efficacy of monoclonal antibodies to *Cryptococcus neoformans* glucuronoxylomannan alone and in combination with amphotericin B. Antimicrob. Agents Chemother. **38:**580–587.
24. **Mukherjee, J., M. D. Scharff, and A. Casadevall.** 1992. Protective murine monoclonal antibodies to *Cryptococcus neoformans*. Infect. Immun. **60:**4534–4541.
25. **Mukherjee, J., L. Pirotski, M. D. Scharff, and A. Casadevall.** 1993. Antibody-mediated protection in mice with lethal intracerebral *Cryptococcus neoformans* infection. Proc. Natl. Acad. Sci. USA **90:**3636–3640.
26. **Murphy, J., and G. C. Cozad.** 1972. Immunological unresponsiveness induced by cryptococcal polysaccharide assayed by the hemolytic plaque technique. Infect. Immun. **5:**896–901.
27. **Reimann, J., A. Rudolphi, and M. H. Claesson.** 1991. Selective reconstitution of T lymphocyte subsets in SCID mice. Immunol. Rev. **124:**75–95.
28. **Sanford, J. E., D. M. Lupan, A. M. Schlageter, and T. R. Kozel.** 1990. Passive immunization against *Cryptococcus neoformans* with an isotype-switch family of monoclonal antibodies reactive with cryptococcal polysaccharide. Infect. Immun. **58:**1919–1923.
29. **Sarosi, G. A., J. D. Parker, K. Doto, and F. E. Tosh.** 1992. Amphotericin B in cryptococcal meningitis. Ann. Intern. Med. **71:**1079–1087.

29a. **Sayles, P. C.** Unpublished data.

30. **Sprent, J., M. Schaefer, M. Hurd, C. D. Sarh, and Y. Ron.** 1991. Mature murine B and T cells transferred to SCID mice can survive indefinitely and maintain a virgin phenotype. J. Exp. Med. **174:**717–728.
31. **Varma, A., J. C. Edman, and K. J. Kwon-Chung.** 1992. Molecular and genetic analysis of URA5 transformants of *Cryptococcus neoformans*. Infect. Immun. **60:**1101–1108.
32. **Vella, A. T., M. T. Scherer, S. Shultz, J. W. Kappler, and P. Marrack.** 1996. B cells are not essential for peripheral T-cell tolerance. Proc. Natl. Acad. Sci. USA **93:**951–955.
33. **Zhong, Z., and L.-A. Pirofski.** 1996. Opsonization of *Cryptococcus neoformans* by human anticryptococcal glucuronoxylomannan antibodies. Infect. Immun. **64:**3446–3450.

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