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# Anti-CD11b Monoclonal Antibody Suppresses Brain Dissemination of *Cryptococcus neoformans* in Mice

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***Abstract*: To elucidate the role of the β2 integrin family of adhesion molecules in the disseminated infection of *Cryptococcus neoformans* from the lung to the central nervous system, we examined the effects of monoclonal antibodies (mAbs) against CD11a, CD11b, CD11c and CD18 on the number of live microorganisms in both the lung and brain of mice three weeks after intratracheal infection. Administration of anti-CD11b mAb partially, but reproducibly, reduced the fungal loads in the brain in three independent experiments, while the lung loads were not affected. In addition, the same treatment significantly decreased the number of live microorganisms in the blood. In sharp contrast, the brain loads one week after intravenous injection with *C. neoformans* were not affected by treatment with anti-CD11b mAb. Finally, administration of mAb against other adhesion molecules (CD11a, CD11c or CD18) failed to affect the fungal loads in the brain as well as in the lung three weeks after intratracheal instillation, except for anti-CD18 mAb which rather increased the brain loads. Our results suggested that CD11b might be involved at least in part in the process of fungal dissemination from lung to brain, although the significance of other β2 integrin family adhesion molecules remains to be substantiated.**

***Key words*: *Cryptococcus neoformans*, Disseminated infection, Brain, CD11b**

*Cryptococcus neoformans*, an opportunistic fungal pathogen, causes a life-threatening meningoencephalitis in severely immunocompromised patients such as those with acquired immunodeficiency syndrome (AIDS). The host defense against this pathogen is mediated by cellular immunity (19), and cytokines secreted from type-1 helper T (Th1) cells play a central role in the establishment of protective immunity (3, 8–11, 13). These mechanisms often succeed in localizing the infection within the lung by preventing the pathogenic organism from disseminating to the central nervous system (CNS) and disseminated infection is accelerated in the presence of defective IL-12 synthesis (13). However, the precise mechanism that allows this fungal pathogen to spread the infection from the lung to the brain remains to be elucidated.

A variety of cell surface molecules are adopted by pathogenic microorganisms for their entry into host cells. Mac-1 (CD11b/CD18), a member of β2 integrin adhesion molecules, is identified as a primary receptor for several fungal pathogens including *Candida albicans* (6), *Histoplasma capsulatum* (1), *Blastomyces dermatitidis* (21) and also *C. neoformans* (2, 17, 18). Dong and Murphy (4) showed the direct binding of cryptococcal capsular polysaccharides to CD18 on human neutrophils. In the present study, to determine the mechanism of dissemination of *C. neoformans* from the primary infected organ to the CNS, we investigated, using a murine model of pulmonary cryptococcosis, the

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*Abbreviations*: CNS, central nervous system; CR, complement receptor; mAb, monoclonal antibody; PDA, potato dextrose agar; Th1 cells, type-1 helper T cells.

involvement of β2 integrins such as LFA-1, Mac-1 and complement receptor type 4 (CR4) in this process by examining the effects of the administration of specific monoclonal antibodies (mAbs) against α and β chains of these molecules.

## Materials and Methods

*Animals.* Female (BALB/c×DBA/2) F1 mice were purchased from SLC Japan (Hamamatsu, Japan) and used in the present experiments at the age of 7 to 10 weeks. All mice were housed in a pathogen-free environment and received sterilized food and water in the Laboratory Animal Center for Biological Science, University of the Ryukyus. All experimental protocols described in this study were approved by the Ethics Review Committee for Animal Experimentation of our university.

Cryptococcus neoformans. A serotype A-encapsulated strain of *C. neoformans*, designated as YC-11, was established from a patient with pulmonary cryptococcosis. The yeast cells were cultured on potato dextrose agar (PDA) plates for 2 to 3 days before use. This strain rapidly multiplies in the lung and hematogenously disseminates to the brain two or three weeks after intratracheal instillation, and resulted in the death of infected mice within six weeks (9).

*Infection of microorganisms.* Mice were anesthetized by intraperitoneal injection of 70 mg/kg of pentobarbital (Abbott Lab., North Chicago, Ill., U.S.A.) and restrained on a small board. In each mouse, a 50-μl suspension of normal saline containing live *C. neoformans* ($1\times10^5$ yeast cells) was inoculated by inserting a 25-gauge blunt needle into and parallel to the trachea. In some experiments, the same number of microorganisms was intravenously inoculated at 100 μl per mouse under ether anesthesia.

*Enumeration of viable* C. neoformans. Mice were sacrificed three weeks after intratracheal infection or one week after intravenous infection, and lungs and brains were homogenized separately in 10 ml of distilled water by teasing with a stainless mesh. The homogenates, appropriately diluted with distilled water, were inoculated at 100 μl on PDA plates, cultured for 2 to 3 days followed by counting the number of colonies. In some experiments, the fungal loads in heparinized peripheral blood were also examined.

*Antibodies.* Anti-CD11a, -CD11b, -CD11c and -CD18 mAbs were purified by a protein G column kit (Kirkegaard & Perry Lab., Gaithersburg, Md., U.S.A.) from serum-free culture supernatants of hybridomas [clones M17/5.2 (rat IgG), M1/70.15.11.5.HL (rat IgG), N418 (hamster IgG) and 2E6 (hamster IgG), respectively, all purchased from ATCC]. Mice were injected intraperitoneally with 200 μg each of mAb at days −3, 0, +3, +7 and +14 after infection with *C. neoformans*. Rat IgG (ICN Pharmaceuticals, Inc., Aurora, Oh., U.S.A.) or hamster IgG (Organon Teknika Co., Durham, N.C., U.S.A.) was used as the control Ab.

*Preparation of pulmonary leukocytes*. Pulmonary intraparenchymal leukocytes were harvested as described previously by our laboratory (7). The cells spun-down onto the glass slides were stained with periodic acid-Schiff (PAS) using a standard staining procedure, and examined under a light microscope.

*Immunohistochemical examination*. Frozen sections of lung were prepared from mice sacrificed 14 days after intratracheal infection with *C. neoformans*. A standard peroxidase/antiperoxidase technique was performed on these sections using anti-CD11b mAb, and the antigen-antibody complexes were visualized with diaminobenzidine. Histopathological examination was carried out to determine positive cells on the sections.

*Statistical analysis*. Differences between groups were examined for statistical significance using ANOVA test with a post-hoc analysis (Fisher PLSD test). A $P$ value less than 0.05 was considered significant.

## Results and Discussion

In our murine model of pulmonary cryptococcosis, the fungal microbe disseminated from the lungs to the brain and caused meningoencephalitis two to three weeks after primary infection (9). To elucidate the mechanism of such dissemination, we examined the effects of mAbs against β2 integrin family adhesion molecules such as CD11a, CD11b, CD11c and their common β chain, CD18, on the brain loads of *C. neoformans* three weeks after intratracheal infection. As shown in Fig. 1A, in three independent experiments, anti-CD11b mAb partially, but consistently, reduced the number of live microorganisms in the brains, compared to mice treated with control rat IgG, although the extent of reduction was not profound. The same treatment did not affect the proportion of CD11b+ leukocytes, including macrophages and neutrophils, obtained from lungs in a flow cytometric analysis (data not shown). In contrast, administration of mAbs against other adhesion molecules did not change the infectious loads of *C. neoformans* in the brain and lung (Fig. 2). These results suggested that CD11b might be involved at least in part in the process of fungal dissemination to the brain, although the significance of other molecules remains to be substantiated.

It is possible that the attenuated brain dissemination by anti-CD11b mAb was secondary to a reduction in the fungal load in the lungs. However, this was not the

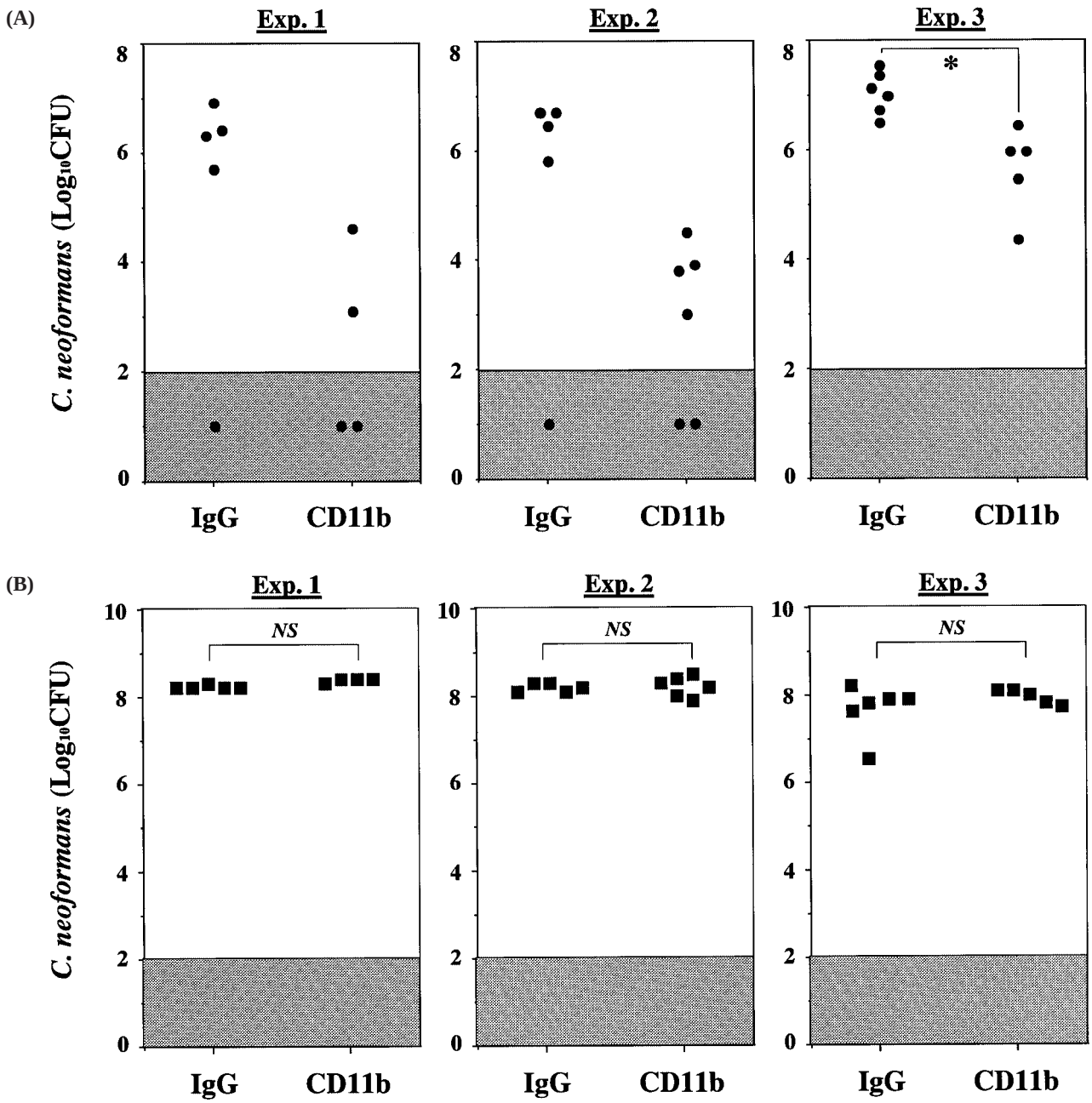


Fig. 1. Anti-CD11b mAb reduces *C. neoformans* counts in the brain, but not in the lung, of intratracheally infected mice. Mice received intraperitoneal injections of anti-CD11b mAb or control rat IgG (200 μg/mouse) at days −3, 0, 3, 7 and 14 after intratracheal infection with *C. neoformans* ($1\times10^5$/mouse). Three weeks later, mice were sacrificed and the number of live microorganisms in brain (A) and lung (B) were counted. Each symbol represents the result of a single mouse and the symbols within shaded area represent mice with zero counts. *, $P<0.05$; NS, not significant.

case, because administration of this mAb did not exert any significant effect on the number of live microorganisms in the lung (Fig. 1B). To further elucidate the mechanism of inhibition of brain dissemination, we examined the effect of anti-CD11b mAb on the fungal loads in the blood of mice infected intratracheally with *C. neoformans*. In addition, the effect of this treatment on the brain loads in mice infected through intravenous injection of the microorganism was examined. As shown in Fig. 3, administration of anti-CD11b mAb significantly reduced the number of live fungal microorganisms in the blood compared to that in mice treated with control rat IgG three weeks after intratracheal infection. In sharp contrast, infectious loads of *C. neoformans* in the brain were not significantly affected by administration of anti-CD11b mAb compared to mice treated with control

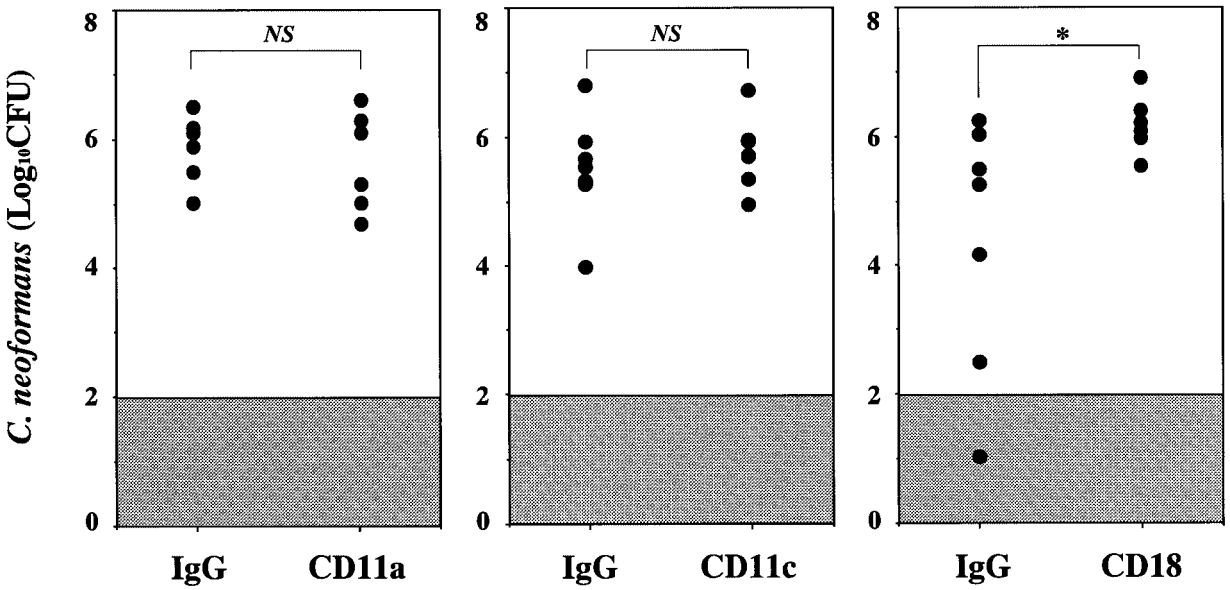


Fig. 2. Anti-CD11a, -CD11c and -CD18 mAb fail to reduce infectious loads of *C. neoformans* in the brain of intratracheally infected mice. Mice received intraperitoneal injections of anti-CD11a, -CD11c, -CD18 mAb or control rat or hamster IgG (200 μg/mouse) at days −3, 0, 3, 7 and 14 after intratracheal infection with *C. neoformans* ($1\times10^5$/mouse). Three weeks later, mice were sacrificed and the number of live microorganisms in brain was counted. Each symbol represents the result of a single mouse and the symbols within shaded area represent mice with zero counts. The experiments were repeated three times with similar results. *, $P<0.05$; NS, not significant.

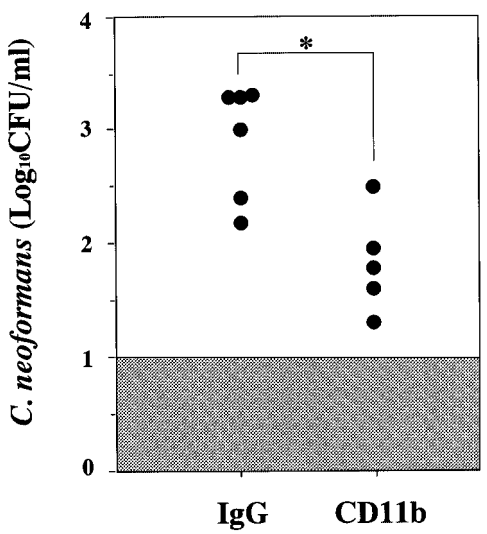


Fig. 3. Anti-CD11b mAb reduces infectious loads of *C. neoformans* in the circulation of intratracheally infected mice. Mice received intraperitoneal injections of anti-CD11b mAb or control rat IgG (200 μg/mouse) at days −3, 0, 3, 7 and 14 after intratracheal infection with *C. neoformans* ($1\times10^5$/mouse). Three weeks later, mice were sacrificed and the number of live microorganisms in blood was counted. Each symbol represents the result of a single mouse and the symbols within shaded area represent mice with zero counts. The experiments were repeated three times with similar results. *$P<0.05$.

rat IgG at day 7 after intravenous infection (Table 1). Considered collectively, these results clearly demonstrate that the α chain of Mac-1 molecule is involved in

Table 1. Anti-CD11b mAb fails to reduce infectious loads of *C. neoformans*[a] in the brain of intravenously infected mice

| Treatments | *C. neoformans* ($Log_{10}$ CFU/brain)[b] |
|---|---|
| PBS | 6.87±0.09 |
| Rat IgG | 6.86±0.08[c] |
| Anti-CD11b mAb | 6.77±0.19[c] |

[a] Mice received intraperitoneal injections of anti-CD11b mAb or control rat IgG (200 μg/mouse) at days −3, 0 and 3 after intravenous injection with *C. neoformans* ($1\times10^5$/mouse). One week later, mice were sacrificed and the number of live microorganisms in brain was counted.

[b] Mean±SD of seven mice.

[c] Not significantly different from PBS-treated mice.

the translocation of *C. neoformans* from the lung to blood, but not from blood to brain.

In preliminary experiments, we determined the number of live *C. neoformans* in the lung, blood and brain of mice instilled intratracheally with the microorganism at various doses in order to elucidate the relationship in fungal loads among these organs. The results indicated that the number of live microorganisms in the lung did not correlate with that in the brain nor in the blood, three weeks after infection [$r=0.24$, $P>0.1$ ($n=23$) and $r=0.21$, $P>0.1$ ($n=17$), respectively] (data not shown). In some mice, a large number of fungal microorganisms were detected in the brain although only a few were found in the lungs. In other mice bearing large numbers of microorganisms in their lungs,

very few or none disseminated to the brain. In sharp contrast, there was a significant relationship in fungal loads between blood and brain [$r=0.75$, $P<0.001$ ($n=17$)]. These data suggest that the translocation of *C. neoformans* from the lungs to bloodstream cannot simply be explained by physical mechanisms, i.e., mechanical pressure on alveolar walls by overgrowing yeast cells. Rather, such translocation is probably controlled by active processes such as receptor-mediated binding. This conclusion is in agreement with the present findings indicating the involvement of Mac-1-mediated mechanism in fungal translocation from the lung to blood, but not from blood to brain.

*C. neoformans* activates the alternative complement pathway (14, 16), which results in the deposition of C3 fragments on the surface of the microorganism, mostly in the form of iC3b (15, 16). iC3b acts as the major opsonic component for phagocytosis of this microorganism by phagocytic leukocytes through binding to CR3 (Mac-1) (2, 17, 18). In the present study, the mAb that recognizes the α chain (CD11b) of CR3 significantly suppressed the dissemination of *C. neoformans* from the lung to the brain. Anti-CD11b mAb with the same clone name (M1/70) was reported to decrease phagocytosis of *Listeria monocytogenes* by macrophages to a level observed in the absence of active complement (5). These findings suggest that the opsonic binding of *C. neoformans* to phagocytes and their subsequent ingestion by these cells may be involved in the translocation of these microorganisms from the alveolar space to the peripheral circulation, that is, the phagocytes may function as carriers of such transmigration. Compatibly with the above hypothesis, *C. neoformans* strain used in the present study was active in complement activation, as indicated by the production of C3a fragment in human plasma, and alveolar macrophage was the only cell population positively stained by this mAb in our immunohistochemical analysis (data not shown).

To examine this possibility, we investigated the effect of anti-CD11b mAb treatment on the proportion of macrophages that engulfed fungal microorganisms in the lungs after intratracheal infection with *C. neoformans*. For this purpose, pulmonary intraparenchymal leukocytes were prepared from mice treated with anti-CD11b mAb or control rat IgG one and two weeks after infection. The harvested cells were stained with PAS and the numbers of macrophages engulfing and non-engulfing fungal microorganisms were counted under a light microscope. The results indicated that there was no significant difference in the proportion of macrophages carrying the fungal organisms to the total number macrophages between anti-CD11b mAb- and control IgG-treated groups [$2.1\pm0.2\%$ vs. $2.3\pm0.5\%$ ($n=3$ each) after the first week and $1.2\pm0.1\%$ vs. $0.9\pm0.4\%$ ($n=3$ each) after the second week]. Thus, in the present study, the suppressive effect of this mAb on the brain dissemination of *C. neoformans* was not ascribed to the hypothetical mechanism described above.

In an alternative explanation, anti-CD11b mAb treatment might cause some positive change in the cytokine production by macrophages, leading to the enhanced host defense against *C. neoformans* in the lung and reduced dissemination to the brain. However, this was not likely to be the case because the clearance of microorganisms from the lungs was not accelerated by this treatment. The local host defense in the lung might rather be attenuated, because IL-12 production by macrophages is strongly inhibited *in vitro* by treatment with anti-CD11b mAb (20, our unpublished results), which should hamper the development of Th1 response. Furthermore, in our mouse model of cryptococcal infection, Th1 response was strongly reduced before anti-CD11b mAb treatment (10), which may result from the attenuation of macrophage IL-12 synthesis by *C. neoformans* (12). Thus, further studies will be necessary to understand the precise mechanism of the preventive effect of anti-CD11b mAb on the disseminated infection to the brain.

In conclusion, we demonstrated in the present study the possible involvement of CD11b in the disseminated infection of *C. neoformans* from the lung to the brain. Our data should be useful for the development of new strategies aimed at limiting the infection of pathogenic fungus within the primary infected organ.

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