INFECTION AND IMMUNITY, Dec. 1997, p. 5354–5357 Vol. 65, No. 12
0019-9567/97/$04.00+0

A Monoclonal Antibody to *Candida albicans* Enhances Mouse Neutrophil Candidacidal Activity

T.-C. CAESAR-TONTHAT† AND J. E. CUTLER*

Department of Microbiology, Montana State University, Bozeman, Montana 59717-3520

Received 10 July 1997/Returned for modification 2 September 1997/Accepted 27 September 1997

A monoclonal antibody (MAb) to *Candida albicans* (MAb B6.1) that protects against candidiasis and the nonprotective MAb B6 were compared for ability to support neutrophil (polymorphonuclear leukocyte [PMN]) candidacidal activity. Both MAbs are immunoglobulin M, and each recognizes distinct *C. albicans* mannan cell wall determinants. PMN candidacidal activity was assessed by transmission electron microscopy and by an in vitro killing assay. The results indicated that MAb B6.1, but not MAb B6, enhances ingestion and killing of yeast cells by PMN in the presence of serum complement.

In previous studies, we described two monoclonal antibodies (MAbs), MAb B6.1 and MAb B6 (7, 8). Both of these antibodies are immunoglobulin M and both agglutinate *Candida albicans* cells, but MAb B6.1 enhances protection against disseminated candidiasis and MAb B6 does not protect. The MAbs are specific for epitopes within the *Candida* phosphomannan complex (PMC); MAb B6.1 is specific for an epitope in the acid-labile part of the PMC, and MAb B6 is specific for the acid-stable part (9). The acid-labile and acid-stable terminology is in accordance with the PMC model as proposed by Shibata et al. (19). We have begun to investigate the mechanism(s) that might explain why MAb B6.1 is protective but MAb B6 is not.

The well-established importance of neutrophils (polymorphonuclear leukocytes) (PMNs) in host defense against disseminated candidiasis (1, 4) stimulated us to investigate whether these MAbs affect the candidacidal activity of mouse PMNs. Although we have shown that MAb B6.1 protects neutropenic mice against disseminated candidiasis, it is important to point out that the neutropenic animals were about 10 times more susceptible to disease than normal mice (8). We hypothesized that under neutropenic conditions, macrophages may play a dominant role in host defense against *C. albicans*. We observed by use of transmission electron microscopy and an in vitro phagocyte candidacidal assay developed in our laboratory (3) that MAb B6.1 enhances uptake and killing of ingested yeast cells by mouse PMNs.

C. albicans A9 (serotype B), previously well described (7, 13), was used throughout the experiments. The fungus was grown as yeast forms to stationary phase in glucose (2%)–yeast extract (0.3%)–peptone (1%) broth for 24 h at 37°C as described before (7, 13). Yeast forms were harvested by centrifugation, washed three times in deionized water, and adjusted to a concentration of 10^5 cells per ml of RPMI 1640 medium (Sigma Chemical Co., St. Louis, Mo.) containing 25 mM HEPES buffer (pH 7.4) without antibiotics. Peritoneal-exudate cells were obtained from 5 week-old female BALB/cByJ mice (Jackson Laboratories, Bar Harbor, Maine) 3 h after an intraperitoneal administration of 2.5 ml of 0.5% glycogen (Sigma) in Dulbecco's phosphate-buffered saline (DPBS) (Sigma Chemical Co., St. Louis, Mo.) as described elsewhere (3). PMNs accounted for at least 75% of the peritoneal-exudate cell population.

MAbs B6 and B6.1 were produced commercially (Montana Immunotech Inc., Bozeman, Mont.) in serum-free medium (HB101; Irvine Scientific, Santa Ana, Calif.), concentrated by 50% ammonium sulfate precipitation, and suspended and diluted in DPBS. The final concentration of the two MAbs, as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Western blot analysis, and protein determination (7, 8), were 3.5 and 3.4 mg of MAb/ml for MAb B6.1 and MAb B6, respectively. At these concentrations, the agglutinin titers against *Candida* mannan-coated latex beads (7, 13) were 1,280 for MAb B6.1 and 640 for MAb B6. For these experiments, the MAbs were diluted in DPBS and tested at 1,700, 13.6, 3.4, and 0.9 μg/ml.

For electron microscopic analysis, *C. albicans* cells were exposed to a 1/500 dilution of either antibody in the presence of 10% fresh normal mouse serum (FMS) and the suspension was added to PMNs on polyethylene rectangles and incubated for either 30 or 60 min at 37°C. Each preparation was rapidly fixed by freeze substitution methods, as described by others (10, 12), but with minor modifications. Briefly, 30-μl volumes of mouse PMNs (10^7 cells per ml of RPMI-HEPES medium with 10% FMS) were placed on the surfaces of rectangles (5 by 7 mm) cut from polyethylene gloves (Fisher brand Poly Gloves; Fisher Scientific, Pittsburgh, Pa.) and incubated for 2 h at 37°C to allow the PMNs to adhere to the polyethylene. Fluid was carefully removed by wicking with Whatman no. 1 filter paper. Thirty-microliter volumes of a yeast cell suspension (10^6 cells per ml of RPMI-HEPES medium with 10% FMS) containing either MAb B6 or MAb B6.1 were added to the PMNs on the polyethylene and incubated for either 30 or 60 min at 37°C. For serum controls, heated (56°C, 30 min) FMS was used instead of untreated FMS. After the incubation, the cells on the polyethylene rectangles were immediately plunged into liquid propane at approximately −190°C. The rectangles were transferred to substitution fluid consisting of 2% osmium tetroxide and 0.05% uranyl acetate in anhydrous acetone (high-performance liquid chromatography grade; J. T. Baker Chemicals) for 72 h at −80°C and then incrementally brought to −20°C for 2 h, 4°C for 1 h, and room temperature. The specimens (i.e., the cells on the polyethylene rectangles) were washed three times in anhydrous acetone and embedded in Epon 812-Araldite 6005 epoxy resin between 2 microscope slides previ-

* Corresponding author. Mailing address: Department of Microbiology, Lewis Hall 109, Montana State University, Bozeman, MT 59717-3520. Phone: (406) 994-2373. Fax: (406) 994-4926. E-mail: umbjc@gemini.oscs.montana.edu.

† Present address: USDA Agricultural Research Service, Sidney, MT, 59270.

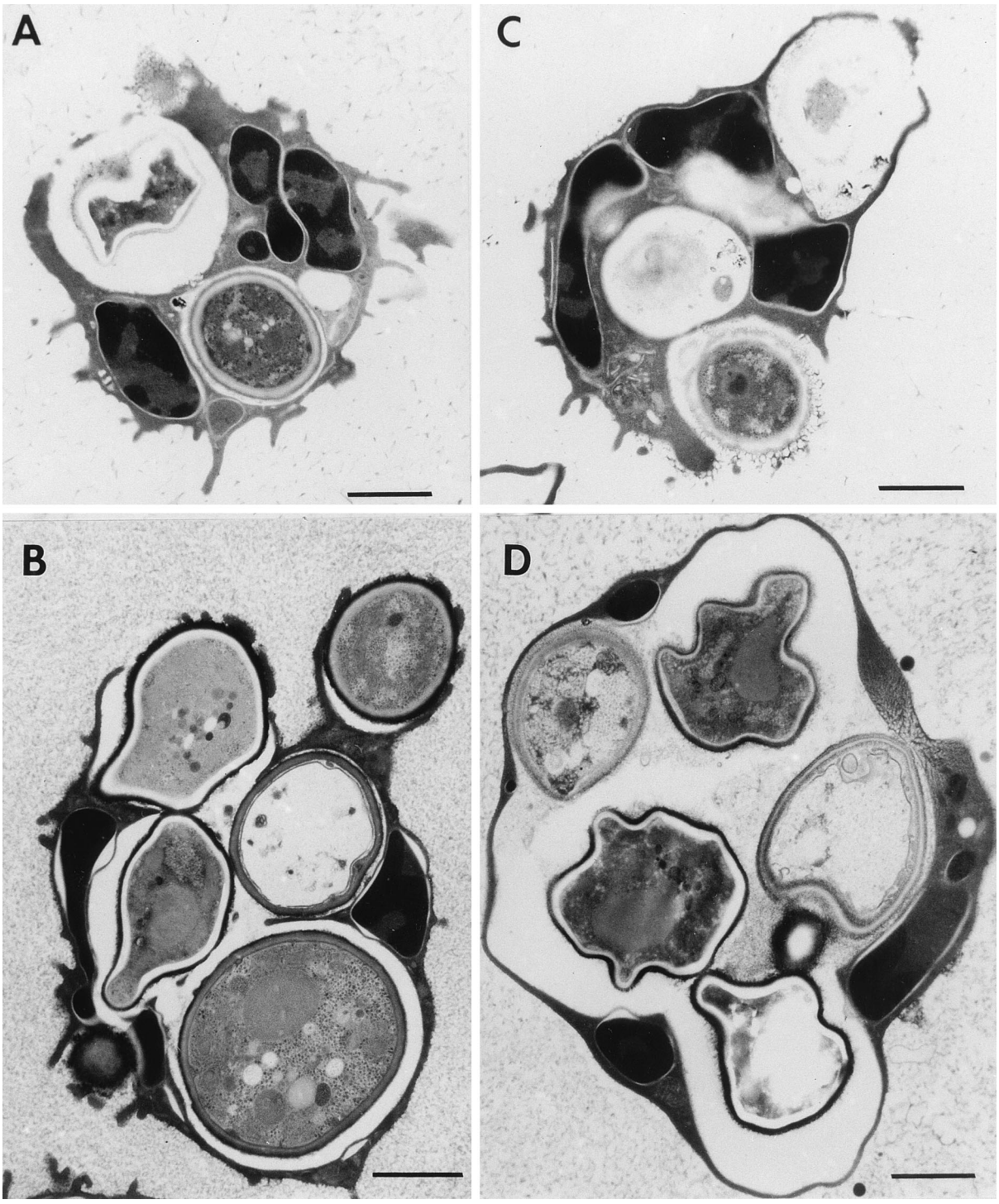

FIG. 1. Transmission electron microscopic evidence that MAb B6.1 enhances the candidacidal activity of mouse PMNs. Freeze-substituted PMNs containing phagocytosed *C. albicans* cells were pretreated with MAb B6 (A and B) or MAb B6.1 (C and D) in the presence of 10% FMS. Note that in the MAb B6-treated sample, one of the two ingested yeast cells in contact with the PMN for 30 min (A) appeared to be dead. At 60 min, five MAb B6-treated yeast cells were ingested by a PMN (B), but only one appeared to have been killed. For MAb B6.1-treated yeast cells phagocytosed by PMNs, at 30 min each PMN contained at least two yeast cells. Many of the captured yeast cells appeared dead (C). By 60 min of incubation, all of the MAb B6.1-treated yeast cells captured by the PMN showed evidence of damage (D). Bars, 2 μm.

ously coated with two layers of Teflon (Fluoroglide; Electron Microscopy Sciences, Fort Washington, Pa.). The thin layer of epoxy resin-embedded cells was removed from the slides, mounted with epoxy resin onto epoxy stubs, and sectioned longitudinally into ultrathin sections 60 to 80 nm thick. The sections were stained with 2% uranyl acetate for 15 min and lead citrate for 2 min and examined with a JEOL 100CX transmission microscope operated at 80 kV. Ultrastructural

features of phagocytosed yeast cells were examined. A combination of the following criteria was used to define dead yeast cells: (i) evidence of granular cytoplasm and electron-translucent areas containing numerous membranous structures and no cellular organelles, (ii) evidence of collapsed cell structures resulting in highly irregular cell borders, and (iii) evidence of a disrupted cell wall. In addition, the number of internalized yeast cells per PMN for each time period was counted and the means and standard deviations of the numbers of dead and live cells were determined.

An in vitro PMN candidacidal assay was adapted from our previous study (3). Two hundred microliters of the mouse PMN suspension was added to each of 96 flat-bottomed wells in microtiter plates (Corning, New York, N.Y.), and the plates were incubated for 2 h at 37°C to allow PMN adherence. While the plates were incubating for 2 h, *C. albicans* yeast forms were suspended in RPMI-HEPES medium (pH 7.4) in the presence of MAb B6.1 or MAb B6 and incubated for 30 min on ice. The yeasts were washed three times in RPMI-HEPES medium and suspended in RPMI-HEPES medium containing 10% untreated FMS or 10% heat-inactivated FMS. At the end of the 2-h incubation, fluid from the microtiter plate wells was discarded, 0.2 ml of the appropriate yeast cell suspension was added to each well to give a PMN/yeast cell ratio of 10:1, and the plate was incubated for 60 min at 37°C. After 60 min of incubation, the fluid was discarded, 0.2 ml of molten (43°C) cornmeal agar (Difco) was added to each well, and the plates were incubated for an additional 1 h at 37°C. Viability was expressed as the average number of germinating forms that developed in each well, as described previously (3). All tests were performed in triplicate, and each experiment was run on three separate occasions. The statistical significance of differences between the effects of MAb B6.1 and MAb B6 was determined by the Fisher exact test.

The ultrastructures of MAb B6- and MAb B6.1-treated yeast cells, which were phagocytosed after 30 and 60 min of contact with PMNs in the presence of 10% FMS, were compared (Fig. 1). Ultrastructural characteristics were observed on sections from 27 different PMNs that ingested yeast cells in the presence of MAb B6.1 and 26 different PMNs that ingested yeast cells in the presence of MAb B6. Figure 1 shows typical results from each. For yeast cells treated with MAb B6 (Fig. 1A and B), there was a mixture of apparently healthy and dead cells within PMNs. By comparison, cells treated with MAb B6.1 showed an increase in the number of ingested dead yeast cells (Fig. 1C and D). At 30 min of contact, each ingested yeast cell was found in an individual phagosome, whereas at 60 min of contact, most of the ingested cells (up to seven) were found within a single phagosome (Fig. 1B and D). The number of pseudopodia developed by PMNs also appeared to decrease after 60 min of contact with the yeast cells. When heat-inactivated FMS was used, no differences were observed between MAb B6.1-treated cells and MAb B6-treated cells with respect to both ultrastructural changes and number of yeast cells ingested (data not shown). These data suggested that MAb B6.1 promotes enhanced PMN candidacidal activity, but the enhanced activity appeared to be complement dependent.

To confirm that MAb B6.1 enhances PMN candidacidal activity, we tested the antibodies in an in vitro PMN candidacidal assay. When yeast cells were preopsonized with 10% FMS, the killing efficiency of PMNs was markedly enhanced at all concentrations of MAb B6.1 tested (Fig. 2). Regardless of the concentration used, MAb B6 did not significantly affect the killing activity of the PMNs. These results were reproduced in two additional independent experiments (data not shown). In the absence of antibodies, untreated FMS supported greater

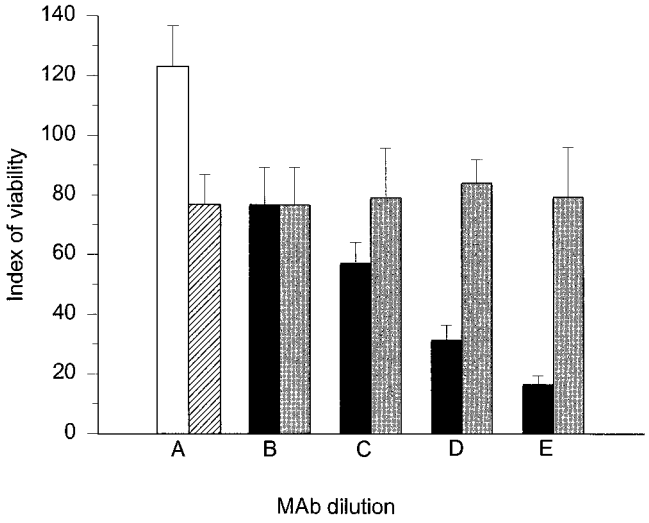

FIG. 2. MAb B6.1 enhances the candidacidal activity of mouse PMNs, as demonstrated by an in vitro assay. Mouse PMNs and yeast cells at a ratio of 10:1 were allowed to interact in the presence of MAb B6.1 or MAb B6 at various concentrations. The index of viability is the percentage of cells that formed germ tubes. Sets A to E, respectively, represent no addition of antibodies and 0.9, 3.4, 13.6, and 1,700 μg of antibodies/ml. White bar, heat-inactivated FMS was used; hatched bar, no antibodies were added and untreated FMS was used; black bars, MAb B6.1 was added; gray bars, MAb B6 was added. For all black and gray bars, untreated FMS was used. The results from one of three individual experiments are shown; variance bars show standard errors. Statistical probabilities of significance are as follows: $P < 0.0001$ for MAb B6.1 versus MAb B6 at 1,700 μg/ml; $P < 0.0291$ for MAb B6.1 versus MAb B6 at 13.6 μg/ml.

candidacidal activity by PMN than did heat-inactivated FMS (Fig. 2). As in the ultrastructural studies, in the presence of heated serum, neither MAb enhanced candidacidal activity beyond the baseline killing effected by fresh serum alone (data not shown). These data suggest that complement is essential for the ability of MAb B6.1 to enhance PMN candidacidal activity.

The apparent complement dependency of the MAb B6.1-enhanced PMN candidacidal activity may explain, at least in part, why MAb B6.1 is protective against disseminated candidiasis in normal mice. Whereas many investigators (6, 15–18) have documented alternative complement cascade activation by *C. albicans*, Kozel et al. (15) studied the kinetics of C3 deposition on the surface of the fungus. Several minutes are required for significant C3 deposition by the alternative complement cascade, but antibody-mediated classical complement activation occurs within 1 to 2 min. We propose that during initiation of fungemia, a protective antibody induces very rapid complement opsonization, which results in an association of yeasts with host phagocytes. Nonprotective antibodies may lead to slower complement activation kinetics. The fungal cell may then become associated with host sites that are more favorable for the fungus, such as extracellular matrix proteins or endothelial cells (2, 5, 11, 14). Related to this hypothesis, MAb B6.1 has been found to cause an almost 10-fold faster activation of complement on the *Candida* cell wall than MAb B6 (20).

We gratefully acknowledge Yongmoon Han for constructive criticism and review of the manuscript.

This work was supported by grants RO1 AI24912 and PO1 AI37194 from the National Institute of Allergy and Infectious Diseases.

REFERENCES

1. **Ashman, R. B., and J. M. Papadimitriou.** 1995. Production and function of cytokines in natural and acquired immunity to *Candida albicans* infection. Microbiol. Rev. **59:**646–672.
2. **Calderone, R. A.** 1995. Recognition of endothelial cells by *Candida albicans*: role of complement-binding proteins. Can. J. Bot. **73:**1154–1159.
3. **Cutler, J. E., and B. D. Thompson.** 1984. A simple and inexpensive method for assessing in vitro candidacidal activity of leukocytes. J. Immunol. Methods **66:**27–33.
4. **Domer, J. E., and R. I. Lehrer.** 1993. Introduction to Candida: systemic candidiasis, p. 49–116. *In* J. W. Murphy, H. Friedman, and M. Bendinelli (ed.), Infectious agents and pathogenesis: fungal infections and immune responses. Plenum Press, New York, N.Y.
5. **Filler, S. G., J. N. Swerdloff, C. Hobbs, and P. M. Luckett.** 1995. Penetration and damage of endothelial cells by *Candida albicans*. Infect. Immun. **63:**976–983.
6. **Gelfand, J. A., D. L. Hurley, A. S. Fauci, and M. M. Frank.** 1978. Role of complement in host defense against experimental disseminated candidiasis. J. Infect. Dis. **138:**9–16.
7. **Han, Y., and J. E. Cutler.** 1995. Antibody response that protects against disseminated candidiasis. Infect. Immun. **63:**2714–2719.
8. **Han, Y., and J. E. Cutler.** 1997. Assessment of a mouse model of neutropenia and the effect of an anti-candidiasis monoclonal antibody in these animals. J. Infect. Dis. **175:**1169–1175.
9. **Han, Y., T. Kanbe, R. Cherniak, and J. E. Cutler.** Biochemical characterization of *Candida albicans* epitopes that can elicit protective and nonprotective antibodies. Infect. Immun. **65:**4100–4107.
10. **Hoch, H.** 1986. Freeze-substitution of fungi, p. 183–212. *In* H. C. Aldrich and W. J. Todd (ed.), Ultrastructure techniques for microorganisms, Plenum Press, New York, N.Y.
11. **Hostetter, M. K.** 1994. Adhesins and ligands involved in the interaction of *Candida* spp. with epithelial and endothelial surfaces. Clin. Microbiol. Rev. **7:**29–42.
12. **Howard, R. J., and K. L. O'Donnell.** 1987. Freeze substitution of fungi for cytological analysis. Exp. Mycol. **11:**250–269.
13. **Kanbe, T., Y. Han, B. Redgrave, M. H. Riesselman, and J. E. Cutler.** 1993. Evidence that mannans of *Candida albicans* are responsible for adherence of yeast forms to spleen and lymph node tissue. Infect. Immun. **61:**2578–2584.
14. **Klotz, S. A.** 1994. Plasma and extracellular matrix proteins mediate in the fate of *Candida albicans* in the human host. Med. Hypotheses **42:**328–334.
15. **Kozel, T. R., L. Weinhold, and D. M. Lupan.** 1996. Distinct characteristics of initiation of the classical and alternative complement pathways by *Candida albicans*. Infect. Immun. **64:**3360–3368.
16. **Morelli, R., and L. T. Rosenberg.** 1971. The role of complement in the phagocytosis of *Candida albicans* by mouse peripheral blood leukocytes. J. Immunol. **107:**476–480.
17. **Morrison, R. P., and J. E. Cutler.** 1981. In vitro studies of the interaction of murine phagocytic cells with *Candida albicans*. J. Reticuloendothel. Soc. **29:**23–34.
18. **Ray, T. L., and K. D. Wuepper.** 1976. Activation of the alternative (properdin) pathway of complement by *Candida albicans* and related species. J. Invest. Dermatol. **67:**700–703.
19. **Shibata, N., K. Ikuta, T. Imai, Y. Satoh, R. Satoh, A. Suzuki, C. Kojima, H. Kobayashi, K. Hisamichi, and S. Suzuki.** 1995. Existence of branched side chains in the cell wall mannan of pathogenic yeast, *Candida albicans*. Structure-antigenicity relationship between the cell wall mannans of *Candida albicans* and *Candida parapsilosis*. J. Biol. Chem. **270:**1113–1122.
20. **Zhang, M. X., et al.** Submitted for publication.

Editor: T. R. Kozel