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Complement Is Essential for Protection by an IgM and an IgG3 Monoclonal Antibody Against Experimental, Hematogenously Disseminated Candidiasis

Yongmoon Han,* Thomas R. Kozel,† Mason X. Zhang,† Randall S. MacGill,† Michael C. Carroll,‡ and Jim E. Cutler2*

The incidence of life-threatening, hematogenously disseminated candidiasis, which is predominantly caused by Candida albicans, parallels the use of modern medical procedures that adversely affect the immune system. Limited antifungal drug choices and emergence of drug-resistant C. albicans strains indicate the need for novel prevention and therapeutic strategies. We are developing vaccines and Abs that enhance resistance against experimental candidiasis. However, the prevalence of serum anti-Candida Abs in candidiasis patients has led to the misconception that Abs are not protective. To explain the apparent discrepancy between such clinical observations and our work, we compared functional activities of C. albicans-specific protective and nonprotective mAbs. Both kinds of Abs are agglutinins that fix complement and are specific for cell surface mannans, but the protective Abs recognize β-mannan, while the nonprotective Ab is specific for α-mannan. By several indirect and direct measures, the protective mAbs more efficiently bind complement factor C3 to the yeast cell than do nonprotective Abs. We hypothesize that the C3 deposition causes preferential association of blood-borne fungi with host phagocytic cells that are capable of killing the fungus. We conclude from these results that the protective potential of Abs is dependent on epitope specificity, serum titer, and ability to rapidly and efficiently fix complement to the fungal surface. The mechanism of protection appears to be associated with enhanced phagocytosis and killing of the fungus. The Journal of Immunology, 2001, 167: 1550–1557.

Candidiasis is a disease of growing incidence that parallels the increasing number of immunocompromised people over the past few decades (1–6). The greater number of people susceptible to this disease, and especially to a form of the illness called hematogenously disseminated candidiasis, is often associated with application of modern medical advances (7–9). In addition to disseminated disease, estimates indicate that at least 50% of women by age 25 yr will have had one or more physician-diagnosed episodes of vulvovaginal candidiasis (10), and mucocutaneous infection at other body locations is occurring with increased frequency for many reasons that include the use of various prescription medications and the incidence of compromising infectious diseases (6, 11–13).

Hematogenously disseminated candidiasis and mucocutaneous forms of candidiasis are sometimes effectively prevented or treated by the use of antifungal drugs, of which fluconazole is the common drug of choice (12, 14, 15). However, even with treatment a large percentage of patients who develop disseminated disease will die (16–18). Although patients with uncomplicated mucocutaneous disease generally respond favorably to drug therapy, chronic and recurrent diseases are problematic. Active and passive immunization measures against candidiasis are not available, but clearly this approach merits consideration.

Over the past several years we (19–24) and others (25–32) have been investigating specific Ab induction as an immunotherapeutic preventive measure against the development of candidiasis. We have developed a vaccine for active immunization and have discovered protective mAbs; both approaches have shown efficacious promise against experimental, hematogenously disseminated candidiasis and against vaginal infection due to C. albicans. The active vaccine consists of a phosphomannanprotein complex isolated from the cell wall of C. albicans, which can be delivered in association with conventional liposomes (20) or conjugated to a protein carrier and given along with an adjuvant (22). Whole serum or Ig fractions from immunized animals can transfer the protection to naïve mice, and the protective Abs are removed by absorption of the antiserum with whole yeast cells (20). mAbs of IgM (mAb B6.1) and IgG3 (mAb C3.1) isotypes are protective against disseminated and vaginal forms of the disease (19, 20, 23). Both these Abs are specific for β-1,2-mannotriose (33), which is a component of the acid-labile portion of the phosphomannan complex on the cell surface of C. albicans. Another IgM mAb (mAb B6) is not protective against disseminated disease and is only slightly effective against vaginal infection (19). mAb B6 is specific for the phosphomannan complex, but the epitope recognition is for mann in the acid-stable part of the complex (33). The acid-labile and acid-stable designations conform to the N-linked phosphomannan complex structure as proposed by Suzuki and coworkers (34).

The idea that Abs can be protective against candidiasis is not without contention. The presence of agglutinins specific for C. albicans in the sera of patients with this disease has led to the general assumption that Candida-specific Abs are not protective (35). Our work with protective and nonprotective mAbs suggests
that epitope specificity is an important consideration as to whether a given Ab will have protective potential. The antigenic complexity of *C. albicans* would probably lead to heterogeneous Ab responses that should be expected to vary among individuals naturally exposed to the fungus. Mere detection of Candida-specific polyclonal Abs in the serum of a patient, then, would tell us very little about the ability of that individual to resist the development of candidiasis. Predictions on resistance would, instead, depend on knowledge about titers of particular Ab classes against specific candidal epitopes.

Our studies show that an Ab-based protective response can be reproducibly obtained by use of a vaccine formulation consisting of a yeast cell fraction enriched for the appropriate mannan epitopes. Induction of such Abs through active immunization or passive administration of protective mAbs should be useful in the prevention and, possibly, therapy of various forms of candidiasis in both normal and immunocompromised patients.

To further our understanding of the criteria necessary for Abs to show protection against candidiasis, we performed experiments designed to reveal the mechanism by which protective Abs work. By use of several approaches, we conclude that protective Abs are those that promote a rapid deposition of a high concentration of C3 onto the surface of *C. albicans*.

**Materials and Methods**

**Organism and culture conditions**

*C. albicans* CA-1, previously characterized (19), was started from frozen glycerol stocks and was grown as hydrophilic stationary phase yeast cells in glucose-yeast extract-peptone broth at 37°C (36). The cells were washed with cold (0–4°C) sterile deionized water, appropriately suspended in cold Dulbecco’s PBS (DPBS; Sigma, St. Louis, MO), and used to infect mice.

For quantification of C3 depression, yeast cells grown as described above were killed by overnight treatment with 1% formaldehyde at 4°C, harvested by centrifugation, washed, and stored in PBS containing 0.02% sodium azide, and stored at 4°C. The cells were washed immediately before use with sodium Veronal (5 mM)-buffered saline (142 mM), pH 7.3.

**Mice**

Female mice deficient in complement component C3 (C3−/− mice) were obtained by homologous recombination in embryonic stem cells as previously described (37). Wild-type B6/129 female mice (parental mouse strain; Charles River Laboratories, Kingston, NY) were used as C3-sufficient control animals. All animals were 6–8 wk old. The absence of C3 in sera from the C3−/− mice and the presence of this protein in B6/129 and BALB/c mice were confirmed by an Ouchterlony double diffusion assay in which anti-mouse C3 (The Binding Site, Birmingham, U.K.) was used as the specific detection reagent. Whereas sera from the total binding with NHS or other serum preparations were standardized to give the same amount of functional Ab of each as previously described (19, 23, 33).

**Monoclonal Abs**

mAbs B6.1 and B6 are IgM Abs (19, 33), and mAb C3.1 is an IgG3 Ab (23). The mAbs were produced in serum-free medium by LigoCyte Pharmaceuticals (Bozeman, MT), and Ab concentrations and purity were estimated as described previously (19, 23, 33). Preparations at <3 mg/ml had no detectable (<3 pg/ml) bacterial endotoxin contamination (E-Toxate Kit, Sigma). Unless otherwise indicated, the starting solutions of the Abs were standardized to give the same amount of functional Ab of each as determined by agglutinin titters against mannann-coated latex beads as previously described (19, 20, 23).

Quantification of C3 deposition on *C. albicans*

Normal human serum (NHS) was prepared from peripheral blood collected from at least 10 normal donors after informed consent, pooled, and stored at −80°C. Potential inhibitors of the classical pathway were removed by absorption with yeast cells (38). To produce yeast-absorbed serum, NHS was incubated for 60 min at 0°C with 1010 yeast cells/ml serum, with mixing every 10–15 min, and separated from the cells by centrifugation. The serum was absorbed a second time, filtered through a 0.45-μm pore size filter, and used immediately.

C3 was isolated from frozen human plasma as described previously (39–41) and stored at −80°C until use. The C3 was labeled with 125I as described previously (42) by use of Iodogen reagent (Pierce, Rockford, IL) (7).

Binding of C3 to candidal yeast cells was analyzed as described by Kozel et al. (43). Briefly, each complement binding medium contained 1) 40% yeast-absorbed serum to which was added an appropriate amount of mAb B6.1 or mAb B6; 2) sodium Veronal (5 mM)-buffered saline (142 mM), pH 7.3, containing 0.1% gelatin, 1.5 mM CaCl2, and 1 mM MgCl2; and 3) 125I-labeled C3 sufficient to provide a specific activity of 50,000 cpm/μg C3 for the mixture of labeled and unlabeled C3 in the serum (assuming that NHS contains 1200 μg C3/ml). The reaction medium was warmed to and kept at 37°C, and 2.0 × 107 yeast cells/ml reaction medium were added to initiate C3 binding. From previous kinetics experiments we determined that C3 binding could be assessed by a 6-min incubation. If incubation was prolonged beyond 6 min, the amount of bound C3 would reflect C3 bound via both the classical and alternative pathways (44). At 6 min 50–μl samples were withdrawn in duplicate and added to 200 μl of a stop solution (PBS, 0.1% SDS, and 20 mM EDTA) in Millipore MABX-N12 filter plates fitted with BV 1.2 μm filter membranes (Millipore, Bedford, MA). The yeast cells were washed five times with PBS containing 0.1% SDS. The membranes were removed, and the amount of radioactivity bound to the yeast cells collected on the membranes was determined with a Packard Autogamma counter (Packard, Downers Grove, IL) (7). Specific binding was determined by subtracting the radioactivity of samples with heat-inactivated serum from the total binding with NHS or other serum preparations.

**Isolation of mouse C3 protein**

Mouse plasma was purchased from Accurate Chemical and Scientific (Westbury, NY), and mouse C3 was isolated by the procedure described by Gyöngyossy and Assimne (45), except that Mono Q and Mono S (Amerham Pharmacia Biotech, Piscataway, NJ) were used for ion exchange chromatography, respectively, in place of QAE and SP Sephadex. Conditions used for ion exchange chromatography with Mono S were identical with conditions for characterization of human C3 (46). Ion exchange chromatography on Mono S separates the hemolytically active form of C3 from inactive C3 and C3 fragments (46). As a consequence, all murine C3 was in the native form. Purified murine C3 had the expected Mr of ~210,000 Da in the unreduced form and bands corresponding to ~130,000 and 70,000 Da in the reduced form as shown by SDS-PAGE (45). The concentration of C3 was determined spectrophotometrically, assuming a value of 10.4 for the E1 cm 1 of mouse C3 (45).

*C. albicans* high dose experiment in mice decomplemented with cobra venom factor (CVF)

One group of BALB/c mice was given CVF (from *Naja haje* venom; Diaomedix, Miami, FL) diluted in DPBS such that 0.2 ml i.v. delivered 12 U CVF/100 g body weight according to the manufacturer’s recommendation. Control mice received DPBS alone. Twenty-four hours later the animals were subdivided into three additional groups to receive 0.1 ml of the protective Ab mAb B6.1 (105 μg/mouse), the nonprotective Ab mAb B6 (100 μg/mouse), or DPBS i.p., respectively. The quantity of mAb B6.1 used in this experiment was the same as the dose previously shown to protect mice against a conventional challenge dose of *C. albicans* (20). Four hours after the Ab treatment all animals were given 0.1 ml containing ~1000 LD50 dose of live *C. albicans* yeast cells (4 × 106 cells/mouse) i.v., and their mean survival times (MSTs) were measured in minutes. In one experiment formalin-killed yeast cells were used instead of viable cells. Formalin killing for this experiment was performed by suspending washed yeast cells, obtained as described above, in a tube containing 10% formalin (J. T. Baker, Phillipsburg, NJ), placing the tube in ice, and gently mixing every 5 min for a total of 60 min. The killed cells were washed three times with cold deionized water, suspended in cold DPBS, and stored at 4–8°C until use. Complete killing was confirmed by culture.

**Abbreviations used in this paper:**

- C, complement factor
- CVF, cobra venom factor
- DPBS, Dulbecco’s PBS
- FNMS, fresh normal mouse serum
- HNMS, heat-inactivated normal mouse serum
- MST, mean survival time
- NHS, normal human serum.
C. albicans high dose experiment in C3<sup>−/−</sup> mice

Similar experiments were performed as described above, except that C3<sup>−/−</sup> mice were used instead of CVF-treated animals, and the protective IgG3 mAb C3.1 was included in the testing. For these experiments mAb C3.1 was tested at one-half the agglutinin titer of the IgM mAbs B6.1 and B6.4, which was previously shown to protect mice against a conventional challenge dose of C. albicans (23). At 3.5 h after Ab treatment the mice were divided into three groups to either receive 0.2 ml pooled whole unheated fresh normal mouse serum (FNMS), heat-inactivated (56°C for 30 min) normal mouse serum (HNMS) from BALB/c female mice, or DPBS, by an i.v. route. Thirty minutes later the animals were infected with the very high dose of C. albicans yeast cells as described above, and their MSTs were measured.

Dose-response protective effect of mAb B6.1 against challenge with a conventional dose of C. albicans

To confirm and extend information on the protective activity of mAb B6.1 against a conventional challenge dose of C. albicans (i.e., 5 x 10<sup>5</sup> viable yeast cells given i.v.), mice were given i.p. doses of varying amounts of the Ab or DPBS 4 h before the inoculation. Protection was assessed by sacrificing the animals 20 h after inoculation and processing their kidneys for determination of Candida CFU as previously described (20).

Protective activity of mAb B6.1 in C3<sup>−/−</sup> mice against challenge with a conventional dose of C. albicans

Groups of C3<sup>−/−</sup> and B6/129 control mice received mAb B6.1 i.p. at the dose described above; 4 h later the mice were challenged with C. albicans i.v. and then followed daily for determination of MSTs. The values were compared with those in groups of each mouse strain that received control Ab (mAb B6) or buffer (DPBS) instead of mAb B6.1. The challenge dose of yeast cells was predetermined as the dose that would not kill control (i.e., non-Ab-treated) animals within 48 h, but would kill all control animals by 14 days after inoculation. This dose was 1.25 x 10<sup>5</sup> live yeast cells/mouse for the C3<sup>−/−</sup> mice and 5 x 10<sup>5</sup> for the B6/129 control mice.

Protective activity of mAbs B6.1 and C3.1 in C3<sup>−/−</sup> mice reconstituted with FNMS

In preliminary experiments varying amounts of FNMS (from BALB/c mice) were given i.v. to C3<sup>−/−</sup> mice before injection of the protective Ab mAb B6.1 and subsequent challenge with yeast cells. An optimal effect occurred in animals that received 0.2 ml FNMS; thus, this volume was selected for subsequent experiments. For the following experiments, appropriate groups of animals were used to separately test mAbs B6.1 (IgM) and C3.1 (IgG3). At 3.5 h after administration of mAb the C3<sup>−/−</sup> mice were given FNMS, HNMS, or DPBS (0.2 ml/mouse) by i.v. injection. Thirty minutes later the animals were infected with C. albicans yeast cells by the i.v. route. Another control group received DPBS instead of Ab and DPBS instead of serum (complement). The MST were unaffected control animals that received only FNMS or DPBS before challenge. For these experiments the same conventional challenge doses of yeast cells were used as in the preceding experiment. The BALB/c mice were challenged with the dose used above for the B6/129 mice. In addition to MST determinations, the mice that survived the observation period were sacrificed, and their kidneys were processed for determination of Candida CFU as described previously (20).

Test protective activity of mAb B6.1 on C3<sup>−/−</sup> mice reconstituted with C3 protein

C3<sup>−/−</sup> mice were given mAb B6.1 i.p. 4 h before an i.v. challenge with 1.25 x 10<sup>5</sup> yeast cells. The mice were arranged into groups to receive i.v. either 0.2 ml isolated C3 protein (2.6 mg/ml), 0.2 ml inactivated C3 protein (2.6 mg/ml), or 0.2 ml DPBS. All were challenged with the yeast cells 30 min later. Inactivation of C3 protein was performed as previously described (47), except that five, instead of eight, freeze-thaw cycles were used. The mice were followed daily for survival data and calculation of MST. Mice that survived the observation period were sacrificed, and kidneys were homogenized and plated for Candida CFU determinations.

Statistics

The statistical significance of difference in survival times was calculated by the Kaplan-Meier test computed with Systat 7.0 (New Statistics for Windows; SPSS, Chicago, IL). In all other analyses Student’s t test was used as a calculation of statistical significance. Differences were considered statistically significant if p < 0.05.

Results

C. albicans high dose experiments suggest that mAbs B6.1 and C3.1 activate complement in vivo more rapidly than mAb B6

In preliminary experiments we found that mice given ~1000 times the conventional doses of C. albicans i.v. died by ~4 h after inoculation; thus, we decided to determine the effect of protective (mAbs B6.1 and C3.1) and nonprotective (mAb B6) mAbs on this rapid death response.

Normal BALB/c mice given either mAb B6 or DPBS and infected with C. albicans i.v. at the very high dose had MSTs of 233 and 258 min, respectively, after inoculation (Fig. 1). However, mice given the protective mAb B6.1 before inoculation died much more quickly, as shown by 27 min MST. To explain this surprising result, we hypothesized that protective Abs are those that cause rapid and efficient complement activation in the presence of yeast cells. Support for this hypothesis was obtained with animals pretreated with CVF to deplete their blood of C3. The shortened MST did not occur if the animals were pretreated with CVF before administration of mAb B6.1 and challenge with the high dose of yeast cells.

Similar trends were obtained with the C3<sup>−/−</sup> mice. Mice that received a combination of mAb B6.1 and FNMS had an MST of ~20 min (Fig. 2), whereas those that were given a combination of mAb B6.1 and either HNMS or DPBS had the more extended survival times of 123 and 142 min, respectively. These latter MST were comparable to those of C3<sup>−/−</sup> mice that received only DPBS before challenge. In addition, C3<sup>−/−</sup> mice that received mAb B6 and either FNMS or HNMS had similar prolonged MST (Fig. 2). As expected, mAb B6 did not significantly affect the survival times compared with those of mice that received buffer (DPBS; data not shown). These experiments were repeated with the IgG3 mAb C3.1 in place of mAb B6.1. MAb C3.1 has the same epitope specificity as mAb B6.1 and is also protective against both disseminated candidiasis and vaginal infection (23). The results with this Ab were essentially the same as those with mAb B6.1. C3<sup>−/−</sup> mice given mAb C3.1 and FNMS died the most rapidly, as indicated by the MST of ~17 min (Fig. 2).

![FIGURE 1](http://www.jimmunol.org/) Indirect evidence that the protective IgM mAb B6.1 causes rapid complement activation upon interaction with a very high dose of C. albicans cells in vivo. Normal BALB/c mice were given the IgM protective mAb B6.1 or the IgM nonprotective mAb B6 i.p. before i.v. infection of a very high dose of C. albicans (4 x 10<sup>5</sup> live yeast cells/mouse). The animals that received the protective Ab (mAb B6.1) had greatly accelerated deaths compared with normal mice that received either the nonprotective Ab or DPBS that was used as a diluent. The accelerated death phenomenon was eliminated if the mice were decompartmentalized by treatment with CVF before administration of mAb B6.1 and subsequent challenge with the yeast cell bolus. Bars show the SE.
To determine whether yeast cell viability is required for the accelerated death phenomenon, essentially identical experiments were performed with formalin-killed *C. albicans* yeast cells. Although the same accelerated death phenomenon was observed with the animals that received FNMS and mAb B6.1, the control C3/−/− mice that received either DPBS or mAb B6 and FNMS survived the entire 3-day observation period when given dead yeast cells compared with ~140 min survival times for such control animals that received viable yeast cells (Fig. 3).

**mAb B6.1 fixes more C3 than mAb B6, but C3 fixation declines at very high Ab concentrations**

To directly determine whether protective Ab fixes more complement than nonprotective Ab, yeast cells were incubated for 6 min in a complement-binding medium containing 40% yeast-absorbed serum supplemented with various amounts of either mAb B6.1 or mAb B6 (0.2–1280 μg/ml binding medium), and the amount of C3 binding to each cell was determined (Fig. 4). The 6-min incubation time was chosen because little or no C3 binding occurs by this time when the classical pathway is blocked by absorption of the serum or by treatment with EGTA, but substantial amounts of C3 are bound when the classical pathway is operative (our unpublished observations) (38). In this way, binding at 6 min can be used as a measure of classical pathway-dependent C3 binding. The dose effect of each mAb on activation of the classical pathway was evaluated as the amount of C3 deposition vs the total input of mAb (Fig. 4). The amount of C3 deposition was positively correlated to mAb concentrations ranging from 0.6 to 40 μg/ml binding medium for mAb B6.1 and from 2.5 to 80 μg for mAb B6. However, we were surprised to find that the amount of bound C3 declined when the concentration of either mAb exceeded 80 μg/ml binding medium. We estimated by use of linear regression that binding of 10^6 C3 molecules/yeast cell required 9 μg mAb B6.1/ml reaction mixture compared with 79 μg/ml mAb B6. As expected, binding of 10^6 C3 molecules/yeast cell at 6 min is less than the maximum binding observed after incubation for 10–20 min (2–2.5 × 10^6 C3/yeast cell), which would represent extended C3 deposition via both the classical and alternative pathways. To place these data in perspective, maximum binding is ~2 × 10^6 C3 molecules/cell of Saccharomyces cerevisiae and 1.8 × 10^5/cell of acapsular Cryptococcus neoformans (43).

**mAb B6.1 protects mice against a conventional *C. albicans* challenge dose, but protection is reduced at high Ab doses**

If C3 binding to yeast cells is involved in the protective effect of Abs, the above in vitro results would suggest that a high in vivo mAb B6.1 titer may be less protective than lower titers. Normal mice were i.p. treated with buffer (DPBS) or varying amounts of mAb B6.1 4 h before an i.v. challenge with a conventional dose (5 × 10^5) of viable yeast cells. Sacrificing the animals 44 h after challenge and determining kidney CFU assessed the effect of the treatments before challenge. At low (26 μg) and intermediate (105 μg) doses of mAb B6.1 protective activity showed a dose response, but this activity declined in animals that received high (420 μg or more) doses of mAb B6.1 (Fig. 5).

**Protective Abs are not functional in C3/−/− mice unless the animals also receive whole serum from normal mice**

To determine whether mAbs B6.1 and C3.1 protect C3/−/− mice against disseminated candidiasis, these animals were given Ab and infected with a conventional challenge dose of *C. albicans* i.v. The most protective doses of mAb B6.1 (19–21) and mAb C3.1 (23)
were used, and the survivals of test and control C3\(^{-/-}\) mice were compared with those of their respective wild-type complement-sufficient normal parental strain, B6/129. The conventional doses of C. albicans were as defined above for C3\(^{-/-}\) and B6/129 mice. C3\(^{-/-}\) mice treated with either mAb B6.1 (Fig. 6A) or mAb C3.1 (Fig. 6B) before challenge did not have a significantly improved chance of prolonged survival compared with animals not given the protective mAb (Fig. 6). However, as expected, mAb B6.1 significantly (\(p < 0.05\)) increased the MST of the complement-sufficient B6/129 control mice against disseminated candidiasis compared with this mouse strain given DPBS instead of the Ab (data not shown).

However, if the C3\(^{-/-}\) mice received FNMS along with mAb B6.1, their MST were increased. Four of seven C3\(^{-/-}\) mice given mAb B6.1 and FNMS survived the entire 76-day observation period after inoculation, whereas all mice given mAb B6.1 and either HMNS or DPBS died by 20 and 12 days, respectively. All mice that received DPBS only died by day 8 after the challenge (Fig. 6A). An additional control group of mice that received HMNS or DPBS, but no mAb B6.1, before challenge had MST of 6.8 ± 1.1 and 5.6 ± 0.5 days, respectively (results not shown). The mice that survived the 76-day observation were sacrificed, and kidney homogenates showed no fungal CFU from three of the animals. The fourth animal had no C. albicans CFU from one kidney homogenate, but a small number of CFU (3.2 × 10\(^3\) CFU/g kidney tissue) grew from the homogenate of the other kidney.

C3\(^{-/-}\) mice that received mAb C3.1 along with DPBS, HMNS, or FNMS had MST of ~8, 17, and 63 days, respectively (Fig. 6B).

The three mice that survived the 90-day observation period were checked for C. albicans CFU; one of the animals was culture-negative for both kidneys, and only one kidney from each of the remaining two animals was positive at 7 × 10\(^2\) and 2.2 × 10\(^2\) CFU/g tissue.

**FIGURE 5.** Dose-response effect of mAb B6.1 on protection against disseminated candidiasis in normal mice. Normal BALB/c mice were given varying doses of mAb B6.1 i.p. or control animals were given buffer (DPBS). Four hours later they were challenged with a conventional i.v. dose (5 × 10\(^5\) yeast cells) of C. albicans, and kidney CFU were enumerated 44 h after challenge. The experiment, repeated twice, showed a dose-response protection in animals given 26 and 105 μg Ab, but higher doses of Ab had less protective activity. Bars show the SE.

**FIGURE 6.** mAbs B6.1 and C3.1 are not protective in C3\(^{-/-}\) mice unless the animals are given FNMS or C3. C3\(^{-/-}\) mice were given either mAb B6.1 (A) or mAb C3.1 (B) i.p., and 4 h later they were challenged i.v. with live C. albicans yeast cells at a dose of 1.25 × 10\(^5\)/C3\(^{-/-}\) mouse. Animals that received the Abs did not have significantly longer survival times than control mice that received only buffer (DPBS) before infection. Mice that received either Ab along with 0.2 ml FNMS obtained from normal BALB/c mice had significantly increased survival times compared with mice that received either HMNS or DPBS (\(p < 0.05\)). Very similar results were obtained when isolated C3 was used to reconstitute the C3\(^{-/-}\) mice instead of FNMS (C). C3\(^{-/-}\) mice that received the Ab and isolated C3 protein (C3) survived an average of 21 days longer than mice from other groups (\(p < 0.01\)). Mice that received freeze-thawed inactivated C3 protein (Inact.-C3) had MST values similar to those of control mice that received buffer only. The SE is given in parentheses.
Confirmation that complement influences the protective effect of mAb B6.1

C3\(^{-/-}\) mice were reconstituted with isolated mouse C3 protein instead of FNMS. The complement-reconstituted C3\(^{-/-}\) mice were given mAb B6.1, followed by an i.v. challenge with a conventional dose (1.25 \(\times\) 10\(^5\)) of C. albin\(\text{c}\)ans. The complement-reconstituted mice survived \(\sim\)20 days longer than mice that either did not receive the C3 preparation or received inactivated C3 (Fig. 6). The four complement-reconstituted survivors were sacrificed on day 48. Kidney homogenates were negative for two of the four survivors and were positive in only one kidney, and at a low count (2.5 \(\times\) 10\(^2\) and 4.2 \(\times\) 10\(^2\) CFU/g kidney), from the other survivors.

Discussion

We previously reported that mAbs B6.1 and C3.1 are protective against a conventional hemogenous dose (5 \(\times\) 10\(^5\)) of C. albi\(\text{c}\)ans yeast cells (20, 23). In preliminary experiments we found that mice succumbed by 4 h to an i.v. dose of a very high number of yeast cells (4 \(\times\) 10\(^3\)) and reasoned that this unconventional model of disseminated candidiasis may yield clues as to the mechanism of protection by Abs. We were thus intrigued that mAb B6.1-treated mice showed an exaggerated lethal response to the very high dose of yeast cells, whereas mice that received the nonprotective mAb B6 were unaffected compared with control animals that received buffer in place of Ab. Our laboratory (43, 48–50) and others (51–53) have previously shown alternative complement cascade activation by C. albi\(\text{c}\)ans yeast cells, but this activation alone would not explain the differential effects of protective and nonprotective Abs. Because the kinetics of alternative complement pathway activation by C. albi\(\text{c}\)ans are slow, taking \(\sim\)8 min for significant C3 deposition (43, 44, 50), we hypothesized that the protective Ab may cause death through rapid classical pathway complement activation and release of pharmacologically active products. Animals depleted of C3 through treatment with CVF were not susceptible to the mAb B6.1-associated enhanced death response due to the very high number of organisms, which provided the first evidence in support of our hypothesis.

We also addressed the alternative explanations that the mAb B6.1-associated rapid lethality is due to CVF toxicities unrelated to C3 depletion or to acute lethality related to metabolically active yeast cells. The acute lethality experiments were repeated in mice genetically incapable of producing C3, thus obviating the need for use of CVF. These animals showed not only essentially identical patterns of increased susceptibility to mAb B6.1 when FNMS was used to replenish complement activity, but mAb C3.1 behaved in a similar fashion. mAb C3.1 is also a protective Ab with apparently identical epitope specificity as mAb B6.1, but mAb C3.1 is an IgG3 (23) and also should be a strong activator of complement (20). A similar observation was made when affinity-purified human anti-mannan IgG was used to restore the complement-activating potential of human serum that had been adsorbed with Candida yeast cells to remove initiators of the classical pathway; reduced binding of C3 was observed at very high concentrations of IgG (38). Although we do not yet have an explanation for the phenomenon, a prediction would be that mAb B6.1 should be less protective in vivo when given at a high concentration to mice.

Protective mAb B6.1 dose-response experiments were conducted to determine whether the Ab is less protective at high concentrations compared with the protective concentration we reported previously (19–21). These experiments were performed in normal mice, and the yeast cell challenge dose i.v. was the conventional concentration use previously (20). Quantification of C. albi\(\text{c}\)ans CFU developing from kidney homogenates as described previously (20, 55) was used to assess the protective activities of the various Ab doses. At all concentrations of mAb B6.1 tested, mice had significantly fewer kidney CFU compared with animals that received buffer diluent (DPBS) instead of Ab. The number of CFU decreased as a function of Ab concentration at Ab doses of 26 and 105 \(\mu\)g/mouse. However, mice that received 420 or more \(\mu\)g Ab developed more kidney CFU than animals that received the lower Ab concentrations. In one experiment a very high dose of Ab was administered (840 \(\mu\)g/mouse), and for reasons not clear to us, these animals produced fewer CFU than mice that received the 420–\(\mu\)g dose, but the CFU were still greater than in mice that received either 26 or 105 \(\mu\)g Ab. Interestingly, the higher, less protective, doses of Ab approximated the same range of Ab that produced the unexpected reduction of C3 deposition on yeast cells as described above. These data support our contention that the mechanism of mAb B6.1 protection involves complement activation. In fact, extrapolation of the complement binding data provides an approximation of the amount of complement binding that would be required for protection. According to the data, knowing that mAb B6 is not protective, and that the optimal protective range of mAb B6.1 lies approximately between 20 and \(>\)100, but <420 \(\mu\)g/mouse (Fig. 5), and assuming that each mouse has \(\sim\)2–2.5 ml blood, we may conclude that there needs to be more than \(~\)1 \(\times\) 10\(^5\) C3 molecules deposited per yeast cell for a protective effect by an opsonic Ab.

To further test the dependence of protective Abs on C3 activation, we determined whether mAbs B6.1 or C3.1 could protect C3 knockout mice. The C3 knockout mice were challenged i.v. with a yeast cell concentration that would cause 100% deaths by 5–10 days after inoculation. Mice given either Ab were essentially unaffected by the treatment. Although there were slight improvements in survival times in mice that received HNMS along with either Ab, the most dramatic increase in survival occurred when the mice received fresh NMS along with either of the protective Abs before inoculation with a conventional dose of viable yeast cells. The slight improvements in animals that received HNMS are, presumably, due to residual intact C3 that survived the heat inactivation. In animals that received fresh NMS along with protective Ab, a majority of mice not only survived the entire observation period, but the kidneys from some of the survivors were culture negative for C. albi\(\text{c}\)ans CFU.

Because of the complexity of normal mouse serum, we specifically tested the role of C3 in Ab protection by testing mAb B6.1 in C3 knockout mice given purified mouse C3. These results were
similar to those for animals given normal mouse serum as a source of complement. As expected, inactivation of the purified C3 by repeated freeze-thaw cycles completely negated the positive effects of C3 administration. As with the use of fresh mouse serum to replenish C3 in these animals, a majority of mice that received mAb B6.1 and C3 survived the entire observation period, and the kidneys from some of the animals were culture negative for C. albicans CFU.

Results of the experiments reported here and our previously reported findings (21, 54–56) support the idea that the mechanism of Ab protection is due to rapid and efficient complement deposition on the fungal cell surface, which leads to preferential uptake and killing by phagocytic cells. Previously we showed that mAb B6.1 protects neutropenic mice, but macrophages were still functional in these animals and presumably accounted for the Ab-associated enhanced resistance (21), as our laboratory (55) and others (57–59) have demonstrated that host macrophages can play an important role in defense against experimental disseminated candidiasis. These host defense cells most likely become of primary importance during episodes of neutropenia, and mAb B6.1 or C3.1 along with a functional complement cascade enhance the delivery of fungal cells to these phagocytes. mAb B6.1 not only efficiently fixes C3 to the fungal cell surface, but does this more rapidly than fixation via the alternative complement cascade (50). Results obtained from in vitro experiments correlate to these conclusions, as mAb B6.1 was shown to enhance neutrophil uptake and killing of C. albicans yeast cells in a dose-response fashion provided that complement was present, whereas mAb B6 did not show this effect regardless of the concentration of Ab added or the presence of complement (56). Upon entry of a C. albicans cell into the circulation of the host, a rapid and efficient C3 deposition may be the critical event that determines where in the body of the host the fungal cell will first associate. If the fungal cell becomes rapidly opsonized with complement, such as within 6 min, interaction of the fungus with a phagocytic cell, rather than with some other host surface, such as on the endothelium, should be favored. An equally important point to be made from these and other studies from our laboratory is that we should not expect to conclude anything about the status of host defense against candidiasis by a simple serum test for the presence of Abs against the fungus. Clearly, more information would be needed, such as the epitope specificities recognized by the Abs, isotype, and titer of the specific Ab.

Considering the antigenic complexity of C. albicans, there is little reason to expect that sensitization of most people to natural infection with the fungus would necessarily lead to the production of protective Ab responses. Development of an efficacious vaccine and protective mAbs may well have a place in medicine. According to our findings, minimum criteria for application of this intervention approach would be individuals who have either normal neutrophil or macrophage functions. Short term protection via passive transfer of protective Abs should be considered for those patients who meet the above criteria and who will undergo medical procedures that might place them at high risk of developing hematogenously disseminated candidiasis.

References


