Brief Definitive Report

Immunoglobulin G3 Blocking Antibodies to the Fungal Pathogen Cryptococcus neoformans

By Gabriel Nussbaum,* RuiRong Yuan,* Arturo Casadevall,‡ and Matthew D. Scharff*

From the *Departments of Cell Biology and Medicine and Microbiology and Immunology of the Albert Einstein College of Medicine, Bronx, New York, 10461

Summary

Vaccination and infection can elicit protective and nonprotective antibodies to the fungus Cryptococcus neoformans in mice. The effect of nonprotective antibodies on host defense is unknown. In this study we used mixtures of protective and nonprotective monoclonal antibodies (mAbs) to determine if nonprotective mAbs blocked the activity of the protective mAbs. Antibody isotype and epitope specificity are important in determining the ability to prolong survival in mice given a lethal C. neoformans infection. Three different nonprotective immunoglobulin (Ig) G3 mAbs to cryptococcal capsular polysaccharide were used to study the interaction between the IgG3 isotype and protective IgG1 and IgG2a mAbs in murine cryptococcal infection. One IgG3 mAb reduced the protective efficacy of an IgG1 with identical epitope specificity. A second IgG3 mAb with different epitope specificity also reduced the protection provided by the IgG1 mAb. The protective efficacy of an IgG2a mAb was also dramatically decreased by still another IgG3 mAb. To our knowledge this is the first report of blocking antibodies to a fungal pathogen. The results have important implications for the development of vaccines and passive antibody therapy against C. neoformans.

The role of antibody immunity in resistance to particular pathogens is frequently determined by correlating the presence of specific antibody with resistance to infection. However, the polyclonal antibody response that is elicited by both vaccination and infection consists of a complex mixture of antibodies differing in isotype, epitope specificity, and protective efficacy. The interpretation of experiments using polyclonal preparations is further complicated by the fact that nonprotective antibodies have been shown to block the activity of protective antibodies in some bacterial infections (1–4). Blocking activity may be due to direct competition for the binding of the same or closely related epitopes (1, 5) or to the enhancement of infection by nonprotective antibodies through a variety of mechanisms (2, 6). This suggests that the relative amounts of protective and nonprotective antibodies elicited by infection or immunization can determine the outcome of infection. Passive administration of mAbs individually and in combination can reveal the impact of nonprotective antibodies on the polyclonal response.

Although it is generally accepted that antibodies contribute to resistance to viruses and bacteria, the role of antibodies in fungal infections is controversial and poorly understood (7). Many of the studies supporting or contradicting antibody efficacy against medically important fungi based their conclusions on data obtained using polyclonal sera. The amount of specific antibody, the isotype composition, and the epitope specificity of the specific antibodies in the polyclonal preparations used in these studies varied and may not have been sufficient to mediate protection. When mAbs were used, protective and nonprotective antibodies were identified to Cryptococcus neoformans and Candida albicans (7, 8).

The difficulty in treating cryptococcal infections has renewed interest in the therapeutic potential of antibody immunity. C. neoformans causes a life-threatening meningoencephalitis in 6–8% of patients with AIDS that is usually incurable despite antifungal therapy (9). The large polysaccharide capsule surrounding C. neoformans distinguishes it from other fungal pathogens and is a major determinant of virulence (10–13). Although protective antibodies could be useful therapeutically, nonprotective (14) and even enhancing (15) mAbs to cryptococcus have also been described, raising the possibility that such antibodies could interfere with the efficacy of the protective antibodies and increase susceptibility to infection. In this study we tested the impact of nonprotective IgG3 mAbs on the ability of
mAbs of the IgG1 and IgG2a isotypes to modulate cryptococcal infection in the mouse. Our results indicate that rather than being simply ineffective, IgG3 antibodies reduce the protection provided by the IgG1 and IgG2a isotypes.

**Materials and Methods**

*C. neoformans.* *C. neoformans* strain 24067 (serotype D) was obtained from the American Type Culture Collection (Rockville, MD) and maintained on Sabouraud dextrose agar (Difco Laboratories Inc., Detroit, MI) slants at 4°C. For mouse infection, *C. neoformans* was grown at 37°C in Sabouraud dextrose broth (Difco Laboratories Inc.). Yeast cells were washed three times in PBS, and the inoculum was determined by counting in a hemocytometer.

*Ascites Preparation.* Ascites was generated by injecting 10⁷ hybridoma cells into the peritoneal cavity of pristane-primed BALB/c mice. Antibody concentration was determined by ELISA relative to isotype-matched standards of known concentration. Although 4H3 is an IgG3 mAb, the 4H3 hybridoma produces a small percentage of antibody with κ light chains (17), and the κ chain containing antibodies was removed by absorbing the ascites three times on a rat anti-mouse κ Sepharose 4B column (Zymed Laboratories Inc., South San Francisco, CA).

*Animal Experiments.* Female A/J mice ages 6-8 wk were used for protection experiments. Mice were obtained from the National Cancer Institute (Rockville, MD) for the intravenous infection experiments and from The Jackson Laboratory (Bar Harbor, ME) for the intraperitoneal experiment, based on availability. A/J mice were chosen because they are highly susceptible to infection with *C. neoformans* (18). Protection experiments were performed as described (19, 20). *C. neoformans* was administered intravenously through the tail vein or by intraperitoneal injection. The inoculum was 2 × 10⁶ cells/mouse in the case of intravenous infection with 2 × 10⁶ cryptococci. Consistent with previous results (15), 3E5 IgG3 did not prolong survival compared with saline controls (P = 0.249), whereas the IgG1 switch variant did (P = 0.0002, Fig. 1). Mice who received 1 mg each of IgG1 and IgG3 were still protected compared with the PBS control group (P = 0.0085); however, protection was significantly reduced compared with mice receiving the IgG1 alone (P = 0.0052). Thus, administration of IgG3 reduced the protective efficacy of an IgG1 with identical epitope specificity in mice infected intravenously with *C. neoformans*.

Effect of IgG3 on a Protective IgG1 with Different Specificity. 4H3 is a nonprotective IgG3a mAb generated in response to infection with *C. neoformans* strain GH (16). 4H3 and 3E5 use different but related H chain variable region genes (V₄₄₄/JH3 and V₄₇₃/JH2, respectively), different L chain variable region genes (V₄₃ and V₄₅, respectively), bind different epitopes on cryptococcal polysaccharide (16), and do not compete by ELISA (our unpublished observations). Administration of 4H3 IgG3 did not protect mice from intravenous infection and reduced survival compared with the control mice (P = 0.0079, Fig. 2). As expected, 3E5 IgG1 prolonged survival (P = 0.0079). Mice who received a mixture of 1 mg each of IgG1 and IgG3 were still protected and did not differ statistically from saline controls (P = 0.249). Mice who received a mixture of 1 mg each of IgG1 and IgG3 were neither protected nor was infection enhanced relative to saline controls (P = 0.4206). Administration of the mixture resulted in a significantly shorter mean survival than administration of 3E5 IgG1 alone (P = 0.0317) and a longer survival than treatment with 4H3 alone (P = 0.0079). Thus, an IgG3 mAb with different epitope specificities, 3E5 IgG3 and 4H3 IgG3, prolonged survival and reduced tissue fungal burden (15). mAb 3E5 IgG3 and its 3E5 IgG1 switch variant have identical variable regions but differ in isotype (15).

**Results**

**Effect of IgG3 on a Protective IgG1 with Identical Specificity.** In previous studies, we demonstrated that 3E5 IgG3 mAb did not protect mice from lethal infection with *C. neoformans* (15, 19). When 3E5 IgG3 was switched to the IgG1 isotype, it prolonged survival and reduced tissue fungal burden (15). mAb 3E5 IgG3 and its 3E5 IgG1 switch variant have identical variable regions but differ in isotype (15). To determine the effect of 3E5 IgG1 and 3E5 IgG3 in combination, 1 mg of each antibody was given alone or in combination to groups of five mice 24 h before intravenous infection with 2 × 10⁶ cryptococci. Consistent with previous results (15), 3E5 IgG3 did not prolong survival compared with saline controls (P = 0.249), whereas the IgG1 switch variant did (P = 0.0002, Fig. 1). Mice who received 1 mg each of IgG1 and IgG3 were still protected compared with the PBS control group (P = 0.0085); however, protection was significantly reduced compared with mice receiving the IgG1 alone (P = 0.0052). Thus, administration of IgG3 reduced the protective efficacy of an IgG1 with identical epitope specificity in mice infected intravenously with *C. neoformans*.

Figure 1. IgG3 blocks protective IgG1 with identical specificity. Survival curves of A/J mice given either mAb or PBS (control) show the effect of IgG3 mAb (3E5 IgG3) administration on an IgG1 mAb (3E5 IgG1) with identical epitope specificity. 1 mg of each antibody was given alone or in combination intraperitoneally 24 h before intravenous infection and reduced survival compared with the control mice (P = 0.0079, Fig. 2). As expected, 3E5 IgG1 prolonged survival (P = 0.0079). Mice who received a mixture of 1 mg each of IgG3 and IgG1 were neither protected nor was infection enhanced relative to saline controls (P = 0.4206). Administration of the mixture resulted in a significantly shorter mean survival than administration of 3E5 IgG1 alone (P = 0.0317) and a longer survival than treatment with 4H3 alone (P = 0.0079). Thus, an IgG3 mAb with different epitope speci-
Figure 2. IgG3 blocks protective IgG1 with different specificity. Survival curves of A/J mice given either mAb or PBS (control) show the effect of IgG3 mAb (4H3) administration on an IgG1 mAb (3E5 IgG1) with different epitope specificity. 1 mg of each antibody was given alone or in combination intraperitoneally 24 h before intravenous challenge with $2 \times 10^6$ C. neoformans. Average survival and standard deviation for the IgG1, IgG3, 3E5 IgG1 + 4H3 IgG3 combination, and PBS groups (n = 5/group) were 11.00 ± 1.58, 4.20 ± 0.84, 7.00 ± 2.24, and 5.98 ± 0.04 d, respectively.

With some of our other IgG3 mAbs, A2F12 was minimally protective ($P = 0.0357$). Animals who received 1 mg each of 7F8 and A2F12 died faster than those who received 7F8 alone ($P = 0.0172$) but survived longer than the group who received A2F12 alone ($P = 0.001$). Thus an IgG3 mAb reduced the protective efficacy of an IgG2a mAb in a murine model of intraperitoneal infection.

Discussion

Three different IgG3 mAbs to GXM were studied, and all reduced the efficacy of protective GXM-binding mAbs. In the first case, we used 3E5 IgG3 and IgG1 isotype switch variants that bind to the same epitope (15). The IgG3 was ineffectual when administered alone and reduced survival provided by IgG1. Since these antibodies may compete for binding to the surface of the organism, the nonprotective IgG3, which binds with higher avidity (15) because of cooperative IgG3 Fc–Fc interactions (21, 22), may block effective opsonization by IgG1. We therefore tested IgG3 and IgG1 antibodies that use different H and L chain variable region genes, bind different epitopes on the cryptococcal capsule (19, 23), and do not compete by ELISA. In this case, the 4H3 IgG3 alone enhances cryptococcal infection. When administered together with IgG1, 4H3 IgG3 abolished the 3E5 IgG1 mediated protection. Finally, we mixed an IgG3 that was minimally protective when given alone with a highly protective IgG2a in a different model of infection. These antibodies use the same variable region genes but have unique somatic mutations that may or may not confer differences in fine specificity. In this experiment, the IgG3 again reduced protection provided by IgG2a.

To our knowledge, this is the first report of blocking antibodies to a fungal pathogen. The term blocking antibodies describes nonbactericidal antibodies that prevent killing by bactericidal antibodies or complement in a wide range of gram-negative organisms. Antibodies that are unable to fix complement effectively or to promote phagocytosis may prevent protective antibodies from binding by competition for the same epitope, or by steric hindrance when they bind to different, but closely located, epitopes. For example, IgG antibodies to the outer membrane protein PIII of Neisseria gonococcus block bactericidal antibody binding to PI and fix complement on sites that do not lead to bacterial death (1, 6, 24). Human convalescent gonococcal and meningococcal antisera can effectively block the natural bactericidal activity of normal serum (1, 2, 5), and preexisting antibody to gonococcal PIII facilitates gonococcal transmission (24, 25). Human IgA1 against N. meningitides blocks IgG bactericidal activity when both are directed against distinct polysaccharide epitopes (2). In Dengue virus infection, virus-reactive antibody enhances cellular infection in vitro (26) and in vivo (27), and severe disease (Dengue shock syndrome and Dengue hemorrhagic fever) occurs most often in patients with a preexisting low level of IgG antibody (for review see reference 28). Nonneutraliz-
Several mechanisms could explain the IgG3-mediated blocking activity. A straightforward competition for antigenic sites or a difference in antigenic density or accessibility is unlikely to explain this phenomenon, since we observed interference with antibodies that bind distinct as well as identical epitopes. For the 3E5 IgG3 and IgG1 pair, antigen density is presumably the same since they have identical variable regions. IgG3 antibodies may modify the structure or flexibility of the capsule, preventing effective opsonization by IgG1 and IgG2a. IgG3 may facilitate phagocytosis by effector cells that are not primed for killing, countering the protective effect of IgG1 and IgG2a. Finally, IgG3 immune complexes could conceivably alter the innate cytokine response to infection, thus suppressing cell-mediated immunity.

Our results indicate that murine IgG3 antibodies to C. neoformans GXM are deleterious to the host by either enhancing infection or interfering with the function of protective antibodies. In contrast, mAbs of IgM, IgG2a, IgG2b, and IgA isotypes have all been shown to be protective (19, 38). The ineffectiveness of IgG3 antibodies against C. neoformans contrasts with the effectiveness of this isotype against other encapsulated pathogens such as Streptococcus pneumoniae (39). Our findings have important implications for the potential use of antibodies in the treatment and prevention of cryptococcal infection. Human correlates of the murine protective and nonprotective isotypes, if they exist, need to be identified and this information applied to vaccine design and the selection of antibodies for passive therapy.

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Address correspondence to Matthew D. Scharff, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461.

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References
1. Joiner, K.A., B. Scales, K.A. Warren, M.M. Frank, and P.A. Rice. 1985. Mechanism of action of blocking immunoglobulin G for Neisseria gonorrhoeae. J. Clin. Invest. 76:1765-1772.
2. Jarvis, G.A., and J.M. Griffiss. 1991. Human IgA1 blockade of IgG-initiated lysis of Neisseria meningitidis is a function of antigen-binding fragment binding to the polysaccharide capsule. J. Immunol. 147:1962-1967.
3. Gutman, R.M., and B.A. Waisbren. 1995. Bacterial blocking activity of specific IgG in chronic Pseudomonas aeruginosa infection. Clin. Exp. Immunol. 19:121-130.
4. Hall, W.H., R.E. Manion, and H.H. Zimmeman. 1971. Blocking serum lysis of Brucella abortus by hyperimmune rabbit immunoglobulin A. J. Immunol. 107:41-46.
5. Apicella, M.A., M.A. Westerink, S.A. Morse, H. Schneider, P.A. Rice, and J.M. Griffiss. 1986. Bacterial antibody response of normal human serum to the lipooligosaccharide of Neisseria gonorrhoeae. J. Infect. Dis. 153:520-526.
6. Rice, P.A., H.E. Vayo, M.R. Tam, and M.S. Blake. 1986. Immunoglobulin G antibodies directed against protein III block killing of serum-resistant Neisseria gonorrhoeae by immune serum. J. Exp. Med. 154:1735-1748.
7. Casadevall, A. 1995. Antibody immunity and invasive fungal
