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Generation of biologically active anti-*Cryptococcus neoformans* IgG, IgE and IgA isotype switch variant antibodies by acridine orange mutagenesis

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SUMMARY

Administration of MoAbs to *Cryptococcus neoformans* capsular glucuronoxylomannan (GXM) can alter the course of infection in mouse models. However, the effectiveness of these antibodies appears to depend on isotype and specificity. Comparison of isotype protection efficacy requires families of MoAbs with identical fine specificity and different constant region domain. The generation of such families by hybridoma technology is not always possible because the immune response produces MoAbs of limited classes or subclasses. In these instances isotype switch variants can be isolated *in vitro*. Unfortunately, standard methods of recovering spontaneous switch variants are often unsuccessful, mainly because of the low frequency of switching. In this study we demonstrate that acridine orange stimulation of an IgG3 anti-*C. neoformans*-producing hybridoma can be used to recover the entire set of isotype switch variants: IgG1, IgG2b, IgG2a, IgE and IgA. All isotype switch variants bind to GXM; fine specificity mapping, using an 11 amino acid peptide polysaccharide mimetope, revealed conservation of binding site specificity. Furthermore, all isotype switch variants reacted with an anti-idiotopic MoAb. The functional activity of this set of MoAbs was demonstrated by their ability to enhance phagocytosis and anti-fungal efficacy of human macrophage-like THP-1 cells, with IgG3 being the most effective and IgE being the least effective.

Keywords monoclonal antibodies isotype switching *Cryptococcus neoformans*

INTRODUCTION

Cryptococcus neoformans is a yeast-like fungus which causes life-threatening infections in 6–8% of patients with AIDS [1]. This fungus is unusual in that it has a large polysaccharide capsule which plays a major role in its virulence. The capsule is anti-phagocytic and host effector cells are unable to bind or internalize the yeast in the absence of opsonins [2]. Antifungal drugs are often unsuccessful in eradicating infection in the setting of severe immunosuppression, making the conventional therapy of *C. neoformans* very difficult [3]. The difficulties associated with treating cryptococcal infections have stimulated interest in the development of preventive vaccines [4] and passive antibody therapy [5]. One option under development is a polysaccharide–tetanus toxoid conjugate vaccine [4] which elicits protective antibodies in mice. Another option under consideration is passive antibody therapy as an adjunct of antifungal chemotherapy [6,7].

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Development of antibody-based strategies against *C. neoformans* has been hindered by a general lack of knowledge of the efficacy and function of antibodies against fungi. In recent years we have employed MoAbs to demonstrate that antibody efficacy against the human pathogenic fungus *C. neoformans* is dependent on isotype [8–11] and precise fine specificity [12]. These studies suggest that the murine IgG3 domain is non-protective. Recently we have shown that an IgG3 anti-*C. neoformans* non-protective MoAb, 3E5.γ3, can become protective by switching its isotype to IgG1 [10]. To better understand the function of each isotype in mediating anti-*C. neoformans* activity we set out to isolate the complete set of anti-*C. neoformans* MoAb switch variants. Unfortunately, the generation of such families has been difficult, mainly because of the low frequency of such events and the fact that switching to some isotypes seems to be restricted *in vitro* (unpublished data). In earlier studies [13] we have demonstrated that acridine orange ICR191 can significantly enhance the frequency of hybridoma isotype switching events *in vitro*. Such treatment allows the isolation of cells that otherwise switch at such low frequency that does not facilitate isolation of switch variants. In this study we

describe the use of ICR191 to generate the full set of IgG subclasses, IgE, and IgA starting from an IgG3 anti-*C. neoformans*-producing hybridoma. These switched antibodies maintain antigen-binding capacity, fine specificity and the ability to mediate specific killing and phagocytosis of *C. neoformans*.

MATERIALS AND METHODS

Cell lines and C. neoformans

Hybridoma cell line 3E5.γ3 was generated from a BALB/c mouse immunized with *C. neoformans* strain 371 GXM, serotype A conjugated to tetanus toxoid [14]. Hybridoma cell lines, 3E5.γ3 and derived switch variants 3E5.γ1, 3E5.γ2b, 3E5.γ2a, 3E5.ϵ and 3E5.α were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), 5% NCTC, 1% non-essential amino acids and 1% penicillin-streptomycin-nystatin. THP-1 is a human derived macrophage-like cell line [15]. THP-1 cells were grown in RPMI medium supplemented with 1% pyruvate, 1% HEPES, 1% penicillin-streptomycin-nystatin and 20% FCS.

Isolation of immunoglobulin isotype switch variants

Isotype switch variants were identified by ELISA spot assay [16] and isolated by the sib selection technique [17]. Briefly, 3E5.γ3-secreting cells were plated in 96-well plates at 2500 cells/well. At 75–90% cell confluency, 50% of the cells were removed to 96-well microtitre plates precoated with a mixture of γ1, γ2b, γ2a, ϵ and α goat anti-mouse heavy chain antibody. After 4–6 h the cells were removed and immunoglobulin spots were determined using biotin-conjugated anti-heavy chain antibody followed by streptavidin coupled to alkaline phosphatase and 5-bromo-4-chloro-3-indolyl phosphate substrate. Cells from wells containing six or more spots were collected and their immunoglobulin isotype was identified by ELISA. Wells containing the largest number of spots were replated at a density of 250–500 cells/well. Cells were grown to 75–90% confluency and tested for the presence of switch variants as described above. Following two to six rounds of enrichment the cells were cloned on 0·4% agar. Mutagenesis with acridine orange was conducted at a concentration known to result in 25–45% cell death in 24 h (0·45 μg/ml). Cells were washed and plated into 96-well plates at 2500 cells/well as described before [13].

Ascites and purification of MoAb

Ascites were collected from pristane-primed BALB/c mice injected with 10^7 cells. IgG3, IgG1, IgG2b and IgG2a MoAbs were purified using Protein G column chromatography. IgA and IgE were isolated over Sepharose 4B conjugated to rat anti-mouse κ light chain column. Antibody concentration and antibody specificity were determined by the ELISA relative to isotype-matched standards of known concentration.

Serology of MoAbs

Serological analysis of the different anti-*C. neoformans* MoAbs was done by ELISA. Briefly, polystyrene microtitre plates were coated with 1 μg/ml GXM in PBS, blocked with 1% bovine serum albumin (BSA) and reacted with serial dilutions of MoAb. Following 2 h incubation, the plates were washed and biotin-conjugated goat anti-heavy chain reagent was added. The binding of each of the antibodies was determined using streptavidin alkaline phosphatase and *p*-nitrophenyl phos-phate substrate. The optical density was recorded at 405 nm with a Biotec Microplate reader. The fine specificity of the immunoglobulin isotypes was studied using 7B8 anti- idiotope MoAb [18] and PA1, an 11 amino acid long peptide (GLQYTPSWLVG) structured following phage display library analysis. PA1 was a gift by Dr P. Valadon and Dr M. D. Scharff (Albert Einstein College of Medicine, NY). 7B8 MoAb was used in antigen binding competition assays. Antibody dilutions known to bind GXM at 50–60% of their maximum were incubated for 2 h with serial dilutions of 7B8 anti-idiotope MoAb. Samples (50 μl) were transferred to GXM-coated plates and the binding was determined following addition of biotin-conjugated goat anti-κ light chain, streptavidin-alkaline phosphatase and *p*-nitrophenyl phosphate. PA1 peptide was used as a peptide–biotin conjugate. Biotinylated peptide was captured to ELISA plates precoated with 2 μg/ml streptavidin. Serial dilutions of MoAb supernatants were incubated for 2 h, followed by the addition of alkaline phosphatase goat anti-mouse κ and *p*-nitrophenyl phosphate substrate.

Biosynthetic labelling and immunoprecipitation

Immunoglobulin was biosynthetically labelled by incubating 10^6 hybridoma cells in methionine-depleted DMEM supplemented with ^{35}S-methionine. Secreted immunoglobulin was immunoprecipitated using affinity adsorbent glutaraldehyde-activated beads coupled to goat anti-mouse κ chain antibody. Labelled immunoglobulin was eluted with SDS sample buffer (0·06 M Tris pH 7·0, 5% glycerol, 1% SDS and 7·5% dithiothreitol) and the size of the heavy and light chains were determined using 10% polyacrylamide gels.

Agglutination

The ability of the 3E5 isotype switch family variants to agglutinate *C. neoformans* was tested as described previously [19]. Briefly, serial dilutions of the MoAbs (ranging from 100 to 0·0003 μg/ml) were made in 96-well microtitre polystyrene plate in 1% BSA in PBS containing 1 mM NaN_3. Yeast cells, 1×10^5/well, were added and the plate shaken for 15 min at 100 rev/min and incubated at 4°C overnight. Agglutination endpoints were determined by microscopic examination of microtitre well contents and the endpoint was the lowest concentration that agglutinated the yeast cells relative to control wells containing no antibody.

Phagocytosis and killing experiments

Phagocytosis and killing studies were done using THP-1, a human macrophage-like cell. To enhance the attachment of THP-1 cells to polystyrene, the plates were treated with 20 μg/ml human fibronectin. Following overnight stimulation with 100 μg/ml phorbol-12-myristate-13-acetate and human recombinant interferon-gamma (IFN-γ), medium was removed and fresh medium containing human IFN-γ (1000 U/ml) lipopolysaccharide (LPS; 30 μg/ml), *C. neoformans* cells and purified MoAb (25 μg/ml) was added. The cells were incubated for 2 h at 37°C, washed with sterile PBS, fixed with cold absolute methanol and stained with 1/20 Giemsa solution. Phagocytosis was determined using a

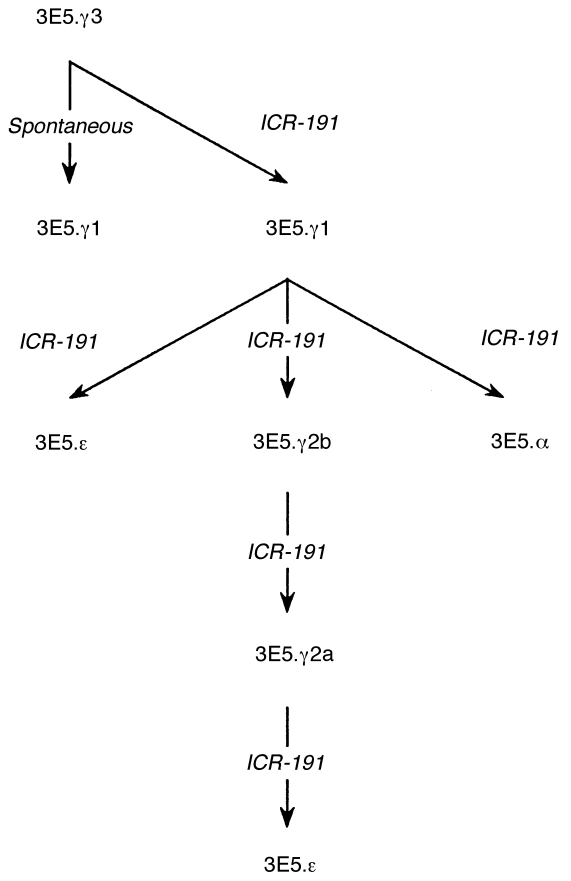

Fig. 1. 3E5.γ3-derived switch variants. Switch variants were identified by the ELISA spot assay and isolated following gradual enrichment and cloning in soft agar.

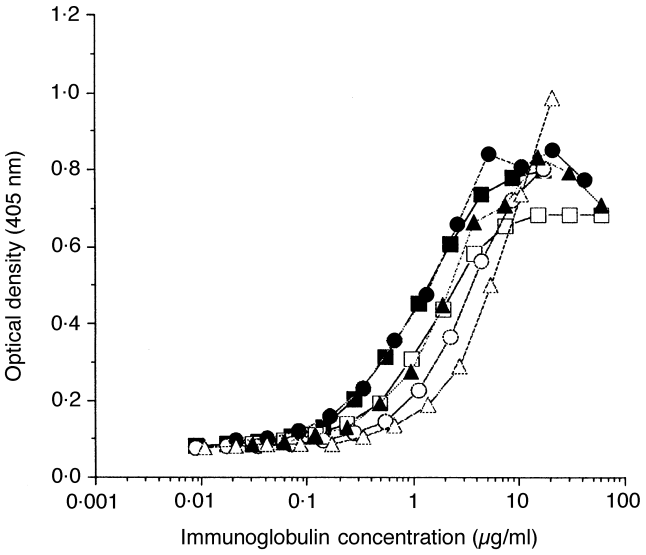

Fig. 2. Antibody titration using constant amounts of GXM (0·5 μg/ml). ELISA plates were coated with GXM followed by serial dilutions of each MoAb; the binding was determined using biotinylated anti-κ mouse light chain, streptavidin-alkaline phosphatase and *p*-nitrophenyl phosphate. ■, γ3; □, γ1; ○, γ2b; ●, γ2a; ▲, ϵ; △, α.

microscope and a 600 × 20 magnification. The phagocytic index is defined as the number of macrophages with internalized yeast cells per number of macrophages per field.

To determine antifungal activity, THP-1 cells were treated as described for phagocytosis at effector to target ratio (E:T) of 10:1. Purified MoAbs were added at 25 μg/ml. Cells and *C. neoformans* were incubated overnight at 37°C. The content of each well was then removed, spun down and cells lysed by adding 100 μl sterile water for 30 min. Supernatants and lysate were then pooled, diluted 1:100 and vortexed for 15 s. Samples of each were diluted again and plated on Sabouraud's dextrose agar plates. Plates were incubated for 2–3 days and the colonies counted.

RESULTS

3E5.γ3 cells spontaneously switch to γ1 but very rarely to other immunoglobulin classes or subclasses

The search for isotype switch variants was initiated by determining the spontaneous frequency of switching of 3E5.γ3-producing cells. This analysis revealed a relatively large number of γ1-secreting cells (663 γ1-producing cells in 10^7 cells), but no γ2a- or α-producing cells. A few γ2b and surprisingly, some ϵ-secreting cells were occasionally identified. Based on previous studies [16], the presence of five to six γ1-secreting cells in 10^5 cells is barely sufficient for the isolation of γ1 switch variants. To enhance the frequency of switching, 3E5.γ3 cells were treated with ICR191. The search for spontaneously γ1-secreting cells continued independently. In contrast to the spontaneous low frequency, ICR191 treatment resulted in a large number of switching cells. Subclass analysis of 20 wells revealed that all contained γ1-secreting cells. Since the frequency of switching of both ICR191 induced and spontaneously arising 3E5.γ1 switch variants to downstream constant region genes was low, the γ1 switch variant cells were further treated with ICR191 to yield γ2b, α and ϵ isotype secreting switch variants. 3E5.γ2a was subsequently obtained after mutagenesis of 3E5.γ2b. In a separate experiment, 3E5.γ2a cells were treated with ICR191. This experiment resulted in a surprisingly large number of ϵ switch variant cells, some of which were isolated (Fig. 1). Heavy supernatants from each switch variant were cross checked by ELISA for each isotype and were found to contain κ light chain and a single heavy chain of the respective isotype.

GXM binding and constant and variable region analysis

Antigen binding of 3E5 MoAb isotype switch variants was tested in a variety of ELISAs. Antigen–antibody reaction of serial dilutions of MoAb on a constant amount of GXM (0·5 μg/ml GXM) resulted in similar curves (Fig. 2). Two methods were used to study the fidelity of switching: anti-idiotope MoAb (7B8), raised against anti-*C. neoformans* MoAb 2H1 (IgG1) and an 11 amino acid long peptide, PA1, which was constructed following phage display library analysis of anti-cryptococcal MoAb. 7B8 MoAb successfully inhibited the binding of GXM to a number of anti-*C. neoformans* MoAbs and seemed to react with both heavy and light chain binding site (Fig. 3a,b). At high concentrations of anti-idiotope MoAb (12 μg/ml), complete inhibition was noted, while at lower concentrations differences were evident. While γ2b and α were still inhibited, there was little or no inhibition of other

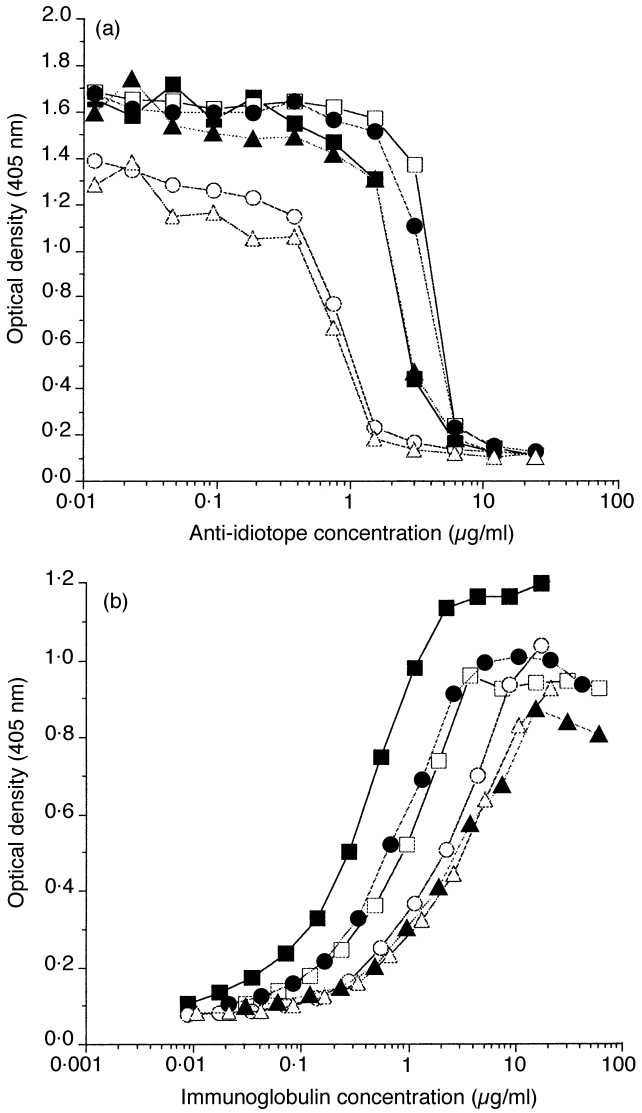

Fig. 3. Fine specificity. The fine specificity of each variant was studied using 7B8 anti-idiotope MoAb (a) and PA1 peptide (b). (a) 3E5-derived switch variant immunoglobulins at a concentration of 60–80% of their maximum GXM binding capacity were incubated with various concentrations of 7B8 immunoglobulin for 2 h. Fifty microlitres were then transferred to GXM-coated ELISA plates. The binding of each immunoglobulin was determined using biotinylated anti-κ light chain, streptavidin-alkaline phosphatase and *p*-nitrophenyl phosphate. (b) Biotin-PA1 was captured on ELISA plates coated with 2 μg/ml streptavidin. Serial dilutions of each 3E5 switch variant immunoglobulin were added and binding determined using the respective anti-heavy chain alkaline phosphatase conjugate and *p*-nitrophenyl phosphate. ■, γ3; □, γ1; ○, γ2b; ●, γ2a; ▲, ϵ; △, α.

subclasses. The binding of PA1 peptide also showed slight differences. In that respect γ3 seemed to bind PA1 more effectively than the other immunoglobulin isotype. These differences and those detected by 7B8 anti-idiotope MoAb could be attributed to the variation in avidity of the antibodies to GXM and/or to the flexibility of the arms of each subclasses. We have also looked at the size of heavy and light chain to determine whether any of the switch variants had undergone deletions. Cells were metabolically labelled, immunoprecipitated with anti-κ light chain and run over

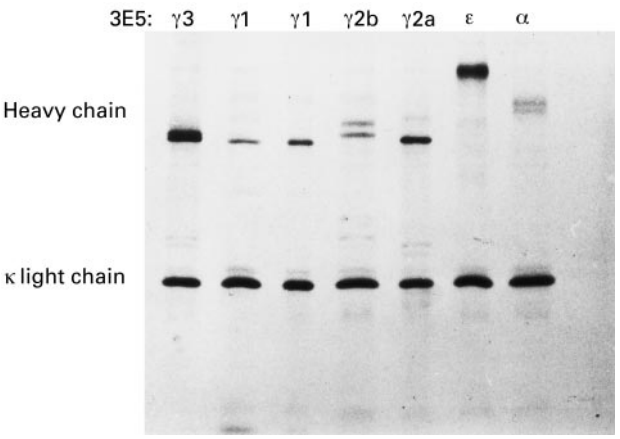

Fig. 4. SDS polyacrylamide gel electrophoresis of 3E5.γ3 and immunoglobulin-derived switch isotype. Methionine-labelled immunoglobulins were immunoprecipitated with glutaraldehyde-activated beads coupled to goat anti-mouse κ light chain antibody. The size of the heavy and light chains were determined using standard molecular weight markers.

10% polyacrylamide gel. The results indicate that all switch variants shared the same size κ light chain, while the size of the heavy chains varied. The parental γ3 heavy chain size was determined at 55 kD and its κ light chain at 26 kD. The size of both the spontaneous and ICR191-induced γ1 switch variants heavy chain was 52 kD, compared with the rest of the family; γ2b, 58 kD; γ2a, 55 kD; ϵ, 74 kD; and α, 61 kD (Fig. 4).

Agglutination and antifungal activity of TPH-1 cells in the presence and absence of 3E5-derived switch variant MoAb

The functional activity of the 3E5-derived MoAbs was tested by measuring their agglutination end point, relative efficacy in enhancing phagocytosis and antifungal efficacy of the macrophage-like cell THP-1. All MoAbs agglutinated strain 24067, with γ1 being the most potent antibody (1·09 μg/ml). γ3, γ2a and α required 6·125 μg/ml while ϵ required between 6·125 and 12·5 μg/ml (Table 1).

In the absence of MoAb, IFN-γ- and LPS-stimulated THP-1 cells did not phagocytose *C. neoformans* cells. However, addition of 3E5-derived MoAbs (at a concentration of 10 μg/ml) resulted in marked enhancement of phagocytic activity with the relative opsonic efficacy being IgG3 > IgG2a > IgG2b > IgA > IgG1 > IgE (Fig. 5). Co-incubation of IFN-γ- and LPS-stimulated THP-1

Table 1. Agglutination of *Cryptococcus neoformans* by 3E5.γ3 and isotype switch variants

3E5 family switch variants	Agglutination end point (μg/ml)
3E5.γ3	6·25
3E5.γ1	1·09
3E5.γ2a	6·25
3E5.γ2b	3·125
3E5.α	6·125
3E5.ϵ	12·5–6·25

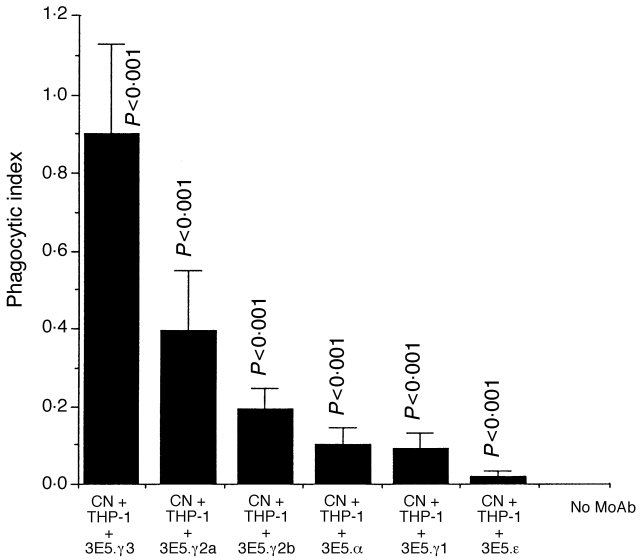

Fig. 5. Phagocytosis indices for the MoAb 3E5 isotype switch variants. 2H1.γ1 MoAb is included as a positive control. Cells were incubated for 2 h at an E:T ratio of 1:1 and MoAb concentration of 25 μg/ml. Each bar is an average of 10 fields. *P* values are in comparison with THP-1 cells without MoAb.

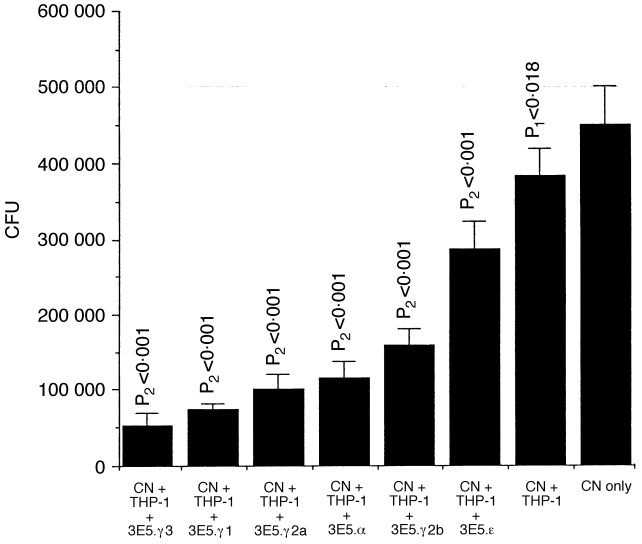

Fig. 6. *Cryptococcus neoformans* (CN) colony-forming units (CFU) following incubation with THP-1 cells in the presence or absence of MoAbs. Cells were incubated for 24 h at an E:T ratio of 1:10 and MoAb concentration of 25 μg/ml. Each bar is an average of eight values. P_2 values are calculated as relative to CFU of THP-1 cells in the absence of MoAbs. P_1 values are in comparison with CN alone.

cells with *C. neoformans* resulted in a reduction in colony-forming units (CFU) relative to wells containing no THP-1 cells. Addition of MoAb to suspensions of THP-1 cells and *C. neoformans* resulted in even greater reduction in CFUs. The relative efficacy of enhancing THP-1 reduction in CFUs was IgG3 > IgG1 > IgG2a > IgA > IgG2b > IgE (Fig. 6). Control experiments where the IgG1, IgG2a, IgG2b, IgG3, IgA and IgE MoAbs were added to *C. neoformans* produced no reductions in CFU, indicating that antibody alone had no effect on fungal cell viability or growth.

DISCUSSION

The inability of existing anti-fungal drugs to eradicate the infection in immunocompromised patients demonstrates the need for a new therapeutic approach. In that respect, *C. neoformans* infection is even more challenging, since the capsular polysaccharide which is released into body fluids and tissues causes in many instances a phenomenon of antibody unresponsiveness similar to the 'immune paralysis' described for pneumococcal polysaccharide. The polysaccharide capsule is poorly immunogenic and inhibits phagocytosis [21]. The potential usefulness of using antibodies in *C. neoformans* infection is suggested by *in vitro* and *in vivo* experiments. Anti-*C. neoformans* antibodies enhance phagocytosis and killing *in vitro* and confer protection *in vivo*. Nevertheless, examination of protective efficacy of several MoAbs suggests that both isotype and epitope specificity are very important, with IgG3 demonstrating only marginal protection [8,12,22,23].

The likelihood of recovery of spontaneous isotype switch mutants from hybridoma cell lines is dependent on the frequency of switching events *in vitro*. Unlike spleen B cells, where switching can be enhanced and targeted to certain isotypes by cytokines [24,25], the fate of hybridoma cells depends on the spontaneous rate of switching for each clone. Frequencies of one to four switch variants per 10^5 cells are common among γ1-secreting hybridoma cells, with IgM-producing hybridomas demonstrating even lower frequencies [16]. Unfortunately, these frequencies often preclude the successful isolation of spontaneous switch variants. Furthermore, some hybridomas preferentially switch only to certain isotypes. For example, IgM- or IgG3-secreting hybridomas usually switch to IgG1, making recovery of IgG2a- or IgG2b-secreting cells difficult. In this study we report the isolation of a complete set of isotype switch variants from an IgG3 hybridoma cell line following treatment with acridine orange ICR191. Since ICR191 is a mutagen, it was important to demonstrate that both the constant and variable regions were structurally and functionally intact. Biosynthetic labelling of hybridoma protein with ^{35}S-methionine followed by SDS–PAGE revealed no differences in the size of the light chain proteins, while the heavy chain proteins were of the expected molecular weight for the isotype in question. The integrity of the variable region was demonstrated by serological assays. Binding curves to GXM were similar for most isotypes. Furthermore, experiments conducted using a 10 amino acid long peptide which is a carbohydrate mimetope with specificity for the 3E5 binding site, or MoAb anti-idiotope indicate that all switched antibodies maintained the same fine specificity. The slight differences noted with respect to the binding of these two reagents could reflect either the higher avidity of IgG3 which results from the ability of this isotype to form Fc–Fc interactions [26], or the flexibility of the different isotypes. In spite of these differences, it is worth noting that the spontaneous γ1 and the parental γ3 variants share identical variable region sequence [10]. The ability of the parental γ3 and the isotype switch variants to react with *C. neoformans* was demonstrated by

direct agglutination and by their potential to promote phagocytosis and enhance the antifungal efficacy of the human THP-1 cell line. In the absence of specific antibody there was little or no phagocytosis of *C. neoformans* by THP-1 cells. Addition of any of the 3E5 isotype switch variants produced a marked increase in phagocytosis of yeast cells.

There were significant differences in the opsonic efficacy of the various isotypes. IgG3 and IgE were the most and least effective isotypes, respectively, in promoting phagocytosis. Isotype opsonic efficacy in this system presumably reflects both the Fc receptor density and the location, orientation, and accessibility of antibody constant regions bound to the capsule of *C. neoformans*. Co-incubation of THP-1 cells with *C. neoformans* in the absence of MoAb resulted in a significant reduction in yeast cell CFUs, indicating extracellular fungicidal and/or fungistatic activity of this cell line. Addition of each of the isotype switch variants resulted in significant enhancement of THP-1 antifungal activity. The IgG3 and IgE MoAbs were the most and least effective iso-types, respectively, in promoting reduction of CFUs by THP-1 cells. For the other isotypes, there was no clear correlation between opsonic efficacy and ability to enhance THP-1 antifungal efficacy. This may reflect temporal differences in the incubation time, since the phagocytic assay was done after 2-h incubation whereas the CFU assay was done after 24-h incubation. In that respect it is worth noting that γ1 MoAb conferred protection in a mouse model, whereas γ3, which was the most potent antibody *in vitro*, was not protective in murine models of infection [8]. The discrepancy between protective (γ1 MoAb) and non-protective (γ3 MoAb) activity and its correlation to antifungal capacity is yet to be studied. Protection experiments in animals lacking components of the normal immune response may elucidate this phenomenon.

To our knowledge 3E5 IgE is the first IgE MoAb to a pathogenic fungus. Little is known of the role of IgE in protection against fungi. Comparison of the IgE MoAb with the IgG MoAbs indicates that the IgE isotype is a less effective opsonin, but this may be only a reflection of a lower prevalence of IgE receptors in THP-1. Nevertheless, the fact that IgE was opsonin and enhanced THP-1 antifungal efficacy suggests that specific antibodies of this isotype may be able to mediate protection against fungal targets *in vivo*. The availability of 3E5 IgE provides the opportunity for further studies to evaluate the potential role of this isotype in protection and pathogenesis during experimental *C. neoformans* infections.

In this study we demonstrate the feasibility of using chemical mutagenesis to facilitate the recovery of functionally active isotype switch variants from a hybridoma cell line. The availability of the full set of anti-cryptococcal antibodies is an essential tool for the comparative study of the role of constant region function in protective efficacy against *C. neoformans*.

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