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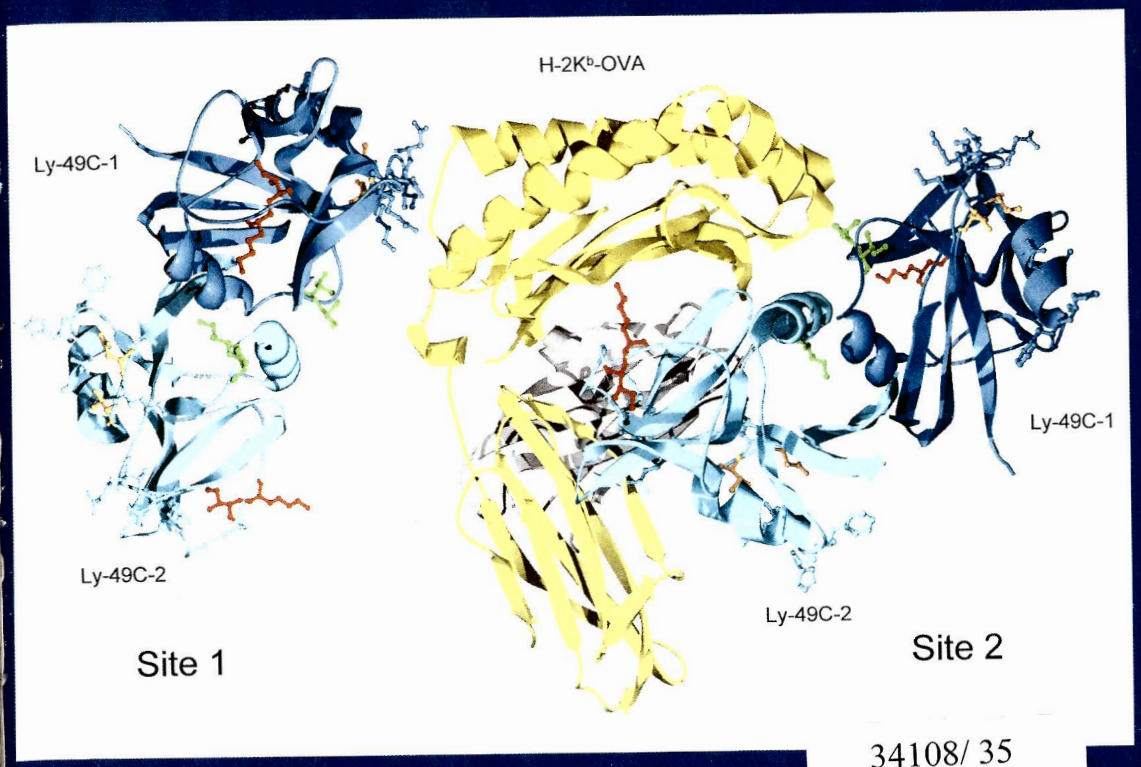

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On the cover: Model of Ly-49C and H-$2K^b$-OVA based on the crystal structure of Ly-49A-H-$2D^d$. Site 1 and site 2 interactions are shown. The H-$2K^b$ H chain is yellow, β_2m is gray, the peptide is orange, the Ly-49C-1 subunit is blue, and Ly-49C-2 is light blue. Ly-49C mutated residues that affected MHC class I binding: N160D/T162K are shown in orange, I195/P196 are shown in green, and K225/I226 are shown in red. Mutated residues that did not affect the Ly-49C-H-$2K^b$ interaction are shown as ball-and-stick molecules in backbone color. Ly-49C was threaded on the crystal structure of Ly-49A and was overlaid in the same orientation as Ly-49A in the Ly-49A-H-$2D^d$ structure (pdb entry 1Q03). The crystal structure of H-$2K^b$-OVA (pdb entry 1VAC) was aligned with the $\alpha_1\alpha_2$ domains of H-$2D^d$ in the crystal. The modeling and image were prepared using the Swiss-PDB Viewer, and the image was refined with the Persistence of Vision Ray Tracer. (Figure 5 from Sundbäck, J., A. Achour, J. Michaëlsson, H. Lindström, and K. Kärre. NK cell inhibitory receptor Ly-49C residues involved in MHC class I binding. *J. Immunol. 168:793*)

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Isotype Can Affect the Fine Specificity of an Antibody for a Polysaccharide Antigen[1]

Gary R. McLean,[2,3]* Marcela Torres,[2]* Natalia Elguezabal,* Antonio Nakouzi,* and Arturo Casadevall[4]*†

Ab specificity is determined by V region sequence. The murine Mab 18B7 (IgG1) binds to the *Cryptococcus neoformans* capsular polysaccharide glucuronoxylomannan and produces annular immunofluorescence (IF) on yeast cells. The heavy and light V regions of 18B7 were expressed with the human C regions μ, γ1, γ2, γ3, γ4, and α1, and the specificity and binding properties of these mouse-human chimeric (ch) Abs was determined. The chIgG1, chIgG2, chIgG4, and the chIgA produced annular IF, whereas the IgM and IgG3 produced punctate IF, despite identical V region sequences. Competition experiments with murine Abs that competed with mAb 18B7 and binding assays to peptide mimetics of glucuronoxylomannan provided additional evidence for altered specificity in some of the ch Abs. Expression of 18B7 heavy V region with murine μ C region produced IgM with a punctate IF, indicating that a change in fine specificity also accompanied the change from murine IgG1 to IgM. Our results show that Ab fine specificity can be a function of isotype. This phenomenon may be most apparent for Abs that bind to Ag with repeating epitopes, such as polysaccharides, where the quarternary structure of the Ag-Ab complex may be influenced by such constraints as Fab-Fab angles, Fc-Fc interactions, Ab size, and solvent accessibility to exposed surfaces. Alterations in Ab fine specificity following isotype change could have important implications for current concepts on the generation of secondary Ab responses to certain Ags and for the isotype preference observed in Abs to polysaccharides. ***The Journal of Immunology,*** **2002, 169: 1379–1386.**

A central concept in our understanding of Ab structure and function is that the V region determines Ag specificity, whereas the C region determines the isotype, effector functions, and pharmacological characteristics of Ig molecules. Ab specificity and affinity are believed to result from the type of interactions made between V region amino acid residues and Ag surfaces. This concept is supported by overwhelming evidence from biochemical, immunological, and crystallographic studies showing that V regions contact Ag and that this interaction is responsible for the specificity of the Ab molecule. Isotype switching from IgM to IgG and IgA is considered to result in Abs that retain Ag specificity with the acquisition of new biological effector functions conferred by the different C region. The C region is not generally believed to contribute to specificity, but can have a major effect on the apparent affinity through its contribution to valency, and hence, avidity. Nevertheless, there is some evidence that C region interactions among Abs bound to Ags with repeating structural motifs can affect the types of complexes formed (1).

The construction of mouse-human chimeric (ch)[5] Abs is predicated on the premise that murine V regions confer and retain Ag specificity in the context of expression with human C regions. (2). Mouse-human ch Abs have been used for comparative studies of human C region function (3, 4) and for the calibration of serological assays as human Ab standards (5). Mouse-human ch Abs are more attractive for use in human therapy than murine Abs because they are less immunogenic, have longer serum half-life, and possess human C region effector function (6). Currently, ch Abs are under clinical evaluation for a variety of conditions. The use of mouse-human ch Ab in clinical and laboratory applications is based on the implicit assumption that they retain the specificity of the parent murine Ab.

The murine mAb 18B7 (IgG1-κ) is currently in clinical trial for evaluation as an adjunct to antifungal therapy in the treatment of *Cryptococcus neoformans* infections (7). This mAb binds to glucuronoxylomannan (GXM), which is the major constituent of the *C. neoformans* polysaccharide capsule. To improve effector cell function for human phagocytic cells and to reduce its immunogenicity in humans, we synthesized ch human-murine mAbs containing the heavy (V_H) and light (V_L) V regions from the murine mAb 18B7 and human C regions for the various Ig isotypes (μ, γ1, γ2, γ3, γ4, and α1; Ref. 3). To our surprise, the 18B7 mouse-human IgM, IgG3, and IgG4 ch Abs revealed differences in fine specificity from the other ch Abs and the parent murine mAb 18B7 despite identical V region sequences. Similarly, expression of 18B7 V_H with murine IgM resulted in altered specificity. The results imply that in certain conditions, the C region can affect the conformation of the V region that may in turn lead to changes in idiotype and fine specificity.

Materials and Methods

Murine and ch Abs

The IgG1 murine mAb 18B7 has been described (7). This mAb binds to the *C. neoformans* polysaccharide and is currently in clinical evaluation. Cloning of the 18B7 V_L regions into pLC and the 18B7 V_H region into pHC μ, γ1, γ2, γ3, γ4, and α1 expression vectors was done as described previously (8). With the exception of IgG3 ch, Abs were expressed in the murine

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[5] Abbreviations used in this paper: ch, chimeric; V_H, heavy V region; V_L, light V region; GXM, glucuronoxylomannan; IF, immunofluorescence.

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myeloma cell line NSO as described (8) and harvested from culture supernatants. Briefly, NSO cells were routinely grown in DMEM supplemented with 10% FCS and clones stably transfected with the pLC vector were selected in the presence of 6 μg/ml puromycin. L chain clones stably transfected with the pHC vectors were selected in the presence of 1.5 mg/ml G418 as described (8). Cells expressing ch Abs were grown in DME with 10% FCS, 10% NCTC 109, 1% nonessential amino acids and 1 mg/ml G418 at 37°C and 10% CO_2. Subsequently, the cell lines were adapted to conditions with 5% FCS. For the chIgG3, protein was obtained by lysis of the transfected cells. The IgM ch Ab was determined to be pentameric from migration of a nonreducing SDS-PAGE relative to native human IgM.

Generation of IgG1 switch variant from IgM hybridoma 13F1

An IgG1 isotype variant of the murine mAb 13F1 was recovered by selecting for a spontaneous IgG-producing hybridoma using the ELISA spot method as described (9).

Expression of 18B7 V_H with murine IgM

RNA was isolated from hybridoma cells using TRIzol reagent (Life Technologies, Gaithersburg, MD). cDNA was generated using reverse transcriptase and the oligonucleotide primer p(dt)15 (Boehringer Mannheim, Indianapolis, IN). DNA containing the V_H was amplified using the primers 5′-ATAAAGCTTGCGGGGATATCCACCATGGAC and 3′-ATAGCTA GCTGAGGAGACTGTGA. The amplified DNA was cloned into the PCR 2.1 vector (Invitrogen, San Diego, CA) for sequencing and subcloned into a murine IgM expression vector, as described (10). The plasmid was then transfected into H chain nonproducing 18B7, 12A1, and 13F1 hybridoma cells by electroporation. Cells were selected with 1.5 mg/ml G418 (Geneticin; Life Technologies) in DME media with 20% of FCS (Harlan, Indianapolis, IN), 10% NCTC-109 (Mediatech, Herndon, VA), and 1% of nonessential amino acid solution (Mediatech).

Immunofluorescence

Glass slides were coated with poly L-lysine (0.1 mg/ml; Sigma-Aldrich, St. Louis, MO), a suspension containing 10^6 yeast cells was placed on the slide surface and the slide was air dried. The slides were then blocked by adding a solution 2% BSA and 0.5% goat serum and incubating for 30 min at 37°C. Abs were then added at a concentration of 5 μg/ml in blocking solution, and incubated overnight at 4°C. FITC-labeled goat anti-human IgG, IgM, IgA, or FITC-labeled goat anti-mouse IgM or IgG1 were added at 10 μg/ml after the incubation with primary Ab, and incubated at 37°C for 30 min. In other experiments, tetramethylrhodamine B isothiocyanate-labeled goat anti-human κ was used. Prior studies have established that the immunofluorescence (IF) pattern is the same regardless of whether one uses FITC-labeled primary or secondary Ab (11). The slides were washed three times with PBS between application of reagents. Finally, 30 ml of mounting medium (0.1 M *n*-propyl gallate–50% glycerol in PBS; Sigma-Aldrich) was added, and coverslips were placed. All labeled reagents were from Southern Biotechnology Associates (Birmingham, AL) except anti-human IgG, which was obtained from ICN Pharmaceuticals (Costa Mesa, CA). The slides were viewed with an Olympus AX70 microscope (Olympus, Melville, NY) equipped with FITC (fluorescein) and tetramethylrhodamine B isothiocyanate (rhodamine) filters. Fluorescent images were recorded with narrow band filter sets to ensure no cross-talk or spillover from one filter set to the others. Gray scale images were merged. This method is equivalent to multiple exposure photography, but has the advantages of wider linear response and dynamic range, optical separation of filters, and digital storage. Images were recorded in black and white. Color corresponding to the filter wavelength was subsequently added back during image reconstruction to reflect the actual color observed.

ELISA

mAb concentration was determined by ELISA relative to isotype-matched standard controls (Sigma-Aldrich). Polystyrene plates were coated with goat anti-human IgM, IgG, or IgA (Accurate Chemical and Scientific, Westbury, NY) blocked with 1% BSA in PBS and incubated with the ch Ab-containing solution. Bound Ab was detected using alkaline-phosphatase or biotin-conjugated goat anti-human IgM, IgG, or IgA (Accurate Chemical and Scientific). Ab binding to GXM was measured as described (6), except that bound Ab was detected using phosphatase-conjugated goat anti-human κ (Caltag Laboratories, San Francisco, CA). Competition ELISAs were done to determine whether murine mAb 12A1 could inhibit the binding of the ch Abs to GXM using the same protocols as described previously (7, 8). For this ELISA, polystyrene plates were coated with 2 μg/ml of GXM and incubated for 1.5 h at 37°C. The plates were then blocked with 1% BSA-PBS and incubated overnight at 4°C. Ch Abs and mAb18B7 were added and serially diluted, followed immediately by the addition of IgM mAb 12A1 (final concentration 1 μg/ml). The plates were incubated overnight at 4°C. Binding of murine IgM was detected by specific alkaline-phosphatase-conjugated goat anti-mouse reagents. Reactivity of ch Abs with the murine anti-idiotypic mAb 7B8 was also measured by ELISA. The anti-idiotypic mAb 7B8 binds to the Ag-combining site of 18B7 (9). Plates were coated with 1 μg/ml of goat anti-mouse κ, blocked with 1% BSA in PBS, and incubated with the ch mAb preparation. The plates were then incubated with 2 μg/ml solution of mAb 7B8 (IgG1) and binding was detected with phosphatase-conjugated goat anti-mouse IgG1. For peptide ELISA, polystyrene plates were coated with 1 μg/ml solution of streptavidin in PBS followed by blocking with 1% BSA in PBS. Biotinylated peptide was then added at a concentration of 2 μg/ml before testing mAb binding by titration. Specific binding of ch18B7 mAb was detected using phosphatase-conjugated goat anti-human κ Ab.

To prepare monomers of IgM, the ch mAb (4 μg/ml) was mixed with an equal volume of a 0.15-M solution of 2-ME in TBS and incubated for 1 h at 37°C immediately before addition to the ELISA plate for GXM binding or anti-idiotype ELISAs. The reduced product of IgM and IgG ch Ab contained both H and L chains by ELISA, indicating that the reduction had not disrupted the monomer.

Phagocytosis assay

J774.16 cells were cultured in DMEM with 10% heat-inactivated FCS, 10% NCTC-109 medium (Life Technologies), and 1% nonessential amino acids (Cellgro, Washington, DC). The protocol for in vitro phagocytosis was as described in earlier studies (11) with minor modifications. Cells were plated in 96-well tissue culture plates (Falcon; BD Biosciences, Franklin Lake, NJ) at a density of 10^5 cells/well in 96-well culture plates and stimulated with 50 U/ml recombinant murine IFN-γ (Genzyme, Cambridge, MA) and LPS (Sigma-Aldrich). Briefly, macrophages were stimulated with 50 U/ml IFN-γ/ml and incubated at 37°C overnight. The medium in each well was then replaced with fresh medium containing 50 U/ml IFN-γ and 1 μg/ml LPS, and *C. neoformans* cells were added at a ratio of 5:1 macrophages to fungi. Phagocytosis was measured in the presence or absence of ch Abs (2 μg/ml). After the addition of *C. neoformans*, the cells were incubated at 37°C for 3 h, washed three times with sterile PBS, fixed with cold absolute methanol, and stained with a 1/20 solution of Giemsa. PBS was used as a negative control and mAb 18B7 (IgG1) was used as a positive control, and phagocytic indices were determined. The phagocytic index is defined by: $PI = P \times F$, where P is the percentage of phagocytic macrophage and F is the average of yeast cells per macrophage. Experiments were done in duplicate and four different fields were counted per well.

Results

V region sequences

The 18B7 V_H and V_L nucleotide sequences have been previously reported based on direct mRNA sequencing (12). The 18B7 V_H and V_L region cDNA was cloned from 18B7 hybridoma cells by RT-PCR and inserted into human Ig expression vectors as described (8). V_H and V_L region sequence analysis revealed several differences from the previously published sequences (7, 12). For 18B7 V_H, there were three amino acid differences resulting from single-base changes. In the CDR1 region, positions 28 and 29 were Ser and Phe rather than Thr and Cys, respectively, and position 65 of the CDR2 was Arg instead of Lys. For the 18B7 V_L, there were two amino acid differences resulting from single-base changes in the cDNA sequence. Amino acid 15 of the first framework region was Leu rather than the original Ala, and CDR1 position 27C was Ala rather than Cys. These updated 18B7 V region sequences are deposited in GenBank under accession nos. AJ309276 and AJ309277. For each cell line producing the ch Abs, the V_H and V_L regions were sequenced and shown to be identical with each other and with those of the parent mAb 18B7. Multiple clones of each V region were analyzed.

Reactivity of ch Abs with GXM

The IgM, IgG1, IgG2, IgG3, IgG4, and IgA1 mouse-human ch Abs bound to GXM immobilized in polystyrene plates by ELISA (Fig. 1). Given that each ch Ab type had identical V region sequence, we

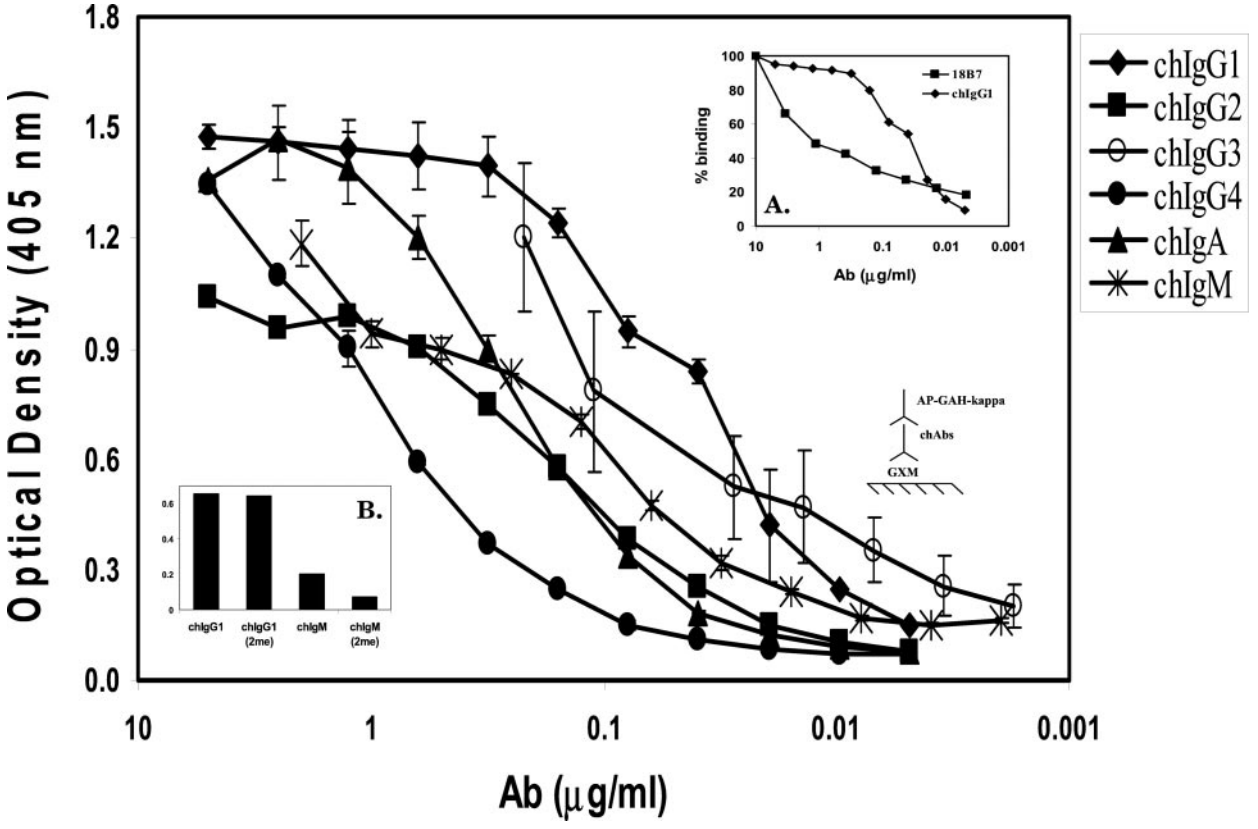

FIGURE 1. GXM binding of ch Abs by ELISA. The chIgG1, chIgG2, chIgG4, chIgA, and chIgM ch Abs bound to GXM immobilized in polystyrene plates by ELISA. The diagram represents the ELISA configuration. Data points are the average of two measurements, and brackets represent SEs. For some points, the error bars are smaller than the symbol used. For chIgG3, the concentration range studied was relatively small because there was insufficient Ab available. The chIgM and chIgG3 are from a different assay than the others, but are plotted here together because very similar results were observed in multiple assays in different days done by different investigators. *A,* A plot of chIgG1 relative to mAb 18B7 to compare their relative binding to GXM. *B,* Reactivity of chIgG1 and chIgM before and after 2-ME reduction.

were surprised to find large differences in binding to GXM (Fig. 1) and to mAb 18B7 (Fig. 1*A*). The relatively weaker binding observed for the chIgM was unexpected, since this molecule was pentameric as indicated by nonreducing SDS-PAGE (data not shown), and the higher valency should have conferred a higher avidity. To explore the potential contribution of valence to the binding of the chIgM, 2-ME was used to dissociate the IgM polymeric structures into monomers (13). Treatment of IgM with 2-ME resulted in complete loss of Ab reactivity, whereas identical 2-ME treatment of chIgG1 had no effect on GXM binding (Fig. 1*B*). Because IgM monomers have the same V region sequence as the IgG subtypes, the 2-ME reduction experiment indicated that the 18B7 V_H-human cμ chimera had significantly reduced affinity for Ag.

Immunofluorescence patterns of C. neoformans *with murine and ch Abs*

Prior studies have shown that the IF pattern resulting from Ab binding to encapsulated *C. neoformans* cells is a sensitive indicator of Ab specificity that correlates with biological activity (11). The IF pattern of mAb 18B7 with *C. neoformans* is annular (Fig. 2) and the expectation was that all the ch derivatives would also produce annular patterns upon binding yeast cells. The IF pattern observed with chIgG1, chIgG2, chIgG4, and chIgA1 Abs was annular (Fig. 2). Nevertheless, we noted differences in the IF pattern for the IgG chs as manifested by differences in fluorescence intensity throughout the capsule (Fig. 2). In particular, the chIgG4 gave a thick annular pattern that was different from the other IgG subtypes. Surprisingly, the IgM and IgG3 ch Ab produced a punctate pattern that was qualitatively different from that observed with the parent murine mAb or the other ch Abs (Fig. 2). The same patterns were obtained regardless of whether the secondary Ab was specific for the human H or L chain. The punctate patterns observed with the IgM and IgG3 chs were similar to that observed with the murine mAb 13F1, which is not protective and not opsonic (14, 15).

Competition experiments and reactivity with peptide mimetics

Because the altered IF patterns for chIgM and chIgG3 suggested an alteration in V region specificity as a result of expression in association with human μ and γ3, we proceeded to explore this possibility by Ab competition assays and reactivity with peptide mimetics. In previous studies, we have used the absence of Ag competition between two Abs as a criterion for different specificity (16). Competition experiments were performed with the murine mAb 12A1 (IgM) that produce annular patterns on binding *C. neoformans* and has similar specificity to 18B7 (17). As is evident in Fig. 3, murine mAb 12A1 inhibited the binding of ch Abs as follows; chIgA1 $>$ chIgG1 $>$ murine 18B7 $\gg$ chIgG2 $>$ IgG4 $\sim$ IgM $>$ IgG3. Absence of competition with an mAb that inhibits the parent mAb 18B7 provides strong evidence for altered specificity in the chIgM, chIgG2, chIgG3, and chIgG4 Abs. Competition experiments between mAb 18b7 and ch Abs revealed that it competed with chIgG1, chIgG2, and chIgG4, but not chIgM or chIgG3 (data not shown). As a second confirmatory method, we measured the binding of ch Abs to two GXM peptide mimetics that

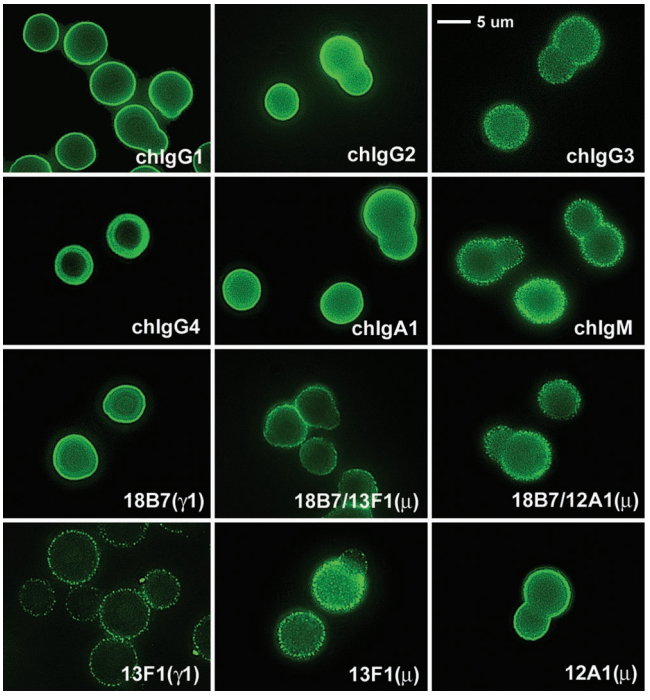

FIGURE 2. Immunofluorescence of *C. neoformans* stained with ch and murine Abs. Each photograph was taken under identical conditions. Cells shown are representative of patterns observed.

have proven useful for identifying small differences in fine specificity among mAbs with identical V region usage (18). Significant differences were noted for the reactivity of the various ch Abs and the parent mAb 18B7 for two peptides, providing additional evidence for alterations in specificity (Fig. 4).

Reactivity of parent and ch Abs with anti-idiotypic mAb

The idiotype of mAb 18B7 is recognized by the murine anti-idiotypic mAb 7B8 (19). The mAb 7B8 interacts with both the V_H and V_L at the Ag combining site (9). The 18B7 Id structure is presumably conferred by the primary sequence folded into the Ag binding site. mAb 7B8 bound strongly to chIgG1, chIgG2, and chIgA1 Abs, but weakly to chIgM, chIgG3, and chIgG4 (Fig. 5). Treating the IgM ch with 2-ME to create monomers did not restore

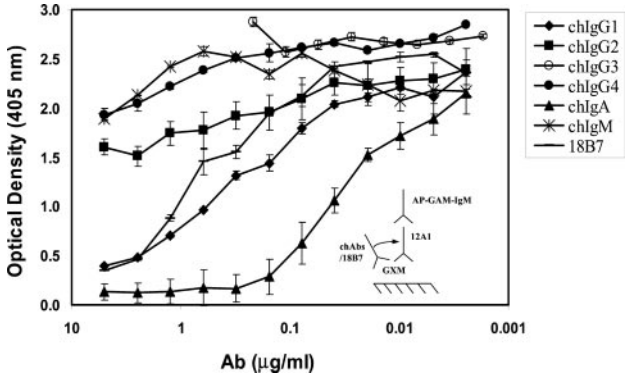

FIGURE 3. Binding of ch Abs and parental mAb 18B7 in the presence of mAb 12A1 by ELISA. The diagram represents the ELISA configuration. Points are the average of two measurements, and brackets represent SEs. For some points, the error bars are smaller than the symbol used. mAb 12A1 strongly inhibits binding of chIgA and chIgG1 and mAb 18B7, but is less effective at interfering with the binding of IgM, IgG2, IgG3, and IgG4 ch Abs.

the 18B7 Id. Hence, there were significant differences in the reactivity of ch Abs with an anti-Id mAb to mAb 18B7 despite identical V region sequences.

Opsonic efficacy of ch Abs

The chIgG1, chIgG2, and chIgG4 were strongly opsonic, whereas chIgA1 had weaker opsonic activity (Fig. 6). The opsonic efficacy of chIgG3 could not be evaluated because we lacked sufficient protein for this experiment. The opsonic activity of chIgG1, chIgG2, and chIgG4 was comparable to that of mAb 18B7. For murine IgM mAbs, the opsonic activity for *C. neoformans* correlates with the type of IF pattern such that Abs with annular IF efficiently promote phagocytosis, whereas those with punctate IF do not (14, 15). For chIgM, the opsonic efficacy was significantly lower than the opsonic murine IgM mAb 12A1, but higher than that observed for the nonopsonic murine IgM mAb 13F1.

Murine isotype and IF pattern

Because punctate IF patterns had previously been observed only with murine IgM mAbs (14, 15), we investigated whether punctate IF was an inherent property of the IgM isotype with certain V regions by carrying out two experiments. First, we selected a spontaneous switch variant from the hybridoma 13F1 that synthesized IgG1. The 13F1 IgG1 variant produced a punctate IF pattern like its parent IgM (Fig. 2). Second, we expressed the 18B7V_H with a murine μ C region to produce IgM. We attempted to express the 18B7 V_H-μ with 18B7V_L, but were unable to obtain an IgM-producing clone after screening >300 clones in three independent transfection experiments. Our inability to express 18B7 V_H-μ with 18B7V_L despite considerable effort suggests that the isotype switch that produced the B cell parent for the 18B7 hybridoma resulted in changes that precluded IgM expression. Instead, we resorted to expressing 18B7 V_H-μ with the V_L of IgM mAbs 12A1 and 13F1. Prior studies had established that the IF pattern is a property of the V_H (10). The 18B7 V_H-μ was expressed with the V_L of mAbs 12A1 and 13F1, which produce annular and punctate IF patterns, respectively, as a function of their V_H sequences (10). Binding of 18B7 murine IgM to *C. neoformans* produced a different IF pattern than that observed with the parent IgG1 mAb in combination with both the 12A1 and 13F1 V_L (Fig. 2). The 18B7/12A1 and 18B7/13F1 Abs each reacted with GXM, but only 18B7/13F1 was recognized by the anti-Id mAb 7B8 (Fig. 7). Competition assays with mAb 18B7 revealed competition with 12A1/13, but not 18B7/12A1 (Fig. 7). These results imply a change in the fine specificity of the 18B7 V_H when expressed with murine μ relative to that observed when expressed as γ1.

Discussion

Each of the mouse-human ch Abs derived from mAb 18B7 bound to the *C. neoformans* capsule and to soluble GXM. Hence, each ch Ab retained the Ag specificity of the parent murine mAb. However, to our surprise, there were differences in the serological properties of the ch Abs with GXM relative to the parent mAb 18B7. Most strikingly, chIgM, chIgG3, and chIgG4 displayed different IF after binding *C. neoformans* cells compared with that of mAb18B7, the other ch18B7 IgG subclasses, and the ch18B7 IgA1. Specifically, the chIgM and chIgG3 produced punctate IF, whereas the other ch Abs and mAb 18B7 produced annular IF. The chIgG4 produced a thick annular IF different from the other Abs. When comparing their binding to GXM, IF patterns, and reactivity with peptide mimotopes, each ch Ab was found to be different from the others and from the parent mAb 18B7. This result was unexpected since sequence analysis revealed that each of the ch

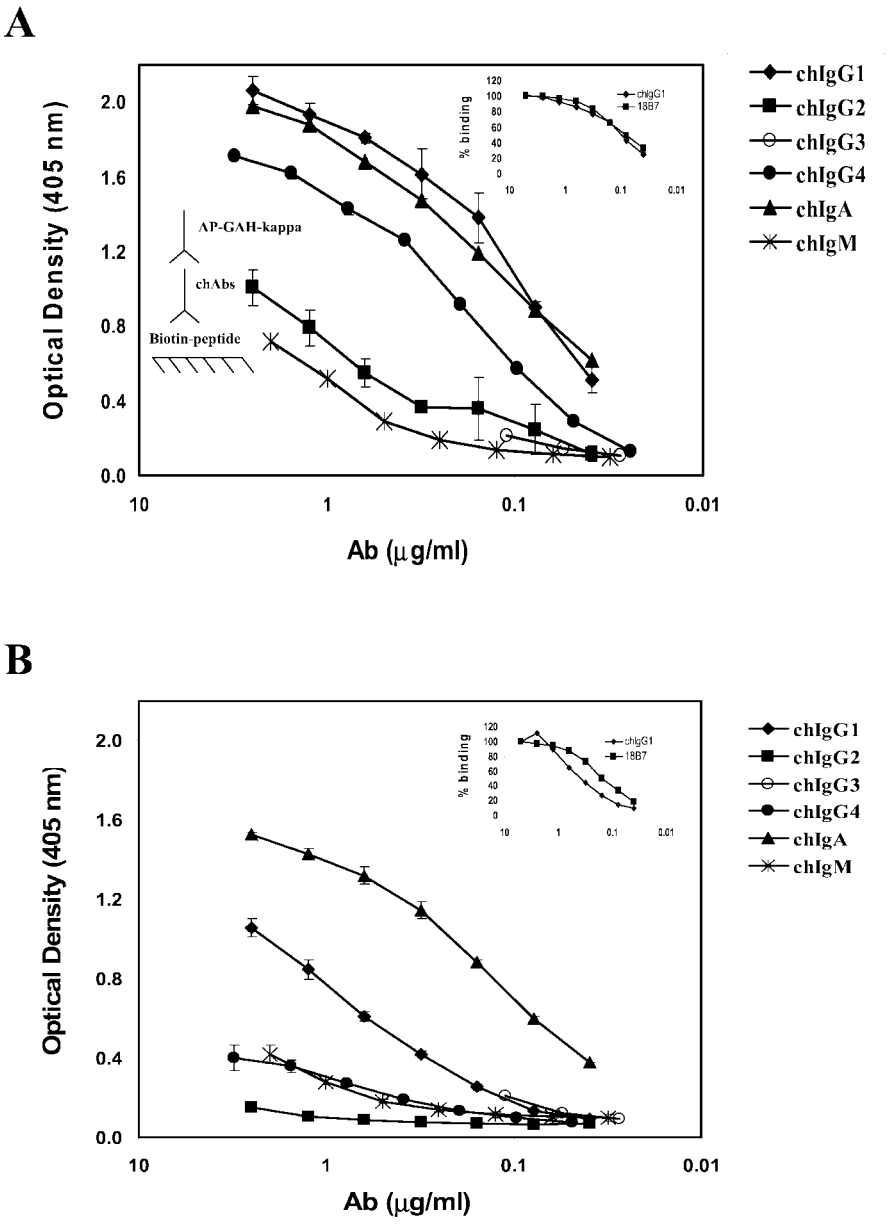

FIGURE 4. Reactivity of mAb 18B7 and ch Abs with GXM peptide mimetics P206.1 (*A*) and P601 (*B*) by ELISA. The diagram represents the ELISA configuration. Data points are the average of two measurements, and brackets represent SEs. For some points, the error bars are smaller than the symbol used. For chIgG3, the concentration range studied was relatively small because limited quantities of this Ab were available. Insets show plots of chIgG1 relative to mAb 18B7 to illustrate their relative binding to these peptides.

Abs had identical V_H and V_L sequence to the parent mAb 18B7. However, V region sequence analysis revealed several amino acid differences between the sequences amplified and cloned from the murine hybridoma 18B7 in this study and that reported previously based on direct mRNA sequencing (7). Because identical sequences were found repeatedly among independently amplified clones in this study, we attribute the differences between the current data and the earlier reports to errors associated with V region sequence determination by direct mRNA sequencing, which is known to be highly error-prone.

The observation that chIgM and chIgG3 gave a punctate IF pattern was intriguing because this type of IF pattern had previously been observed only with two nonprotective IgM murine mAbs (15, 16), which are not opsonic (14). We do not currently know the molecular structure of the carbohydrate domain recognized by the Abs binding in either punctate or annular IF. However, the differences in protective efficacy (15, 16), complement activation (20), and opsonic capacity (14) noted for mAbs that bind in annular in punctate IF patterns provide strong evidence that these represent different epitopes. For this discussion, we use the term "specificity" to denote the binding of Ab to the polysaccharide Ag GXM as manifested by reactivity with GXM, and the term "fine specificity" to denote the nature of Ab binding to GXM as manifested by the interaction of complexes formed by different Abs with GXM that is demonstrated by differences in IF and other serological assays. We are cognizant of the theoretical and practical difficulties involved in defining the terms specificity, fine specificity, and epitope in the absence of structural information (21, 22). Furthermore, we caution that Abs binding in annular or punctate IF are not necessarily binding at exactly the same site on the GXM or forming the same types of Ag-Ab complexes, a notion that finds support in this study.

We investigated whether expression of the 18B7 V_H with the human C regions altered Fab structure by evaluating the integrity of the 18B7 Id structure on the ch Abs. The mAb 7B8 demonstrated strong reactivity with chIgG1, chIgG2, and chIgA1, and weaker reactivity with chIgM, chIgG3, and chIgG4. Because the V region sequences of all the chs are identical, this result indicates that expression of the murine 18B7 V_H in the context of the human IgM, IgG3, and IgG4 altered the idiotypic structure, possibly a

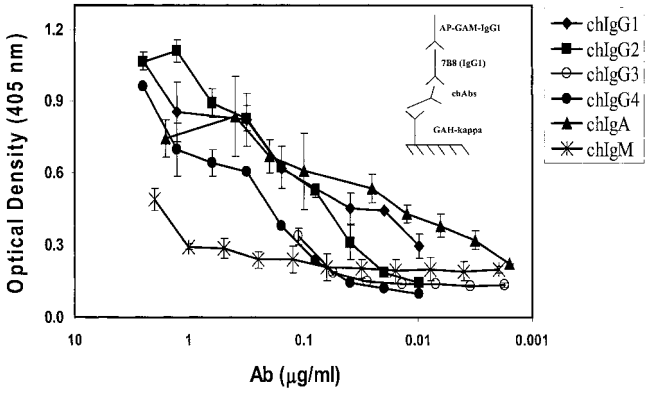

FIGURE 5. Reactivity of parent and ch Abs with the anti-idiotypic mAb 7B8 by ELISA. The diagram represents the ELISA configuration. Points are the average of two measurements, and brackets represent SEs. For some points, the error bars are smaller than the symbol used. For chIgG3, the concentration range studied was relatively small because there was insufficient Ab available. The chIgM and chIgG3 are from a different assay than the others but are plotted here together because very similar results were observed in multiple assays on different days done by different investigators.

result of differences in protein folding. This observation is consistent with the report that syngeneic V regions are more immunogenic, as IgM molecules when compared with IgG molecules, suggesting isotype-related differences in V region folding and exposure of antigenic epitopes (23).

Reduction of chIgM with 2-ME resulted in complete loss of Ag binding. This was unexpected because IgM monomers should bind to GXM with similar specificity and affinity as the IgG isotypes given identical V region sequences. Because the IgM C region sequence was that expected for this protein (8), mutations or deletions in the c_H domains can be excluded as an explanation for the altered fine specificity or the inability to bind as a monomer. Hence, for the chIgM, the change in fine specificity was accompanied by a reduction in apparent affinity and Id recognition consistent with an alteration in the protein structure at the Ag binding site.

Prior studies had shown that the punctate IF fine specificity was conferred by V_H (10), but we investigated whether expression of this pattern required the IgM valency structure. The approach was to generate an IgG1 isotype switch variant from a murine IgM that has punctate IF and to express 18B7 V_H as a murine IgM. An IgG1 isotype-switched variant was generated from the murine IgM mAb 13F1 that displays punctate IF. Like the parent 13F1 IgM, the 13F1 IgG1 isotype-switched variant revealed punctate IF, a finding indicating that the IF pattern was not restricted to the IgM class. However, expression of the 18B7 V_H with the murine μ-chain to generate a murine IgM produced a different IF pattern than the parent IgG1 mAb 18B7. In fact, the pattern of the 18B7 IgM was very similar to the punctate IF observed with chIgM, chIgG3, and mAb 13F1. These results indicate that punctate IF patterns can be observed with both IgM and IgG isotypes. For the specific case of 18B7 IgM, the change from annular to punctate observed for IgG1 and IgM, respectively, may reflect a change in fine specificity resulting from greater avidity. This phenomenon may be similar to that observed to occur for murine IgG3 to streptococcal polysaccharide which can form different types of Ag-Ab complexes than other V region identical IgG isotypes because of ability to aggregate and increase its avidity (1).

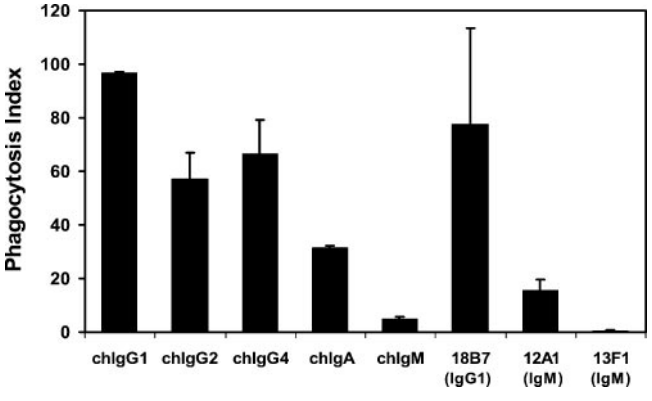

FIGURE 6. Opsonic efficacy of murine mAbs and ch Abs for *C. neoformans* with J774.16 cells. Values represent the average of eight measurements and brackets represent SD.

chIgG3 also produced a punctate IF pattern, but retained reactivity with the anti-Id mAb, suggesting that steric and/or flexibility constraints may also contribute to the altered fine specificity observed for both IgM and IgG3. Among human IgG subclasses IgG3 is the most flexible, as evidenced by its ability to form ring dimers with anti-Id mAbs (24). IgG3 complexes also demonstrated the largest Fab-Fab angles among IgG subclasses bound to anti-Id mAbs, presumably due to their extended hinge region (24). However, human IgM monomers are even more effective than IgG3 at forming ring dimers, suggesting that it is the most flexible Ab class (25). Because GXM is a polysaccharide with repeating epitopes and both human IgM and IgG3 are multivalent ligands which display great flexibility for their Fab domains, it is possible that the punctate patterns reflect the formation of Ag-Ab complexes in the capsule with different geometry than those associated with the annular IF pattern. Consistent with this view is the observation that Abs which produce annular IF on wild-type *C. neoformans* strains produce punctate IF when binding to mutants that express *O*-acetylation-deficient GXM, which is more flexible polysaccharide structure and may form different types of Ag-Ab complexes in the capsule (26). Another factor that may contribute to the type of complexes formed is the nature of the exposed surfaces of the various isotypes. In this regard, liquid partition studies have shown that human IgM and IgG3 also appear to have different surface properties from each other and from other IgG subclasses which could conceivably affect the microenvironment in which the complexes are formed (27). For chIgG4, the alteration of the IF pattern to a thick annular type may reflect the ability of IgG4 molecules to self interact, a phenomenon proposed to account for both its apparent monovalence and its capacity to form bispecific molecules (28).

In summary, we observed a change in IF pattern from annular to punctate when 18B7 V_H was expressed with murine and human μ C region and with human γ3 c and γ4 c. For each Ab, the explanation for the change in fine specificity may be different. For chIgM, this phenomenon may reflect an altered Fab structure (as evidenced by loss of Id reactivity and reduced affinity), greater intrinsic Fab flexibility (25), or different Ig surface properties (27). For the murine 18B7 IgM, the change in specificity may reflect a difference in avidity as reported for Abs to streptococcal polysaccharide (1). For the chIgG3, the change in specificity may reflect the enhanced Fab flexibility of this isotype relative to other IgGs (24), or different Ig surface properties (27). Furthermore, we did note variations in IF after binding *C. neoformans* and in the reactivity with GXM and peptide mimetics for the IgG1, IgG2, and IgG4 chs, suggesting that more subtle changes in fine specificity may have also occurred in the other ch Abs.

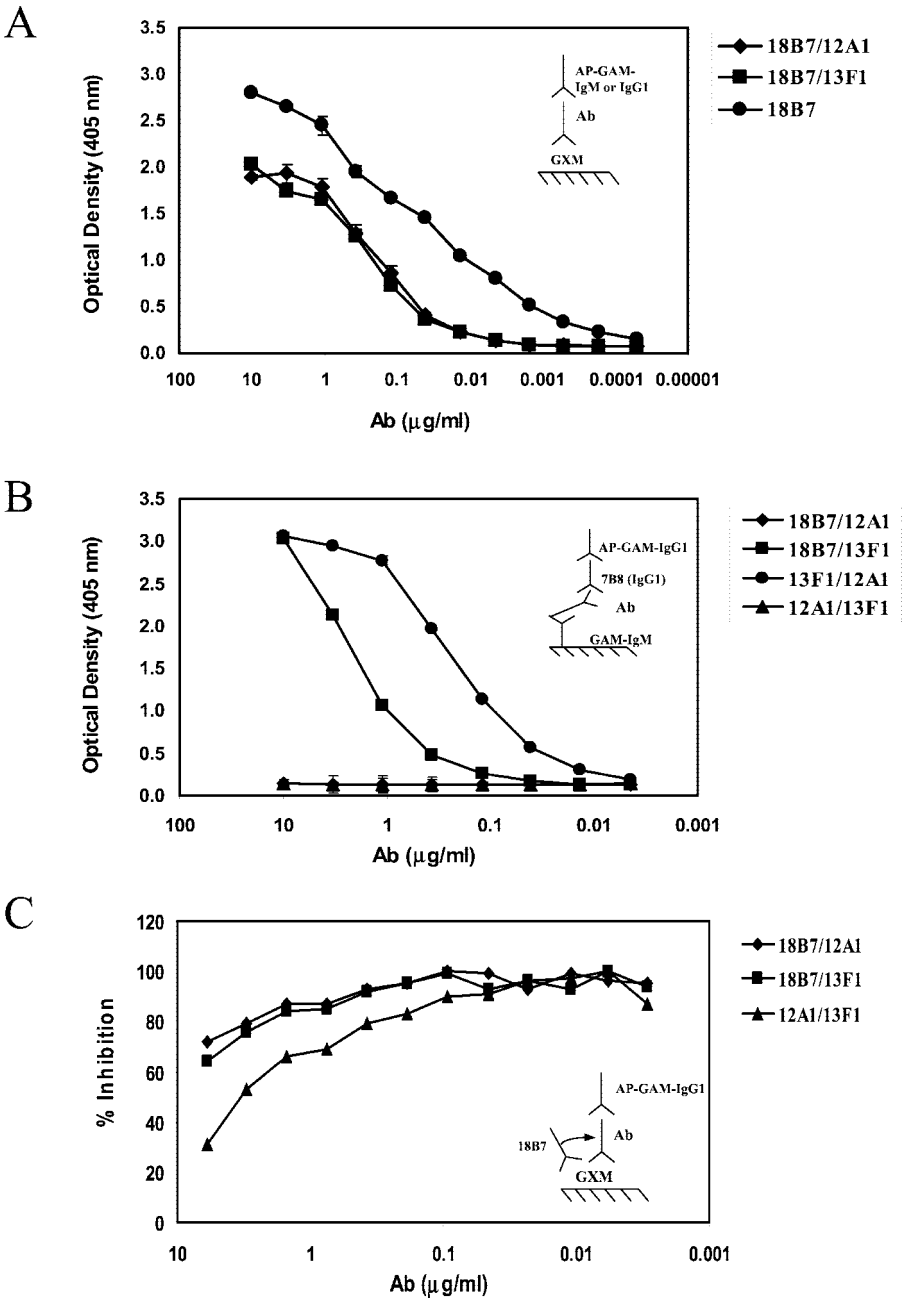

FIGURE 7. Reactivity of 18B7 V_H expressed as IgM with L chains of mAbs 12A1 and 13F1 with GXM (*A*), anti-idiotypic mAb 7B8 (*B*), and GXM in competition with mAb 18B7 (*C*).

The results of this study also shed light on the requirements for generation of the punctate IF pattern. Before this study, the punctate IF pattern had been observed only with two murine IgMs known as 13F1 and 21D2 (15). Recently, we used site-directed mutagenesis to establish that changing two amino acids in the in V region of an IgM mAb could change the IF pattern from annular to punctate (10). In this study, we demonstrate that the same V can produce annular or punctate patterns depending on which human or mouse C region it is associated with. Hence, the type of IF pattern is dependent on both the primary structure of the V region and the conformation of the Ag combining sites as determined by the associated C region.

Our observations add to the growing body of evidence that in certain circumstances, the Ig C region can affect the fine specificity of an Ab molecule. There are at least two potential mechanisms for this phenomenon. First, the C region could affect V region folding. This mechanism could explain isotype-related differences in Id immunogenicity (23) and the simultaneous loss of Id recognition for the chIgM and altered IF pattern on binding *C. neoformans* noted in this study. Second, the C region could influence the specificity of an Ab for an Ag with repeating epitopes through effects on segmental flexibility and the ability of certain Fc regions to associate (1). This phenomenon was demonstrated for a family of murine mAbs to group A streptococcal polysaccharide with identical V region which show isotype-related differences in their ability to bind bacterial strains expressing different epitope densities (1). Furthermore, there is evidence that C regions can affect the neutralizing activity of V-region identical Abs to human cytomegalovirus, possibly as a result of differences in the binding domain of the Ab to the target glycoprotein as a consequence of Fc-related effects (29). Another study reported that changing the C region of a viral enhancing murine mAb from IgG3 to a human IgG1 abrogated its deleterious activity in vitro, possibly as a result of changing the three-dimensional structure of the virus-Ab complex (30).

The observation that the C region can affect the specificity of an Ab raises the possibility that isotype switching in the immune response can be accompanied by a change in specificity. If this is the case, then isotype switching may lead to loss of recognition of the original Ag as well as the recognition of new epitopes. In fact, this could suggest an explanation for the observation that the primary and secondary response to certain Ags can arise from different precursors (31). A variation of this theme was suggested by the observation that IgA1 bind tubulin with significantly greater affinity than IgG1 as a consequence of c_H segmental flexibility, raising the possibility that affinity maturation can occur by isotype switching alone (32). Furthermore, our findings imply that certain types of Ag-Ab interactions may be limited to certain C regions and suggest a potential explanation for the biased preference of certain isotypes in polysaccharide responses. From a practical consideration, our observations suggest that fine specificity changes may be a relatively common phenomenon when ch or humanized Abs are constructed. This phenomenon may be limited to certain murine V regions, and/or may apply primarily to V regions that bind to Ags with repeating epitopes such as polysaccharides. Furthermore, the results are consistent with the view that differences in hinge angle and flexibility, possibly in combination with other variables such as C region size and glycosylation can affect the type of Ag-Ab complexes made. Our findings provide additional validation for the theory proposed by N. S. Greenspan (33) that valency can influence the specificity of the Ag-Ab interaction. Given the increasing use of mouse-human ch Abs in human therapy, it is critical that each ch Ab be carefully tested for specificity to insure the fidelity of the Ab engineering process.

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