The Mouse Antibody Response to Infection with Cryptococcus neoformans: $V_\text{H}$ and $V_\text{L}$ Usage in Polysaccharide Binding Antibodies

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Summary

Cryptococcus neoformans is a ubiquitous fungus that can cause serious infections in humans. The fungus has a polysaccharide (C. neoformans capsular polysaccharide; CNPS) capsule that contributes to its pathogenicity and can elicit an antibody response. Nevertheless, only 4 of 60 BALB/c mice chronically infected with C. neoformans had a detectable increase in serum anti-CNPS. The sera of three responder mice contained both IgM and IgG anti-CNPS antibody, and the titers of $\lambda$ and $\kappa$ anti-CNPS antibody were approximately equal. Eight IgM and one IgG3 monoclonal antibodies (mAbs) were generated from the spleen of one responder mouse, and one IgA was generated from the spleen of another mouse. Seven of the IgMs, the IgG3, and the IgA mAb had $\lambda$ light chains and were specific for serotype D CNPS. Molecular analysis confirmed that this was a highly restricted antibody response. All of the D-specific antibodies used $V_\text{H}441$, $J_\text{H}3$, and either $V_\text{L}2/J_\text{L}2$ or $V_\text{L}1/J_\text{L}1$, and all had the same heavy chain CDR3 amino acid sequence, even though there were differences in the nucleotide sequence of the N/D segment. One IgM mAb reacted with both serotype A and D CNPS, and this mAb used different $V_\text{H}$ and $J_\text{H}$ genetic elements and had $\kappa$ light chains. All the anti-CNPS mAbs used $J$ proximal $V_\text{H}$ gene elements that have previously been shown to bind dextran and other polysaccharides. Sequence and Southern blot analysis indicate that the serotype-D CNPS-specific mAbs arose from only a few precursor B cells.

The fungus Cryptococcus neoformans can cause serious infection in humans, and immunocompromised individuals are at particular risk (1). C. neoformans causes disease in up to 10% of individuals with AIDS (2). In the setting of AIDS, cryptococcal infections are usually incurable and often fatal (3). C. neoformans has a large polysaccharide capsule that inhibits phagocytosis by macrophages (4). The capsular polysaccharide is poorly immunogenic and causes the phenomenon of immune paralysis in mice (5–7). Structural differences in the capsular polysaccharides allow the grouping of cryptococcal strains into five serotypes, A, B, C, D, and AD (8, 9). Serotypes A and D cause the majority of infections in AIDS patients (10).

Cellular immunity is believed to provide the primary host defense against cryptococcosis (1). The role of humoral immunity to the C. neoformans capsular polysaccharide (CNPS) in protection is uncertain. Favoring an important role for antibodies are the observations that: (a) individuals with cryptococcal infection have a better prognosis if they have serum antibodies (11); (b) antibody enhances phagocytosis by macrophages (12), mediates fungistasis by NK cells (13), and facilitates leukocyte killing (14, 15); and (c) passive administration of an IgG1 murine mAb produced a sixfold increase in the survival of lethally infected complement-deficient mice (16), and potentiated the therapeutic action of Amphotericin B (17). However, certain observations are not consistent with an important role for humoral immunity. For example, B cell-deficient mice are not especially susceptible to cryptococcal infection (18); vaccination with immunogenic polysaccharide glyconjugates has not been protective in mice (19); and passively administered mAbs failed to protect mice in another model (20). Thus, many in vitro observations indicate an important role for antibody by enhancing cellular immunity, whereas some in vivo experiments have confirmed a protective effect and some have not. The finding that AIDS patients lack anti-CNPS IgG (21) raises the possibility that lack of antibody contributes to their marked susceptibility to cryptococcus.

The overall aim of our work has been to find mouse mAbs to CNPS that are protective against cryptococcus. This paper reports the serologic characterization of the serum response to cryptococcal infection and the generation of mAbs to CNPS.

1 Abbreviation used in this paper: CNPS, Cryptococcus neoformans capsular polysaccharide.
from these animals. Protection experiments using these mAbs are continuing and the results will be described elsewhere.

We have determined the primary structure of the anti-CPSNs mAbs from their mRNA sequences and studied the Ig gene rearrangements in the hybridomas to gain a more detailed understanding of the genetic and molecular basis for the responses.

Materials and Methods

C. neoformans. The strain used to infect mice was isolated from an AIDS patient with cryptococcal meningitis at Bronx Hospital Medical Center, and is referred to as "GH". Standard serotype strains A, B, C, and D (nos. 24064, 24065, 24066, and 24067, respectively) were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). The strains were maintained in Sabouraud's agar slants at 4°C. CNPS was prepared as described by others (22, 23). The concentration of polysaccharide was determined by the phenol-sulfuric acid method (24).

ELISA. ELISA plates (no. 25801; Corning Glass Works, Corning, NY) were coated with CNPS by incubating 50 µl of a 10 µg/ml solution of CNPS in 0.02 M PBS, pH 7.2, in each well at room temperature overnight. The concentration of protein in GH CNPS was determined with a protein assay (Bio-Rad Laboratories). Plates were blocked with a solution of 1% BSA in PBS. Alkaline phosphatase-conjugated goat anti-mouse IgM, IgG1, IgG3, IgG2a, IgG2b, IgA, X, and A reagents (Fisher Biotech) were used to develop the ELISA.

Mice. BALB/c mice were obtained from the National Cancer Institute (Bethesda, MD). The mice were infected with cryptococci intraperitoneally. Before inoculation, the yeast was washed with PBS and counted in a hemocytometer. Mice were bled from the retro-orbital sinus, and sera were separated by centrifugation and stored at −20°C.

Monoclonal Antibodies. mAbs to GH CNPS were made from chronically infected BALB/c mice with high serum titers. The use of spleens from infected mice posed the potential problem of hybridoma cell culture contamination with cryptococci. We avoided this problem by treating the mice with Amphotericin B and the hybridoma cultures with Nystatin. The mice were treated with Amphotericin B intraperitoneally (5-15 mg/kg total dose) during the week before harvesting the spleen to decrease the number of cryptococci in their tissues. We cultured the brain, heart, lungs, liver, and kidney from a mouse that had received 15 mg/kg of Amphotericin B, and found cryptococci only in brain tissue. Hybridomas were made by fusing splenocytes with NSO myeloma cells at a 4:1 ratio with polyethylene glycol by a protocol described previously (25). Nystatin (Gibco Laboratories, Grand Island, NY) was added to the hybridoma cultures at a concentration of 100 U/ml 1 d after the fusion. Hybridomas were screened by ELISA using plates coated with 50 µl of 10 µg/ml GH CNPS. Cells from positive wells were cloned in soft agar.

The mAb isotype and light chain type were determined using goat anti-mouse isotype and light chain-specific alkaline phosphatase-labeled antibodies. Hybridoma supernatants containing the mAbs were used for binding studies. The mAb concentration was determined by ELISA relative to standards of the same isotype and of known concentration for all antibodies except 4H3. Because the goat anti-IgG3 reagents were of low affinity, the 4H3 mAb was purified using an anti-mouse IgG column, dialyzed against PBS, and its concentration was determined by a protein assay (Bio-Rad Laboratories) using a myeloma IgG3 as a standard rather than by ELISA.

mRNA Sequencing. Total cellular RNA was prepared by the guanidinium method, and poly(A)+ mRNA was isolated using an oligo-dT affinity column. The nucleotide sequence of the antibody mRNAs was determined using a modification of the method of Gelebter et al. (26). The following oligonucleotides were used as primers: TGGATGGTGGAAGATG (α), TCTCGGAGGAGC- GAGGAGGA (µ), CCGGAAAGTCTGACAGAACA (VA), TGT- TCTGGCATITGCTCAG (Ve, Ge150.1), TACACGGATTTT- GGGCTT (V, 441), CGTGGAGGGCAGCGCCCTTGC (5' VA), GAGTTGTACCGGATGGATGGGT (Coa), GAGACCGAAATA- AACTCC (V, 5.1), TGGACAGGAGTTTCACG (5' Ve, 441), GGGTGGGAGCCGCGCTTGC (5' VA), GACCCCGAGA- AACGGGTT (5' V, 5.1), ACACAGGACGTACCTT (J2), CACCATGTTTACAGA (µ), AAGAAGCCTGAGAACAA- GCA (γ3). The oligonucleotide primers were made in the DNA synthesis facility in our institution. For 4H3, in addition to sequencing directly from the mRNA, Vµ cDNA was made using the primer AAGTAGCCCTTTTGACAGCCA and then amplified by the PCR using that same primer and GAGTTGAGCTTCCTGAGTCT. The total PCR product was then sequenced using the Sequenase kit (United States Biochemical Corp., Cleveland, OH) to confirm the 4H3 CDR3 sequence.

Southern Blot Hybridization. DNA was extracted from the NSO myeloma, the hybridomas, and BALB/c liver. Restriction enzyme fragments were separated in 0.8-1.0% agarose gels and transferred to modified Nylion membranes (GeneScreen) by blotting using 10× SSC (1× SSC is a 0.15 M NaCl and 0.015 M sodium citrate solution). Blots were prehybridized in a solution of denatured salmon sperm DNA (0.5-1.0 mg/ml) in 5× SSC, 7% SDS, 10× Denhard's reagent, 10% dextran sulfate, in 20 mM sodium phosphate buffer (pH 7.2) at 65°C. Hybridization was performed under the same conditions with DNA probes labeled with γ-[32P]-dCTP using random primers (Boehringer Mannheim Biochemicals). After hybridization, blots were washed initially with a solution of 3× SSC, 5% SDS, 10× Denhard's reagent, 10% dextran sulfate, in 20 mM sodium phosphate buffer, pH 7.2, and finally in a solution of 1× SSC and 1% SDS. Blots were washed at 65°C.

Immunofluorescence. A suspension of yeast (10⁶ to 10⁷ yeast/ml) in PBS was air dried on glass slides. The slides were first blocked with 1% BSA in PBS, then incubated with cell supernatants containing mAb at a concentration of 1 µg/ml, washed, and then incubated with fluorescein-labeled anti-mouse IgM.

Results

Mouse Antibody Response to Infection. The sera of BALB/c mice infected intraperitoneally with sublethal innocula of 10⁴ to 10⁶ cryptococci (GH) were assayed for the presence of antibody to GH CNPS. The antibody titer was measured by ELISA after serial dilution on plates coated with GH CNPS. Uninfected animals usually had serum titers of 1:50 to 1:100, which probably reflect the presence of crossreactive antibodies. Of 60 infected mice, only four had titers of anti-CNPS antibody >1:200. Fig. 1 shows the serum titers of IgM, IgG, IgA, X, and λ at several times after infection for three of the mice that had high titers of anti-CNPS. The fourth animal was killed early to fuse its spleen. These data show that (a) antibody titers peak between days 11 and 18 and then slowly decline with time even though these animals are chronically
infected; (b) both IgM and IgG are present; (c) in many of
the bleedings, the titer of X is roughly equivalent to that of t.

Monoclonal Antibodies. Spleens from four infected mice
were used in this study. Two of the spleens came from the
mice with the highest titers of antibody to CNPS. Since it
was possible that the low serum titers measured for the
majority of mice were the result of antibody sequestration
in complexes with polysaccharide, we also fused splenocytes
from two mice with low titers of antibody to CNPS (<1:200).
Each spleen was handled separately. Hybridomas producing
mAb to CNPS were obtained only from the spleens of the
animals with high serum titers of anti-CNPS antibody. One
of the two productive fusions used a spleen harvested on day
33 of infection (mouse 3 in Fig. 1), and it yielded seven IgM-
and one IgG3 mAb-producing hybridomas. The other produc-
tive fusion used a spleen harvested on day 17 of infection,
and it yielded only one IgA hybridoma—producing mAb.

Serological Characterization of the Monoclonal Antibodies. The
mAbs were initially characterized for heavy chain isotype,
light chain type, and binding to CNPS from standard ATCC
A, B, C, and D serotypes and the GH strain (Table 1). Al-
though the serotype of the GH strain used in this study was
not initially known, the reactivity of our panel of mAbs with
GH CNPS indicates that GH belongs to either the D or A/D
serotypes (9). With the exception of 21D2, all the mAbs had
κ light chains and reacted only with serotype D CNPS. The
21D2 mAb had a λ light chain and it reacted with CNPS
from both serotypes A and D. This indicates that 21D2 recog-
nizes a different epitope than the other mAbs.

Binding of 14A12 (μλ) or 21D2 (μκ) mAbs to cryptococci,
followed by staining with fluorescein-conjugated anti—mice
IgM, produced capsular fluorescence like those reported pre-
viously for both immune polyclonal sera (19) and for other
anti-CNPS mAbs (23) (data not shown). This confirms that
these mAbs bind to the cryptococcal capsule.

Fig. 2 shows binding curves of 14A12 (μλ) and 21D2 (μκ)
to GH CNPS. The binding curves of the other IgMκ antibiot-
ics, 7B13, 11E2, 12G5, 20B5, and 20C5, are indistinguish-
able from those of 14A12 and are not shown here. Since we
found no significant differences in the binding of these IgMκ
mAbs to GH CNPS or in their mRNA nucleotide sequences
(see below), 14A12 was designated as the prototype for the
IgMκ group. The binding curves of 14A12 and 21D2 are

Table 1. Characteristics of CNPS Binding Antibodies

| mAb    | Class | A | B | C | D | GH | Vw | Jw | VL | JL |
|--------|-------|---|---|---|---|----|----|----|----|----|
| 21D2   | IgMκ  | + | - | - | + | + | 50.1 | V_5.1 | J_1 |
| 14A12  | IgMλ  | - | - | + | + | + | V_441 | 3 | V_2 | J_2 |
| 11E2   | IgMα  | - | - | - | + | + | V_441 | 3 | V_2 | J_2 |
| 7B13   | IgMλ  | - | - | - | + | + | V_441 | 3 | V_2 | J_2 |
| 12G5   | IgMλ  | - | - | - | + | + | V_441 | 3 | V_2 | J_2 |
| 20C5   | IgMλ  | - | - | - | + | + | V_441 | 3 | V_2 | J_2 |
| 20B5   | IgMλ  | - | - | - | + | + | V_441 | 3 | V_2 | J_2 |
| 4H3    | IgG3λ | - | - | - | + | + | V_441 | 3 | V_1 | J_1 |
| 15C6   | IgAλ  | - | - | + | + | V_441 | 3 | V_2 | J_2 |

Class, light chain usage, reactivity with CNPS of serotypes A, B, C, D, and GH, and the Vw and Vl usage for the anti-CNPS mAbs. The symbols + and − denote binding and lack of binding, respectively, to ELISA plates coated with 10 μg/ml of CNPS from the different sero-
types. The 15C6 mAb is separated from the others by a space because
it was generated from the spleen of a different mouse. The Vw, Jw, and JL were determined from the Ig mRNA sequences. The V_50.1 and V_441 are gene elements belonging to the 7183 and X-24 gene fami-
lies, respectively (29). V_5.1 has been reported in antiprenolol mAbs
(32). The N/D segment sequences are shown in Fig. 4.
Figure 2. ELISA binding data of the 14A12 and 21D2 mAbs to GH CNPS. The graph shows a plot of OD_{405} vs. GH CNPS concentration, where the mAb concentration is kept constant at 1 μg/ml and the CNPS concentration is varied. The binding curves of the other IgMA antibodies, 7B13, 11E2, 12G5, 20B5, and 20C5, were like that of the 14A12 mAb and are not shown here. Note that the hybridoma supernatants were screened using plates coated with 10 μg/ml of GH CNPS.

Figure 3. Inhibition of mAb binding to CNPS-coated ELISA plates by soluble CNPS. The plates were coated with a solution of 10 μg/ml of GH CNPS. The antibody and CNPS were incubated for 1.5 h at 37°C. We calculated apparent binding constants (k_{a}) of 8 x 10^{6} M^{-1}, 2 x 10^{6} M^{-1}, 7 x 10^{5} M^{-1}, and 8 x 10^{5} M^{-1} for the 4H3, 21D2, 15C6, and 14A12 mAbs, respectively, using the method described by Nieto et al. (27). For the ak calculation, we assumed a molecular mass of 800,000 daltons for the polysaccharide (41). For 4H3, an IgG3 mAb, we observed considerable variability in the shape of the curves, and suspect that this variability reflects cooperative binding effects that can occur with this subclass (26). The significance of the different slopes is not understood.

different for CNPS concentrations of <10 μg/ml (Fig. 2). Since the 14A12 (μκ) and 21D2 (μκ) mAbs bind to different epitopes (Table 1), the differences in binding shown in Fig. 2 could reflect variation in epitope density, a higher intrinsic affinity for the 21D2 mAb, or both.

In addition to the seven IgM mAbs described, we also obtained an IgG3λ (4H3) and an IgAλ (15C6) mAb. These two mAbs have the same serotype specificity as those of the 14A12 class, binding only to serotype D and GH CNPS (Table 1). Soluble CNPS inhibited mAb binding to CNPS-coated ELISA plates, as shown in Fig. 3 for 21D2 (μκ), 14A12 (μλ), 4H3 (γλ), and 15C6 (αλ). The binding curves are not directly comparable since the two IgMs, 21D2 and 14A12, bind to different epitopes, and since the 14A12, 4H3, and 15C6 mAbs are of different isotypes that differ in avidity (Table 1). Of the two IgMs, 21D2 requires 10^{2} less CNPS to inhibit its binding than 14A12, and hence, it has higher apparent affinity. The 4H3 (γλ) mAb is inhibited by the lowest concentration of soluble CNPS, and hence, this mAb has the highest apparent affinity of the four antibodies. Using the method of Nieto et al. (27), we have calculated apparent binding constants (k_{a}) in the range of 10^{6} to 10^{8} M^{-1} for these four mAbs (see Fig. 3 legend). The three isolates in this study, namely IgM, IgG3, and IgA, all can bind antigen more strongly than expected from the intrinsic binding constants of their individual binding sites, as a result of either higher avidity due to polymer formation (IgM and IgA) or because of cooperative binding through Fc interactions (IgG3) (28).

V Region Use and Antibody Sequences. The mAb mRNAs were sequenced to determine antibody structure and variable gene usage. Based on the nucleotide sequence data, our mAb set can be classified into two groups that correlate with their serotype specificity (Table 1). One group consists of the 14A12 class (μλ), 4H3 (γ3λ), and 15C6 (αλ), which bind only serotype D CNPS. The second group consists of 21D2 (μκ), which binds both A and D CNPS.

All of the serotype D-specific λ mAbs have a heavy chain variable region (V_{H}), encoded by V_{H}441 (V_{H}Gal39.1) (29), a small "diversity" segment consisting of four codons, and J_{H}3 (Table 1; Fig. 4). The light chain variable region (V_{L}) is encoded by V_{L}2/J_{L}2 for the 14A12 class and 15C6 and by V_{L}1/J_{L}1 for 4H3. The fact that all of these mAbs have a variable region structure that is identical or nearly identical indicates that all recognize the same epitope. No somatic mutations were found in the sequences of the V_{H}441, J_{H}3, V_{L}2, and J_{L}2 genetic elements used in the mAbs of the 14A12 class (μλ) (data not shown). The members of the 14A12 class also have the same sequences in their diversity segments (Fig. 4). We could not identify the D segments in 14A12, 4H3, or 15C6 among reported germline D sequences even if we looked for unusual reading frames, inversions, or deletions. Since these sequences contain many G residues, they may be largely or completely N-sequence, and hence, we refer to this segment in our mAbs as N/D.

Five differences distinguish 4H3 (γ3λ) from the 14A12 (μκ) group: (a) the presence of a silent mutation in codon 64 (CTA to CTT) of the V_{H}441 (data not shown); (b) a replacement mutation in codon 104 (GCT to TCT) resulting in a valine to serine change in J_{H}3 (Fig. 4); (c) the codon GGT instead of GGG at position 97 (in the N/D sequence), which represents either a somatic mutation of the 14A12 N/D sequence or a different N/D segment reflecting a different
clonal origin of the B cell encoding 4H3 (Fig. 4); (d) an additional codon at the 3' end of the JH3, which is not encoded by either Jα3 or Cγ3 (30) (Fig. 4). The origin of this extra codon is not known. One possibility is that somatic mutation occurred in the intervening sequence (31) resulting in altered RNA splicing. The first three nucleotides in the intervening sequence downstream of JH3 are GGT (30), and a single somatic mutation could have changed this codon to GCT; and (e) the 4H3 Vκ uses Vκ1/Jκ1 instead of Vκ2/Jκ2 (Table 1). The Vκ1 of 4H3 has two somatic mutations, one resulting in replacement of alanine by valine at position 57 (GCT to GTT), and one silent mutation in the Jκ1 at position 107 (CTC to CTG) (data not shown).

The 15C6 (αλ) mRNA was generated from a different mouse than the other anti-CNPS mAbs. The construction of 15C6 used the same gene elements as used in the 14A12 group, namely VH441, JH3, Vκ2, and Jκ2. Remarkably, 15C6 has the same CDR3 protein sequence as the mAbs in the 14A12 class and 4H3, even though its mRNA sequence has three base differences in the N/D segment (Fig. 4). No somatic mutations were found in the 15C6 Vκ441, Vκ2, Jκ2 genetic elements, but one silent mutation occurred in the first codon of Jκ3 (Fig. 4).

As expected from its different serotype specificity, the construction of the 21D2 A-D-reactive Vκ and Vλ used different genetic elements than those found in the serotype D-specific mAbs (Table 1). The Vκ of 21D2 is composed of Vκ50.1 (29) (a member of the 7183 gene family), an unidentified D, and
Jα2. The D segment of 21D2 has seven codons (CGGGAC-AGCTCAGGAGCATAC), and thus is larger than that found in the serotype D–specific mAbs. The light chain of 21D2 is composed of the Vκ5.1 (32) and Jκ2 gene elements (data not shown). Although no somatic mutations were identified in the genetic elements used in the 21D2 mAb, the last codon of the Vκ5.1 sequence (at the VJ junction) was CCA instead of CCT (32), resulting in a silent substitution.

In summary, the sequence data revealed: (a) a marked restriction in variable region gene usage in the serotype D–specific set of mAbs; (b) a few somatic mutations in the IgG3 and IgA mAbs; (c) the use of Vκ gene elements from 3' proximal gene families that have previously been reported in antipolysaccharide antibodies (29); and (d) N/D segments with different base sequences but identical amino acid sequences in the serotype D–specific mAbs.

**Southern Blot Analysis of the Ig Gene Rearrangements.**

The sequence data suggested that the antibodies from the first fusion were the products of at least three different B cell clones that served as precursors to the 21D2 (μκ), the 4H3 (γλ), and the 14A12 group (αX) hybridomas. To further examine the clonal relationship of the members of the 14A12 class and 4H3, we studied the restriction patterns of their productive and nonproductive alleles. Fig. 5 shows an autoradiograph of a Southern blot of HindIII DNA restriction fragments of DNA from BALB/c liver, the NSO fusion partner, and the various anti-CNPS hybridomas after hybridization with the Jκ1-5 probe. All hybridomas have a band at 6.6 kb that comigrates with the single band in NSO DNA, and presumably comes from the fusion partner. In addition, the blot reveals three types of Jκ locus rearrangements in the 14A12 group: (a) 7B13 and 20B5 have two bands each at 12 and 2.5 kb; (b) 11E2, 12G5, and 14A12 have two bands each at 2.7 kb (suggesting that at least one allele is in the germline configuration) and at 9.4 kb; and (c) 20C5 has only the band at 6.6 kb corresponding to that of NSO, indicating that the Jκ locus was lost either in the precursor B cell or after hybridoma formation, making it impossible to assign to either group. The 4H3 (γλ) hybridomas have different Jκ locus rearrangements than those found in the 14A12 group, having one band at 5.2 kb and another band >23 kb. Also shown in Fig. 6 is the 21D2 (μκ) hybridoma, which has two bands at 4.0 and 9.4 kb in addition to the NSO fusion partner band.

**Discussion**

The decision to generate anti-CNPS mAbs from cryptococcus-infected mice was made with the premise that such antibodies would be elicited by biologically relevant epitopes. Eight mAbs (seven IgM, one IgG3) were generated from the fusion of one spleen, and one IgA mAb was generated from another spleen. The serum antibody titers and the isotype distribution of the mAbs generated suggests that the hybridomas obtained generally reflect the animals total B cell response. Since IgM has an intravascular half life (t½) of 8.5 h, whereas both IgG1 and IgG3 have t½ of ~200 h (34), the ratio of IgM to IgG hybridomas obtained in the first fusion is consistent with the IgM and IgG anti-CNPS serum titers of 1:400 and 1:800, respectively, measured on the day before the fusion. Most of the mAbs had λ light chains, consistent with the λ-rich serum antibody response. However, despite the fact that the serum titers of κ and λ anti-CNPS antibodies were roughly equal, only one κ mAb (21D2) was isolated from two protective fusions. The paucity of κ anti-CNPS hybridomas might be explained by either a preferential association of κ light chains with IgG (which have much longer serum t½) or the absence of κ anti-CNPS B cells in the spleen lymphoid compartment.

The following lines of evidence indicate that our mAbs recognize polysaccharide epitopes. (a) The mAbs are serotype specific and serotype specificity has been assigned to polysaccharide structural determinants (9, 35–37); (b) immunofluorescence shows that 14A12 and 21D2 bind to the polysaccharide capsule; (c) the polysaccharide preparations used in the ELISAs had an extremely low level of protein relative to polysaccharide concentration (<2 ng/ml, or <1:5000 ratio of protein/polysaccharide by weight), and this amount of protein is below the usual detection threshold in the ELISA (38); and (d) all the mAbs use gene elements that have been associated with antipolysaccharide antibodies such as Vκ441 and VκGa150.1 (28, 39, 40).

mAbs to CNPS have been generated by three other groups using animals immunized with either CNPS (22), CNPS con-
jugated to sheep erythrocytes (41), or CNPS conjugated to BSA (35). Our mAbs came from B cells stimulated during cryptococcal infection. They are different from previously described mAbs in that most are serotype D–specific antibodies that use \( \lambda \) light chains. Using polyclonal antisera and sequential absorptions, Ikeda et al. (9) developed a numerical taxonomy system for categorizing CNPS serotype according to eight antigenic factors, and concluded that serotype D was determined by only a few antigenic determinants. In the Ikeda scheme, antigenic factor 8 is a D–specific antigen, and factor 3 is found shared by the A and D serotypes. Since the 14A12 group, 4H3, and 15C6 bind only serotype D, they probably recognize antigenic factor 8. Since the 21D2 mAb binds to both serotypes A and D, it probably recognizes antigenic factor 3. The specificities of our mAbs are thus consistent with and support the Ikeda (9) classification.

The molecular characterization of the mAbs revealed a striking degree of restriction in variable gene element usage in the serotype D–specific mAbs. In fact, the large proportion of \( \lambda \) antibodies in the serum antibody response to GH CNPS suggests a restricted response since \( \lambda \) antibodies are relatively rare in the mouse and comprise only 3–5% of the total antibody pool. There are only two \( V_{\lambda} \) genes and they share a high degree of homology (33). All of our \( \lambda \) antibodies were specific for serotype D CNPS. Restriction in variable gene element usage has been reported in responses to some carbohydrates such as 3-fucosyllamine (40). However, other carbohydrates such as group A streptococcal polysaccharide (42) and dextran (43) elicit antibodies using a variety of variable gene elements.

The \( V_{\lambda} \) of all of the serotype D–specific antibodies used \( V_{\lambda}441 \), an N/D segment consisting of four codons, and J\( _{\lambda}3 \). The \( V_{\lambda}441 \) is a member of the X-24 \( V_{\lambda} \) family, which is the smallest \( V_{\lambda} \) family and consists of only two gene elements (44). \( V_{\lambda}441 \) has a potential glycosylation site in the CDR2 domain. \( V_{\lambda} \) glycosylation has recently been shown to increase the affinity of an antidextran antibody (45) and may be important in the binding to CNPS. \( V_{\lambda}441 \) usage has been reported in several anticoagulants mAbs that recognize very different carbohydrate structures (29, 40, 45). Since most of these mAbs use \( V_{\lambda}441 \) in its germline sequence, it has been proposed that the fine specificity of these mAbs for carbohydrate epitopes resides in their CDR3 and/or light chains (39, 40). The fact that the N/D segments of all serotype D–specific mAbs, including antibodies from two different animals, encode the same protein sequence suggests that the structure of CDR3 is important for binding. In addition, serotype D epitope specificity may require the use of \( \lambda \) light chains.

Since VDJ recombination and the introduction of N sequence usually occur before light chain rearrangements (46), the most conservative interpretation of the sequence and Southern blot data is that there was a single pre-B cell precursor for all members of the 14A12 group (\( \mu \lambda \)) and for 4H3, which assembled its heavy chain gene and underwent subsequent cell divisions. Two such subclones then carried out different nonproductive \( \kappa \) rearrangements and went on to use the same \( V_{\lambda} \) and J\( _{\lambda} \) elements to form identical productive light chains resulting in the 14A12 group. Since 4H3 has different \( J_k \) rearrangements and uses different \( V_{\lambda}/J_{\lambda} \) gene elements than the 14A12 group, its ancestor may have been a third subclone of the original B cell precursor. In this interpretation, the one base difference in the N/D segment of 4H3 compared with those of the 14A12 group is attributed to somatic mutation. It is noteworthy that the response that generated the 14A12 group (and possibly 4H3) appears to be a second example where a single pre-B cell gave rise to antigen-specific B cells with different light chain gene rearrangements (47). However, if one base difference in the 4H3 N/D sequence arose during VDJ formation rather than as a result of somatic mutation, then this fact, along with the differences in \( V_{\lambda}/J_{\lambda} \) gene usage and in \( \kappa \) rearrangements, would suggest that 4H3 arose from a completely different B cell precursor. Thus, the B cell precursors for the hybridoma group specific for the D serotype comprised at least two, probably three, and possibly five different pre-B cell clones, all with the same N/D amino acid sequence.

We found only a few mice (4/60) that responded to infection with high circulating anti-CNPS titer. The lack of serum antibody in most mice is consistent with an earlier observation that anti-CNPS antibodies are not detectable in infected mice (48). A similar phenomenon has been described in humans, where most individuals with cryptococcal disease lack circulating antibody (11). This lack of response may be due to the immunological paralysis that has been reported to follow immunization with CNPS (5–7). CNPS has properties of a T cell–independent (TI) type 2 antigen (49). There is evidence that the antibody response to CNPS in mice, like the response to pneumococcal polysaccharide (50), is regulated by suppressor T cells (7). Additional studies have shown that cryptococcal antigen preparations can induce suppressor T cells (51). Our inability to obtain hybridomas producing anti-CNPS mAbs from the spleens of two infected animals with low serum titers to CNPS (1:200 or less) is consistent with few antibody-producing cells in those spleens at the time fusion.

Given that CNPS has only a few immunogenic determinants (9) and that the antibody response to some of these determinants is highly restricted, one explanation for the paucity of responder mice is that only occasional mice have precursor B cells that can respond to CNPS. However, this is unlikely since most animals do respond to optimal doses of polysaccharide immunization (49) and to conjugates of CNPS and protein (19). This leads us to believe that the few animals that responded did so because, before infection, they had expanded the number of B cells that could produce CNPS-specific antibodies. It is possible that such an expanded pool of B cells could escape from immunological paralysis either because of the greater number of B cells or because the expanded pool contained cells that could no longer be suppressed. The fact that we were able to recover six IgM hybridomas with identical CDR3 sequences from one animal certainly indicates that this particular B cell clone was greatly expanded at the time of the fusion, and raises the possibility that it might have been expanded before infection. This could have
resulted indirectly from an idiotype-antiidiotype network or directly from exposure to a crossreactive antigen. This latter possibility is supported by the fact that most of the responders were older mice: 3 of 12 9-12-mo-old mice had a high titer response, whereas only 1 of 48 adult mice that were <6 mo old responded. Another study that showed unresponsiveness to infection used 3-4-mo-old mice (48).

If the responder mice were able to make an antibody response because they had prior exposure to a cross reactive antigen, that antigen could have been another polysaccharide (52), or even a protein, since protein epitopes can resemble carbohydrate (53). Since CNPS is a TI antigen (49), it is presumably unable to induce a secondary immune response (54). However, TI antigens are capable of activating pre-existing memory cells (54–56). The IgM hybridomas lack somatic mutations and reflect a TI response to a D-specific epitope in CNPS. However, the 4H3 hybridoma has somatic mutations in both V_H and V_L, which may have occurred in the response to a T cell-dependent (TD) stimulus prior to memory B cell activation by CNPS. In this scenario, CNPS stimulates memory cells and produces an antibody response with characteristics of both TI and TD antigens.

Thus, the analysis of the antibodies produced by the responder mice suggests explanations for why some animals were able to respond. They also suggest that priming either with a glycoconjugate (56, 57), an antiidiotype, or other TD crossreactive antigens could increase the number of responder mice in infection. It is interesting to note that dextran, which elicits antibody responses in mice that use some of the same genetic elements that we have described above (58), has been used by AIDS patients as an antiviral agent (59). Given that the ability to mount an antibody response to CNPS in infection may depend on prior exposures to other antigens, it is conceivable that the administration of dextran to AIDS patients could influence their ability to make antibody to CNPS.

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