Variable Region Primary Structures of a High Affinity Anti-fluorescein Immunoglobulin M Cryoglobulin Exhibiting Oxazolone Cross-reactivity*

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Previous studies of murine IgM hybridoma protein 18-2-3, derived from an (NZB/NZW)F1 mouse following hyperimmunization with fluorescein (Fl)-conjugated keyhole limpet hemocyanin, demonstrated a high affinity for Fl ($K_a = 2.9 \times 10^{10} \text{ M}^{-1}$) and cryoprecipitation that was abrogated upon Fl binding to the antibody-combining site. V region sequences of 18-2-3 were determined by Edman degradation and nucleotide sequence analysis. The $V_H$ region of 18-2-3 was encoded by a gene $V_{H}J(B)$ of the QS2 $V_H$ family with $96\%$ homology to anti-oxazolone antibody NQ7.5.3 but utilized a larger D region ($D_{Q5}$ plus N region). The $V_L$ region of 18-2-3 was encoded by a gene $V_{L}IV$ with an amino acid sequence $97\%$ homologous to that of anti-oxazolone antibody NQ11.1.18. Although monoclonal anti-Fl antibodies 18-2-3 and 4-4-20 possessed similar binding affinities and quenched bound fluorescein to the same extent ($Q_{max} > 96\%$), they utilized different $V_H$, $D$, $V_L$, and $J_L$ genes, but the same $J_H$ gene segment ($J_{H4}$). Solid-phase analyses showed that 18-2-3 was not idiotypically related to 4-4-20 and 9-40, prototypic anti-Fl antibodies. Fine specificity binding patterns of Fl analogues by 18-2-3 IgM and IgG were distinct from other anti-Fl antibodies. Monoclonal antibody 18-2-3 bound phenyloxazolone bovine serum albumin with a lower affinity than for Fl-bovine serum albumin. The first hypervariable region of the 18-2-3 light chain showed homology to human cryoglobulins. This is the first variable region sequence of a murine IgM which self-aggregates at low temperature.

Cryoglobulins reversibly precipitate at temperatures below $37^\circ C$ and have been classified into three types based on the molecular composition of the aggregate (Brouet et al., 1974). Murine IgM 18-2-3 is a Type I cryoglobulin consisting solely of the monoclonal 18-2-3 component. Cryoglobulins have been observed in normal BALB/c mice, but occur at increased levels in autoimmune-prone strains (NZB, NZB/NZW, MRL/1) correlated with age and disease severity (Andrews et al., 1978). Data suggest that autoreactive B cell precursors are in a proliferative state in these autoimmune strains since they show abnormally high spontaneous polyclonal B cell activation (Izui et al., 1978).

A high affinity ($K_a = 2.9 \times 10^{10} \text{ M}^{-1}$) murine monoclonal anti-fluorescein IgM antibody 18-2-3 displaying low temperature insolubility in the absence of bound ligand has served as a model to study Type I cryoprecipitation. Antigen binding site involvement was indicated since the presence of fluorescein prevented cryoprecipitation (Ballard et al., 1983). Antibody 18-2-3 was originally derived from an (NZB/NZW)F1 mouse, a strain showing a high incidence of autoimmunity. Studies by Ballard et al. (1986) suggested that 18-2-3 was derived from a relatively rare B cell progenitor since examination of 37 IgM and IgG monoclonal antibodies of similar origin and specificity did not reveal low temperature insolubility or high binding affinity for fluorescein. Previous results indicated that cryoprecipitation occurred via electrostatic interactions involving 18-2-3 antibody-combining sites with interactive sites in the Fc region of the homologous IgM (Dombrink-Kurtzman and Voss, 1988).

In the present study, variable region sequences of heavy and light chains derived from 18-2-3 have been determined through cloned cDNA synthesized from mRNA templates. Three DNA segments ($V_{H}$, $D_{H}$, and $J_{H}$) encode the $V_H$ region, while two DNA segments ($V_L$ and $J_L$) encode the $V_L$ region (Seidman et al., 1978; Sakano et al., 1978; Schilling et al., 1980). $V_{H}$ and $V_L$ polypeptides both contribute to antigenic binding specificity of antibodies. The $V_L$ of 18-2-3 was nearly identical to that of BALB/c anti-oxazolone antibodies (Berek et al., 1985), whereas the $V_H$ of 18-2-3 was highly homologous to other BALB/c anti-oxazolone antibodies (Griffiths et al., 1984). Although anti-fluorescein antibodies 18-2-3 and 4-4-20 had similar high binding affinity and fluorescence quenching of bound fluorescein, they differed in $V_H$ and $V_L$ gene usage, idiotypic and metatypic relatedness, and fine specificity. CDR1 of the 18-2-3 $V_L$ gene segment closely resembled the human $V_L$ sub-subgroup IIIb, which has been preferentially used by a group of human monoclonal IgM-RF cryoglobulins (Kunkel et al., 1973).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J04609 and J04610.

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EXPERIMENTAL PROCEDURES

RESULTS

Determination of NH₂-terminal Amino Acid Sequences—
The NH₂-terminal amino acid sequence (43 residues) of 18-2-3 light chain was determined by repetitive Edman degradation. After deblocking the amino-terminal residue of the heavy chain of 18-2-3 with pyroglutamate aminopeptidase, the NH₂-terminal sequence (30 residues) was identified. Monoclonal antibody 18-2-3 utilized a VH gene of the Q52 family and a V₅ gene from the V₅ subgroup of Potter et al. (1982) or V₄V subgroup of Kabat et al. (1987). Southern blot hybridization was utilized to identify J usage. Restriction fragments obtained from separate digests of 18-2-3 DNA with CfoI and EcoRI indicated J₅ was being used (data not shown). Heavy Chain Sequences—Six oligonucleotides were synthesized as primers in sequencing the heavy and light chain variable regions. Fig. 1 describes the oligonucleotides and locations to which they hybridized. The nucleotide and amino acid sequences of the VH segment of 18-2-3 are described. Primers numbered 1311, 2542, 1798, 1648, 2507, and 2515 are oligonucleotides designed on the basis of sequencing data obtained using primers 2507 and 2515. Specific location within the genes where the oligonucleotides bound is shown together with the direction and extent of the sequencing information obtained. Key to the oligonucleotides: 1311, 5'-dCTTCAGGCATC GGTGACC-3'; 1648, 5'-dCTCTACCTCCTCAACTAATC-3'; 1798, 5'- dATGAATTCCGCACGGACG-3'; 2507, 5'-dGTGATGGTGAGTGAA GATG-3'; 2515, 5'-dCAGGAGACGGGGAGAATC-3'; 2542, 5'-dAAC TATGGTGTCACCTG-3'. The 17-mer, M13 sequencing primer (−20) was also used.

Fig. 1. DNA sequencing strategy. The synthetic oligonucleotide primers used to sequence the clones of the heavy and light chain variable regions of 18-2-3 are described. Primers numbered 1311, 1648, 1798, and 2542 are oligonucleotides designed on the basis of sequencing data obtained using primers 2507 and 2515. Specific location within the genes where the oligonucleotides bound is shown together with the direction and extent of the sequencing information obtained. Key to the oligonucleotides: 1311, 5'-dCTTCAGGCATC GGTGACC-3'; 1648, 5'-dCTCTACCTCCTCAACTAATC-3'; 1798, 5'- dATGAATTCCGCACGGACG-3'; 2507, 5'-dGTGATGGTGAGTGAA GATG-3'; 2515, 5'-dCAGGAGACGGGGAGAATC-3'; 2542, 5'-dAAC TATGGTGTCACCTG-3'. The 17-mer, M13 sequencing primer (−20) was also used.

31 determined by amino acid sequencing corresponded to those deduced from the nucleotide sequence. The VH region of 18-2-3 was encoded by the VH chromosome segment closely resembling the D₉₂ gene segment (Sakano et al., 1981; Kurosawa and Tonegawa, 1982), as shown in Fig. 4, but did not express the germline sequence Gin-Leu-Gly since it differed at two bases, resulting in a sequence of Arg-Leu-Glu. Additionally 18-2-3 exhibited variation in the length of the CDR3 segment. Eight non-coding bases (N region; Alt and Baltimore, 1982) appeared to be present between D and J₆, resulting in a D region of six amino acids (Fig. 4). The precise boundaries between VH and D gene segments and D and J gene segments will only be known when germ line D and J gene segments are cloned.

A comparison of heavy chain variable region amino acid sequences is also shown in Fig. 3. Interestingly, the 18-2-3 sequence had much greater homology with antibodies of another specificity, anti-oxazolone (V₄H-Oxl and NQ7.5.3) than with anti-Fl antibodies (4-4-20 and 3-13). Monoclonal antibody 4-4-20 had an affinity comparable to that of 18-2-3 and quenched bound fluorescein to the same degree (Qmax > 96%). The only similarity in various gene segments between the three anti-Fl antibodies was that they contained J₄, J₆, and J₇ genes (Sakano et al., 1980). The VH genes used by 18-2-3, 4-4-20, and 3-13 belonged to V₅H(15), V₅H(11), and V₆H(A), respectively. The D region of 4-4-20 was truncated (Bedzyk et al., 1989), while that of 3-13 was unusually long, suggesting...
that it may be derived from D-D joining (D_{FL.4.2} plus D_{SP.2.5}) (Liu et al., 1987).

**Light Chain Sequences**—The nucleotide and amino acid sequences of the $V_L$ segment of 18-2-3 are presented in Figs. 5 and 6, respectively. Dideoxy sequencing of cDNA, which had been synthesized from mRNA, was used to determine the amino acid sequence. Amino acid residues 1–43 determined by amino acid sequencing of pure light chains were identical.
Fig. 4. Nucleotide sequences of D regions of immunoglobulin heavy chains using DQ52. Sequences arc aligned for maximal homology with the DQ52 germ line gene segment. 18-2-3, 4-4-20, and 3-13 are anti-fluorescein antibodies. S43 and 28.8 are anti-4-hydroxy-3-nitrophenyl and anti-poly-(Glu-Ala-Ala-Tyr) (GAT) antibodies, respectively (Bothwell et al., 1981; Roth et al., 1985). Q52J is a myeloma clone (Sakano et al., 1981), 04-01 is an anti-ssDNA autoantibody (Smith et al., 1988), and V_{H} Ox1 is an anti-oxazolone antibody.

3-13

\[ \text{Germline DQ52} \]

| ACG TGG GGC GAC |

\[ \text{Hybridoma, Myeloma} \]

| 18-2-3 - G - - - - A - G A A T C T T T T |

| 22.8 - - - - - - A T C |

| Q52J - - - - - - - - A A G G |

| 04-01 G A T - - - - - - G |

| V_{H} Ox1 G - T - G - - - - G |

| 4-4-20 T C T |

| 3-13 G G G G C A - A C A G C T C G G G C T A C |

Fig. 5. Nucleotide and deduced amino acid sequences of the 18-2-3 V, gene segment, including leader peptide and 5'-untranslated region. Numbering of the amino acids and complementarity determining regions is according to Kabat et al. (1987).

Reactivity of 18-2-3 with Anti-idiotype Antibodies—Anti-idiotypic antisera were produced against anti-fluorescein antibody 4-4-20, 9-40, and 5-27. Each idiotype-anti-idiotype interaction was ligand-inhibitable, indicating that the idiotypic reagents may be interacting specifically with active site determinants or that tertiary structure was altered upon antigen binding. Idiotype interactions were also inhibitable by nonradioiodinated homologous proteins. Fig. 7, in the Mini-print, shows inhibition of the idiotype-anti-idiotype interactions with increasing concentrations of unlabeled 4-4-20, 9-40, and 5-27. One-half nanogram of unlabeled 4-4-20 inhibited the 4-4-20-anti-4-4-20 interaction 50% while 1 ng of 9-40 inhibited the 9-40-anti-9-40 interaction and 10 ng of 5-27 inhibited the 5-27-anti-5-27 interaction to the same extent.

Inhibition of the idiotype-anti-idiotype interactions by increasing concentrations of 18-2-3 IgM protein is shown in Fig. 7. In general, 18-2-3 did not appreciably inhibit homologous 4-4-20 or the 5-27 idiotype-anti-idiotype interactions over a wide protein concentration range. Only at the highest concentration tested (10 \( \mu \)g) in the 9-40-anti-9-40 interaction did 18-2-3 show any significant inhibition (40%). This represented a much higher concentration (>10,000 times) of 18-2-3 than required using homologous 9-40 protein. Thus, the effect seen regarding 9-40 appeared to be nonspecific.

Fine Specificity—Fine specificity binding patterns for 18-2-3 IgM and IgM_{1} were determined using five analogues of F1 covalently conjugated to BSA at similar epitope densities (erythrosin{\text{I}}-BSA, eosin{\text{II}}-BSA, tetramethylrhodamine{\text{15}}-BSA, rhodamine{\text{18}}-BSA, substituted rhodamine{\text{18}}-BSA), plus dinitrophenol (dinitrophenol{\text{11}}-BSA). Table I, in the Mini-print, lists analogue concentrations required to inhibit binding 50% to solid phase F1-BSA. Inhibition patterns for 18-2-3 were distinct from the other anti-F1 antibodies, indicating that monoclonal antibody 18-2-3 possessed a nonidentical antigen-combining site. The only analogue showing 50% inhibition was tetramethylrhodamine{\text{15}}-BSA which inhibited the binding of 18-2-3 IgM and IgM_{1} at concentrations of 440 and 600 \( \mu \)M, respectively. The highest concentration of F1 analogues tested was 1 mM, while the highest concentration of dinitrophenol tested was 10 \( \mu \)M.

Binding of 18-2-3 to Fl-BSA and phOx-BSA—A direct binding assay was used in studying the binding of 18-2-3 to phOx-BSA because in preliminary inhibition assays it was not possible to inhibit the high affinity binding of 18-2-3 to solid-phase Fl-BSA with fluid-phase phOx-BSA. Binding of 18-2-3 to phOx-BSA (Fig. 8, in the Mini-print) appeared to be low affinity. Although the heavy and light chain variable

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region sequences utilized by 18-2-3 have been observed in different anti-oxazolone antibodies, no single anti-phOx antibody used both variable sequences found in 18-2-3. Structurally, the epitopes differ. The xanthenone portion of fluorescein is planar (Voss et al., 1976), whereas phenyloxazolone has two nonrigid aromatic rings. Chemical structures of F1-BSA and phOx-BSA are shown in Fig. 9 (in the Miniprint).

**DISCUSSION**

Data reported here represent primary structural determinations of gene segments encoding the variable domains of murine IgM 18-2-3, an antibody that self-aggregates in the absence of its cognate antigen Fl. Antibodies 18-2-3 and 4-4-20 had similar high intrinsic binding affinities ($K_a = 2-3 \times 10^{10} \text{M}^{-1}$) and quenched bound fluorescein to the same extent ($Q = 96\%$), yet they utilized different VH, D, V, and J, gene segments. The only similarity in gene segment usage between the two antibodies was that they contained JH~. Gene segments utilized by the 18-2-3 heavy chain variable region appeared to be $V_{H1}(B)$ (a member of the $V_{H}Q52$ family), $D_{002}$, and $J_{H4}$. Antibody 18-2-3 appeared to be using $V_{H}$ and D gene segments from families residing most proximal to D and J, respectively. Preferential utilization of D-proximal $V_{H}$ gene families has been observed in murine pre-B-cell lines (Yancopolous et al., 1984) and in hybridomas derived from non-immunized 6-day-old BALB/c mice (Holmberg, 1987).

A comparison of heavy chain variable region sequences indicated that 18-2-3 and anti-phenyloxazolone antibodies used homologous $V_{H}$ genes. The highest degree of homology (96% at the nucleotide and amino acid levels) with known sequences was with anti-phOx antibody NQ7.5.3 which had been obtained 14 days following primary immunization. Meek et al. (1987) suggested that novel mechanisms were involved in generation of D segments in autoantibodies. Although 18-2-3 utilized a gene segment resembling $D_{Q53}$ there were eight additional noncoded nucleotides between the D and JH~ segments. The GAATCTTT sequence was probably not attributable to imprecise joining since it did not represent flanking regions nor was D-D joining indicated. The D segment was interesting in that the N segment was A,T-rich rather than

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**FIG. 6. Comparison of light chain immunoglobulin variable region amino acid sequences.** 18-2-3, 4-4-20, and 9-40 are anti-fluorescein antibodies. $V_{L}0x1$ and $NQ7.1.18$ are anti-oxazolone antibodies. 04-01 is an anti-single stranded DNA autoantibody. Gaps are indicated by dots.
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G,C-rich. Extra nucleotides may be a product of the activity of terminal deoxynucleotidyltransferase (Alt and Baltimore, 1982), although this enzyme, which polymerizes random deoxynucleotides at 3' ends, show a preference for dG residues. Alternatively, the possibility exists that the N segment was generated-encoding since germ line D genes have not yet been isolated.

The J£ segment of 18-2-3 was identical to the J£ germ line gene segment of the BALB/c strain (Sakano et al., 1980), except for one silent substitution at the third base of codon 102 (T for C). Since 18-2-3 was derived from a NZB/NZW mouse, this difference may be an allelic form of the J£ gene. The entire J£ segment was used by 18-2-3. Because anti-Fl antibodies 18-2-3, 4-4-20, and 3-13 all showed a high degree of fluorescence quenching of bound fluorescein (Table 11, in the Miniprint), this property may be related to utilization of the J£ germ line segment.

The sequence of 18-2-3 light chain indicated that the V£ segment was encoded by a gene belonging to the V,5 subgroup (Potter et al., 1982). The deduced amino acid sequence of the 18-2-3 V£ segment was almost identical to the sequence of light chains in antibodies against 2-phenyloxazolone from BALB/c mice. The light chain of anti-phOx antibody NQ11.18.1 utilized a V£ gene that was 98% homologous at the nucleotide level and 97% at the protein level to the V£ gene used by 18-2-3. Hybridoma NQ11.18.1 had been obtained following a secondary immunization 8 weeks after the primary with phenyloxazolone-conjugated chicken serum albumin.

Both gene segments could have been derived from two germ line genes in the same family or they could be related by somatic mutation of the same gene. Somatic mutations have been observed in IgM, but are more restricted than in IgG or IgA (Chua et al., 1987). Alternatively, slight differences in homology may indicate that different allelic genes were being utilized since 18-2-3 was derived from an (NZB/NZW)F1 mouse and the anti-phOx antibody NQ11.18.1 was from a BALB/c mouse.

The J£ gene segment utilized by 18-2-3 was J£5, a relatively uncommon occurrence, since 80% of splenic B cell V/J rearrangements utilized either J£1 or J£2 (Wood and Coleclough, 1984; Nishi et al., 1985). Anti-oxazolone antibodies preferentially utilized J£5 (Griffiths et al., 1984). It is possible that the V£ segment utilized by 18-2-3 and anti-phenyloxazolone antibodies preferentially rearranged to J£5.

The ability of monoclonal antibody 18-2-3 to bind to phOx may correlate with self-aggregation at low temperatures (Dombrink-Kurtzman and Voss, 1988). The structure of phOx may simulate dipetidyl conformational or sequential epitopes (e.g. Phe-His) in the Fc region of 18-2-3 to which the antigen binding region of 18-2-3 can bind. Chemical modification studies have indicated that histidines are involved in the Fc region of human IgG and tyrosines on both antigenic and antibody sides of the interactions of two IgG-rheumatoid factors (RFs) (Nardella et al., 1985).

Although RFs are typically IgM and form immune complexes by binding Fc determinants on IgG molecules, self-association of IgM (Tsai et al., 1977), and IgG antibodies (Pope et al., 1974; Nardella et al., 1981) has been observed. In such cases each molecule serves as an antigen as well as an antigen, as with 18-2-3. Antibody 18-2-3 did not appear to have RF activity since it did not bind to IgG molecules, but bound to both human and murine IgM molecules.

Interestingly, there was a high degree of homology between the CDR1 region of 18-2-3 V£, and that of human V£ subgroup IIIb, which is utilized by cryoimmunoglobulin RFs having anti-IgG activity. Ten of twelve amino acid residues were the same. Differences were at positions 27 (Ser and Gln) and 34 (His and Ala) for 18-2-3 and V£IIIb, respectively. Additionally, molecular modeling of the antigen binding site of 18-2-3 has indicated the presence of aromatic residues (tyrosines and tryptophan). Tyrosine residues have been shown to be involved in the combining site of RFs (Nardella et al., 1985). The sequence V£ listed as a murine V£IV gene in Kabat et al. (1987) appears to have been a mistaken classification as a murine gene because V£4 has been reported by Pech and Zachau (1984) to be related to human V£, subgroup IIIb. Thus, in accord with the proposed evolution of human V£ genes and murine V V genes (Barker et al., 1972), human V£ sub-group IIIb and murine V£ IV could be considered related phylogenetically. Moynihan et al. (1985) observed restricted association of the V,IIIb light chain subgroup with 4-heavy chain in normal human serum and suggested that the KlIb-4 combination could represent a signal to prevent class switching. This may represent a way of generating high affinity IgM antibodies, as seen with 18-2-3.

X-ray crystallographic analyses of F(ab'2)2 fragments (Amzel and Poljak, 1979) have indicated that the tertiary and quaternary structures of the antigen binding site can be significantly influenced by the chemical nature of the amino acid at position 96 of the light chain. This residue occurs at the V-J junction in CDR3 and is encoded by V and J genes. A conserved leucine was located at position 96 in both anti-oxazolone antibodies and 18-2-3.

Genes utilized by autoantibodies appear to be present in normal individuals as well as in autoimmune patients. It has been suggested that differences in the complex regulatory pathways of the immune system are responsible for the expansion in autoimmune patients of clones that would be down-regulated in normal individuals (Sanz and Capra, 1988). Findings indicate that autoantibody production in NZB mice results because NZB marrow-derived immature B cells abnormally resist tolerance induction due to defective clonal inactivation (Cowdery et al., 1987). During the secondary immune response in normal humans and animals, IgM RFs are regularly synthesized. Rheumatoid factors may have been maintained during evolution because they have the ability to remove opsonized bacterial and parasites (Clarkson and Mellow, 1981).

Studies investigating the genetic origin of murine autoantibodies have indicated that autoimmune mice do not possess unique IgMh genes (Koffler et al., 1985b). The genetic elements (V, D, J segments) used to encode autoantibodies and antibodies against foreign antigens are not obviously different (Koffler et al., 1985a; Manheimer-Lory et al., 1986). Although somatic mutations can be a contributory factor (Diamond and Scharff, 1984), germ line genes can encode autoantibodies (Naparstek et al., 1988). Recent findings indicate that unmodified or scarcely modified human V£4 germ line genes encode systemic lupus erythematosus-derived anti-DNA autoantibodies (Dersimonian et al., 1987). Studies based on idiotypic and structural characteristics of human monoclonal cryoglobulins with RF activity have indicated that different V£ genes are utilized, but only a limited set of V£ genes are present (Kunkel et al., 1973). An inherent restriction in the immune response to self-antigens was suggested by the preferential association of KlIb light chains with monoclonal human IgM, RF autoantibodies (Ledford et al., 1983). The high degree of primary structure homology and cross-reacting idiotypes indicated that the majority of human IgM RF light chains

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were derived from a single germ line V, gene or a family of closely related V,III germ line genes (Gońi et al., 1985).

For murine RFs, no clear consensus exists. Part of the divergence is due to the variety of strains and conditions (e.g., unmanipulated, polyclonally activated, antigen-injected) used in the different studies. An additional consideration is the actual number of genes that comprise a family. Originally the J558 VH gene family was thought to have ~60% of the approximately 100 germ line VH genes (Brodeur and Riblet, 1984). Recent evidence indicated that 500–1000 genes exist in the J558 family (Livant et al., 1986).

Since the anti-fluorescein response is diverse, it was not surprising that antibodies 18-2-3 and 4-4-20 used different VH and V, genes, had unrelated idiotype and metatypic structures (Voss et al., 1988) and demonstrated different fine specificities regarding structural analogues. Yet similarity exists between these two antibodies since they both exhibit high intrinsic affinity for fluorescein and >96% quenching of bound ligand. Although differing in primary structure, the three-dimensional structure of their respective antigen binding sites may be similar. X-ray crystallographic studies are in progress to determine such correlations.

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**Variable Region Sequences of an Anti-F1 Cryoantibody**

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**Supplementary Material**

Variable Region Primary Structures Of A High Affinity Anti-F1 Cryoantibody

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**EXPERIMENTAL PROCEDURES**

**Cell Line and Tissue Purification**

Hybridomas 18-2-3 and 18-2-2 were produced by polyethylene glycol fusion of the mouse cell line NS-1 (Galfre et al., 1977) with murine immunoglobulins from a male CBA/W (Jax), female surviving secondary with F175 (C57BL/6J x SJL/J) 2,4-dinitrophenyl (DNP) IgG, as described elsewhere (Fiebich et al., 1982). Purified IgG was isolated from the ascites fluid of each hybridoma using ammonium sulfate precipitation. The yield of IgG was 40-80% of the total cell lysate. The IgG was further purified using C-40 column chromatography.

**Preparation of Cell Extracts**

Cell extracts were prepared by homogenization in a buffer containing 10 mM Tris, 100 mM NaCl, 0.5% NP-40. The extracts were clarified by centrifugation at 10,000 x g for 10 min and stored at -80°C.

**Immunization of Mice**

Female C57BL/6 mice were immunized subcutaneously with 1 mg of purified IgG in complete Freund's adjuvant. The mice were bled 2-3 weeks after the last immunization and the sera were tested for antibody to F1 by ELISA.

**Harvesting Hybridoma Supernatants**

Supernatants were harvested from cultures of hybridoma cell lines grown in 100-mm dishes. The supernatants were clarified by centrifugation at 20,000 x g for 10 min and stored at -80°C.

**Immunofluorescence Microscopy**

The hybridoma lines were cultured in 96-well microtiter plates. The cells were fixed in 3% formaldehyde in PBS and stained with anti-F1 monoclonal antibodies followed by rhodamine-conjugated goat anti-mouse IgG. The slides were examined by fluorescence microscopy.

**Complement Dependent Cytotoxicity Assay**

The assay was performed using a 96-well microtiter plate. The cells were incubated with varying dilutions of hybridoma supernatants and complement for 1 hour at 37°C. The number of surviving cells was determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay.

**Antigenic Determinant Mapping**

The antigenic determinants on the F1 protein were mapped by a competitive ELISA using synthetic peptides as competitors. The peptides were synthesized on a solid support and then cleaved from the resin with acid. The peptides were then purified by high performance liquid chromatography.

**Production of Anti-F1 Antibodies**

The hybridoma lines were cultured in 100-mm dishes and the supernatants were harvested after each 4 days. The supernatants were clarified by centrifugation at 10,000 x g for 10 min and stored at -80°C.

**Results and Discussion**

The hybridoma lines were tested for their ability to produce antibodies against F1 protein by enzyme-linked immunosorbent assay (ELISA) and Western blotting. The hybridoma lines 18-2-3 and 18-2-2 produced antibodies that were reactive with purified F1 protein.

**Conclusions**

The hybridoma lines 18-2-3 and 18-2-2 were shown to produce antibodies against F1 protein. These antibodies were useful for mapping the antigenic determinants on the F1 protein and for studying the immune response to F1 protein.
Variable Region Sequences of an Anti-F1 Cryoantibody

Primary antibody was added to E. coli 956 cells (Bethesda Research Laboratories) which had been made competent by a modification of the procedure of Hahne (1981). Frozen bulk cells were thawed, gently mixed and 10 µl were aliquoted into sterile tubes (Falcon polypropylene tubes, Becton Dickinson and Co., Lincoln Park, N.J.). The tubes were incubated at 37°C overnight in a shaking waterbath at 100 rpm to increase the number of viable cells per tube. The next day, 100 µl of each cell suspension was mixed with 100 µl of the antibody in a 1:1 ratio and incubated for 2 hr at 37°C. Following this, the tubes were mixed and 100 µl was added to each of the tubes. The tubes were then incubated at 37°C for 30 min. After centrifugation at 10,000 g for 3 min, the supernatant was removed from each tube and the resulting pellets were resuspended in 50 µl of PBS solution (pH 7.2). The pellets were then placed on ice and stored at -20°C until use.

Preparation of Primary Antibody: The supernatants from the primary antibody were used directly for the second antibody. The antibody was affinity-purified by a modification of the procedure described by Hahne et al. (1983). Briefly, 15 µg of IgG antibody was cross-linked to a Affigel-10 column (Bio-Rad Laboratories, Richmond, Ca., USA). After washing the column with 20 ml of PBS, the antibody was eluted with 20 ml of 0.1 M glycine-HCl (pH 2.5). The eluate was neutralized with 2 M Tris-HCl (pH 8.5) and concentrated to 2% (w/v) by lyophilization.

Preparation of Secondary Antibody: The secondary antibody, a rabbit anti-mouse IgG, was used to detect the primary antibody. The antibody was purified by affinity-purification on a Protein A-Sepharose column (Pharmacia Fine Chemicals, Uppsala, Sweden). The antibody was then dialyzed against PBS and stored at -20°C until use.

Table 1: Concentration of immunogen giving 50% inhibition of binding of the Fab and F1 in PBS in 30 minutes.

| Fab or F1 | 1/10,000 | 1/1,000 | 1/100 | 1/10 | 1/1 |
|-----------|---------|--------|-------|------|---|
| PBS       | 100%    | 100%   | 100%  | 100% | 100%|
| 0.1 M NaCl| 100%    | 100%   | 100%  | 100% | 100%|

The data in Table 1 indicate that the secondary antibody reacts with the primary antibody at a concentration of 1/100 in PBS. The optimal concentration of primary antibody in PBS was determined to be 1/100 for optimal detection of the secondary antibody.

Table 3: Isoform, affinity and Qmax of anti-cryoprotective antibodies.

| Isoform | Affinity | Qmax (E) |
|---------|----------|----------|
| Fab     | 1/100    | 100%     |
| F1      | 1/100    | 100%     |
| 0.1 M NaCl | 1/100  | 100%     |
| PBS     | 1/100    | 100%     |

The data in Table 3 indicate that the affinity of the primary antibody is optimal at a concentration of 1/100 in PBS. The optimal concentration of primary antibody in PBS was determined to be 1/100 for optimal detection of the secondary antibody.
Figure 7. Inhibition of 4-6-20, 4-40 and 5-27 idiotope-anti-idiotypic interactions by unlabeled homologous or heterologous (4-4-2) affinity purified anti-fluorescein haptens. Rabbit anti-idiotypic reagents (anti-4-6-20, anti-4-40 and anti-5-27) coated on solid phase were incubated with 50 μl unlabeled anti-fluorescein antibody in the presence of [125I]-4-6-20, [125I]-4-40 or [125I]-5-27 (5 x 10^4 c.p.m.). The percent inhibition values represent the mean of triplicate determinations.

Figure 8. Direct binding of 18-2-3 to solid phase FI-BSA and phox-BSA. FI-BSA or phox-BSA was absorbed onto wells and incubated with 50 μl affinity purified anti-fluorescein antibody 18-2-3. Bound antibody was detected with [125I]-anti-IgG Fab chain specific (5 x 10^4 c.p.m.).

Figure 9. Structure of fluorescein compared to 2-phenylazochrome covalently coupled to bovine serum albumin.