SPECIFICITIES AND V GENES ENCODING MONOCLONAL AUTOANTIBODIES FROM VIABLE MOTHEATEN MICE

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Mice homozygous for recessive mutations at the "motheaten" locus on chromosome 6 (1) are an important experimental model for studying the origin, immunochemistry, and molecular properties of autoantibodies. Two such allelic mutations have arisen spontaneously in the C57BL/6J strain: the original motheaten (*me*) (2), and the later viable motheaten (me^v) (3) mutations, whose homozygotes live to average ages of 3 and 9 wk, respectively. Both types of mice develop a rapidly fatal disease characterized by hyperglobulinemia, production of a variety of autoantibodies (including antithymocyte and anti–red blood cell), and a number of lesions suggestive of an autoimmune pathology: neutrophilic dermatitis, thymic atrophy, glomerular immune complexes, interstitial hemorrhagic pneumonitis (which is usually the immediate cause of death), and swollen limb joints (1).

The B cells of viable motheaten mice have been shown to be all or nearly all of the Ly-1^+ phenotype (Ly-1 B cells) (4). Ly-1 B cells are thought to represent a distinct lineage of B cells possessing an immature phenotype (5) and containing many or most of the precursors of autoantibody forming cells (6).

Because of the severity of the autoimmune syndrome in these mice, including the elaboration of autoantibodies exhibiting a variety of specificities, we examined and here report the immunochemical specificities and V gene family usage of mAb secreted from hybridomas obtained from viable motheaten mice. The results obtained show that the autoantibodies expressed in these mice exhibit a wide range of single and multiple reactivities to self antigens and are not derived from a restricted set of V gene families.

Materials and Methods

Mice

1–2-mo-old viable motheaten (C57BL/6JSmn-me^v/me^v) and control (C57BL/6JSmn) mice were used.

Hybridomas

Hybridomas were obtained from 1- or 2-mo-old viable motheaten mice by fusion of splenocytes with the Sp2/0 cell line in accordance with a previously described technique

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(7). Hybridomas secreting autoantibodies were cloned under stringent limiting dilution conditions (0.5 cell/well).

Antigen Specificity of Monoclonal Antibodies

Antigens. A panel of autoantigens were used to determine the specificity of *me*^{v} mAbs. These antigens are generally thought to be involved in autoimmune diseases. Intrinsic factor (IF)[1] was purchased from ICN Biochemical Inc. (Irvine, CA); thyroglobulin (TG) and cardiolipin from Sigma Chemical Co. (St. Louis, MO), and murine transferrin (TR) from U.S. Biochemical Corp. (Cleveland, OH). Immunoglobulins of the γ3, γ1, γ2b, or γ2a isotypes (each bearing λ light chains) were purified from culture supernatants of the J606 myeloma, or the 88-C692, BA6, or HOPC1 hybridomas, respectively. Acetylcholine receptor (AcR) was a gift from Dr. A. Penn (Columbia University, New York, NY); Sm, extracted from MRL/lpr thymocytes, was a gift from Dr. H. Dang (University of South Texas, San Antonio, TX); and myelin basic protein (MBP) from seven different species was from Dr. Hashim (St. Luke's Roosevelt Hospital, New York).

Radioimmunoassay. RIA was used to detect antibodies reacting with bromelain-treated RBC membrane, guinea pig MBP, murine TR, murine aggregated IgG, porcine TG, murine Sm, torpedo AcR, murine type II or type III collagen (CII or CIII), IF, cardiolipin, and thymocytes (Thy). Microtiter plate wells were coated with 50 μl of 10 μg/ml of soluble autoantigen in carbonate buffer, pH 9.0, except that Sm was used at 1 μg/ml, MBP at 20 μg/ml, and cardiolipin (in 95% ethanol) at 50 μg/ml. After overnight incubation at 4°C, the plates were incubated for 1 h at 4°C with 1% BSA-PBS and then washed three times. 50 μl of hybridoma cell culture supernatant or chromatographically purified antibody (10 μg/ml) was added per well for 3 h at room temperature; plates were then washed and incubated for 2 h with ^{125}I–rat anti–murine κ mAb (50,000 cpm/well); all hybridomas producing autoantibodies had been previously shown to use κ light chains. After extensive washings, the radioactivity remaining in the wells was counted in a Biogamma TM radiation counter (Beckman Instruments, Inc., Fullerton, CA). Specificity was further studied by competitive inhibition RIA. In these studies, 50 μl (at 10 μg/ml) of a given mAb was preincubated for 2 h at room temperature with 50 μl of 10 or 30 μg/ml autoantigen and then transferred to microtiter plates coated with the antigen as previously noted. The binding was determined by RIA, as above. Antithymocyte antibodies were detected by RIA as above, using 100,000 thymocytes per well.

Immunoperoxidase Assay. Immunoperoxidase staining was used to detect binding activity against human skin and murine lung cryostat sections. Cryostat sections were incubated for 1 h at 4°C with 0.5 ml of 10 μg/ml chromatographically purified antibody, washed several times, and then incubated for 1 h with horseradish peroxidase–conjugated rabbit anti–mouse Ig antibodies.

Immunofluorescence. Rat stomach and kidney frozen sections were used to detect antinuclear, antimitochondrial, anti–smooth muscle, and anti–glomerular basal membrane antibodies according to a previously described technique (8). Antithymocyte and anti-RBC antibodies were detected by incubating 10^6 cells (from control C57BL/6J mice) for 45 min on ice with 50 μl of 1:2 diluted culture supernatant, washing three times, and then incubating for 45 min on ice with 20 μl of 1:10 diluted fluoresceinated goat anti–mouse Ig (Southern Biotechnology Associates, Inc., Birmingham, AL). After three washes, the fluorescence was measured using a cytofluorograph (model 50H; Ortho Diagnostic Systems Inc., Westwood, MA).

V Gene Probes. Plasmids of interest were transfected into *Escherichia coli* HB101: HB101 were grown to a Δ Klett of 40–60, pelleted, and resuspended in 0.5× volume of 50 mM cold $CaCl_2$ and incubated on ice for 40 min; bacteria were repelleted and resuspended in 0.2× volume of 50 mM cold $CaCl_2$ and incubated on ice for 10 min. 5–10 ng of plasmid were mixed with 0.2 ml of bacteria and incubated 15 min on ice, then 2 min at 37°C, and finally 10 min at room temperature. After addition of nonselective

[1] *Abbreviations used in this paper:* AcR, acetyl choline receptor; CII or CIII, murine collagen type II or III; IF, intrinsic factor; MBP, myelin basic protein; RF, rheumatoid factor; TG, thyroglobulin; TR, transferrin.

LB medium and incubation for 1 h at 37°C, the transformation mixture was plated with 100 μg/ml ampicillin. Single colonies were expanded in bulk cultures and plasmids were prepared by the alkali lysis method (9), except that 3 M sodium acetate brought to pH 4.8 with glacial acetic acid was used. After CsCl density gradient centrifugation (9), purified plasmids were digested with appropriate restriction enzymes (New England Biolabs, Beverly, MA, or IBI), after which probes were isolated by electroelution (200 V, 30 min; 30-s reverse current) in 0.5× TBE (9) from slices from 5% polyacrylamide gels (9).

Purified probes (100 ng) were nick translated with 60 μCi of α-[^{32}P]dCTP (3,000 Ci/mol, Amersham Corp., Arlington Heights, IL) to a sp act of ≥1 × 10^6 cpm/μg. The reaction contained 2 μl each of 10–25 nM dATP, dTTP, and dGTP (Pharmacia Fine Chemicals, Piscataway, NJ), 1 μl of 0.1 ng/ml DNAse I (Worthington Biochemical Corp., Freehold, NJ), and 5 U Kornberg DNA polymerase I (New England Biolabs) in 1× nick translation buffer (50 mM Tris–HCl pH 7.8, 5 mM $MgCl_2$, 10 mM 2-ME, 50 μg/ml BSA). Incubation was at 14–16°C for 75 min, after which 2 μl of 0.5 M EDTA and 10 μg of tRNA (Sigma Chemical Co.) were added. Probe was separated from unincorporated label using a G-50 Sephadex spin column in a 3-ml syringe packed in H_2O and prerun with 1 ml of TE, pH 7.4, containing 100 μg of salmon sperm DNA (Sigma Chemical Co.) (9).

The following five V_H gene probe constructs (prepared in the laboratory of F. Alt, Columbia University) are in SP65 plasmids: the V_H J606 probe is a Hind III/Eco RI fragment containing the 300-bp Hind III/Bgl II fragment of a rearranged A/J anti-GAC V_H gene (10); the V_H 36–60 probe is a 350-bp Eco RI/Pvu II fragment containing the 140-bp Bam HI/Eco RI fragment of the V_H 36–60 gene (11); the V_H NP.B4 probe (V_H family J558) is a 315-bp Pst I/Eco RI BALB/c germline V_H gene fragment (12); the V_H S107 probe is a 213-bp Dde I fragment, isolated from a rearrangement, which was subcloned after addition of Eco RI linkers (13); and the V_H QPC52 probe is a 300-bp Pvu II/Hae III fragment subcloned after addition of Eco RI linkers (14). The V_H81X probe (V_H family 7183) (kindly given by G. Yancopoulos and F. Alt, Columbia University) is a 280-bp Pst I/Eco RI fragment containing the Pvu II/Pst I fragment of the germline V_H81X(G) gene cloned in SP64 (15). The V_H 441-4 probe (V_H X24 family) (graciously given by A. B. Hartmann and S. Rudikoff, National Cancer Institute, NIH, Bethesda, MD) is a 500-bp Eco RI fragment of a germline gene cloned into pUC8 (clone Gal 39.1) (16). The V_H 36–09 probe (kindly provided by D. J. Capra, Texas Medical Center, Dallas, TX) is a 1.6-kb Eco RI/Bam HI fragment in pUC12 (17).

The V_κ1 probe (generously given by C. Schiff and M. Fougereau, Centre d'Immunologie, INSERM, CNRS, Marseille, France) is a 300-bp Dde I fragment from the LXIX27 cDNA cloned into pBR322 (18). The V_κ4 probe (generously supplied by M. Heller and S. Rudikoff, NCI) is a 260-bp Pvu II/Ava I fragment of a pBR322 clone containing a 400-bp cDNA. The V_κ8 probe (kindly provided by P. Gearhart, Baltimore, MD) is a 360-bp cDNA fragment cloned into the Sma I site of pUC9 (18a). The V_κ10 probe (clone pC3386, obtained through the generosity of M. Shapiro and M. Weigert, Institute for Cancer Research, Philadelphia, PA) is a 900-bp Eco RI/Hind III fragment of a nonproductive κ gene rearrangement cloned into pUC12 (19). The V_κ19 probe is a 400-bp Eco RI/Hind III fragment from a pBR322 plasmid into the Pst I site of which the Pvu II/Hpa II fragment of the MPC11 V_κ gene cDNA was subcloned (20). The V_κ21 probe (generously provided, along with the Vκ19 probe, by D. Kelly and R. Perry, Institute for Cancer Research) is a 700-bp Hind III/Hha I fragment from a pBR322 plasmid containing cDNA sequence of MOPC321 (21). The V_κ22 probe (a gift from P. Gearhart) is a 1.2-kb Xba fragment of the V_κS107A rearranged genomic clone in pBR322 (21a). The V_κ24 probe (kindly given by P. Gearhart) is a 900-bp Hind III germline gene fragment from BALB/c mice cloned into the Hind III site of pBR322 (22). The V_κ2 probe is a 350-bp Pst I/Mbo II fragment of a pBR322 clone of the M603K2 1-kb cDNA (23). The V_κ9 probe (kindly supplied along with the V_κ2 probe F. Alt, Columbia University) is a 350-bp Pst I/Mbo II fragment of a pUC9 clone of the M41K1 950-bp cDNA (23).

TABLE I

Frequency of Ig Secretory Hybridomas Obtained from Motheaten Mice

	Number of hybrids obtained	Number of Ig secretors	Number of Ig nonsecretors	Fraction of autoantibodies	Number of multispecific autoantibodies
1st Fusion (2 mo old)	14	6	8	5/6	3/5
2nd Fusion (1 mo old)	385	41	344	12/27*	8/12

* 14/41 Ig secretors were lost before they could be analyzed.

Preparation of Total RNA and Northern Blot Analysis

Total RNA was prepared from 3–5 × 10^7 cells using the guanidinium thiocyanate extraction procedure (9). Northern blotting was performed by electrophoretically fractionating the RNA on a 1.2% agarose gel (6% formaldehyde) in 40 mM 4-morpholinopropanesulfonic acid, 20 mM NaOAc, and 2 mM EDTA. The gel was blotted overnight (without pretreatment) onto nitrocellulose using 20× SSC (3 M NaCl, 0.3 M sodium citrate) (8). Blots were baked in a vacuum oven 2 h at 80°C.

Prehybridization was carried out at 42°C in 50% formamide, 5× SSCPE (20× = 2.4 M NaCl, 0.3 M sodium citrate, 0.2 M KH_2PO_4, 0.02 M EDTA), 5× Denhardt's, 500 μg/ml sheared denatured salmon sperm DNA. Probe was added to 10^6 cpm/ml in hybridization solution (50% formamide, 5× SSCPE, 1× Denhardt's, 100 μg/ml salmon sperm DNA, and 10% dextran sulfate). Blots were hybridized overnight at 42°C. Blots were washed three times for 15 min in 2× SSC, 0.1% SDS at 68°C and exposed to Kodak XAR film using Dupont Lightning Plus intensifying screens at −70°C for several days. V_H blots were rewashed in 0.1× SSC, 0.1% SDS at 68°C for 15 min after the initial exposure, reexposed, and the relative decreases in signal were noted.

Results

Preparation of Hybridomas

It proved difficult to obtain successful fusions from 1- and 2-mo-old *me*^{v} spleens. The *me*^{v} splenic cell population contains increased numbers of cells from the monocytic and granulocytic lineages (2), which may explain the overgrowth of our first fusion with nonlymphoid cells and the limited number of hybrids we were able to obtain (the S2 series). A number of approaches were taken to overcome this problem for the second fusion: a thymocyte feeder layer was used, fused cells were plated at a fivefold greater dilution than is normally used, and hybrids were transferred from the plates 5–7 d after fusion, before overwhelming overgrowth had occurred. A panning procedure to select for sIg$^+$ cells before fusion yielded only one hybrid (A-23-10), while all other hybrids of the second fusion resulted from cells that were not subjected to the panning procedure (the *un*selected UN series). The second fusion yielded hybrids in nearly every well, resembling the yield from fusions of polyclonally activated spleen cells.

More than 350 hybrids obtained were tested for Ig secretion using anti-κ or anti-λ antibodies in a sandwich RIA. Surprisingly, very few (12% of the hybrids) were secretory (see Table I). This low percentage could be due to the fusion of nonlymphoid cells or Mott cells (nonsecreting B cells with intracellular Ig deposits), or it could result from possible B cell defects resulting in a high incidence of unproductive rearrangements or truncated Ig message. Analysis of the nonsecreting hybrids will be addressed in future studies.

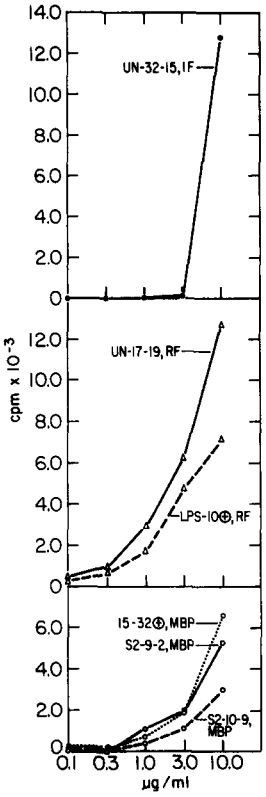

FIGURE 1. Dose-effect relationship of the binding activity of various concentrations of mAb (0.1–10 μg/ml) to microtiter plates coated with either IF (*top*), heat-aggregated IgG2a (*middle*), or guinea pig MBP (*bottom*). LPS-10 is a monoclonal RF and 15-32 is a monoclonal anti-MBP (positive controls).

Frequency of Self-reactive Antibodies Produced by me^v Hybridomas

In preliminary screening experiments, the binding activity of Ig found in hybrid cell supernatants was tested against a panel of autoantigens. A high frequency (17/33) of autoantibodies was observed (see below). The autoreactive hybrids were cloned and their antibodies were chromatographically purified on a rat anti–mouse κ chain–Sepharose 4B column (as all positive antibodies had already been isotyped as bearing κ light chain). The purified mAbs were used in the subsequent studies on antigen specificity, and two groups of antibodies were identified based on these studies.

Monospecific Antibodies. Among this panel of autoreactive antibodies, six were found to be specific for a single antigen among those tested. Three mAbs bound to a single soluble antigen in a dose-dependent manner (Fig. 1). UN-32-15 bound to IF, UN-17-19 exhibited rheumatoid factor (RF) activity, and S2-9-2 was specific for MBP. UN-17-19 bound to a determinant common to various classes of murine IgG but had its highest binding activity to IgG2b (Table II). The binding of S2-9-2 and S2-10-9 to MBP obtained from seven different mammalian species was compared with that of the MBP mAb 15-32 (obtained from an SJL/J mouse immunized with spinal cord extract) (Table II). Striking differences were observed, in that 15-32 exhibited high binding activity to mouse MBP, while binding activities of both *me^v* antibodies were low. In contrast, the *me^v* antibodies both showed high binding activity against human, monkey, and rabbit MBP.

TABLE II

Fine Specificity of Rheumatoid Factor– and Myelin Basic Protein–specific Viable Motheaten mAbs

	BSA	IgG3	IgG1	IgG2b	IgG2a
A. Rheumatoid factor					
LPS10-1	1,174 ± 32*	6,572 ± 31	8,884 ± 212	13,512 ± 185	14,752 ± 1,417
UN-17-19	148 ± 1	1,732 ± 107	1,104 ± 134	4,218 ± 1,143	1,825 ± 309

	BSA	Murine	Bovine	Rat	Human	Monkey	Rabbit	Guinea pig
B. Myelin basic protein								
15-32	442 ± 116	6,293 ± 335	1,561 ± 59	5,345 ± 1,504	23,667 ± 885	21,297 ± 1,410	23,326 ± 1,210	3,929 ± 993
S2-10-9	220 ± 1	684 ± 191	315 ± 30	1,056 ± 126	3,826 ± 458	5,475 ± 346	5,236 ± 338	2,576 ± 412
S2-9-2	431 ± 7	963 ± 85	1,032 ± 162	2,881 ± 40	7,294 ± 513	8,926 ± 749	10,388 ± 1,897	2,336 ± 963

* cpm: Average of triplicates ± SD. Microtiter plates coated with 10 μg/ml antigen (IgG or myelin basic protein) in carbonate buffer, pH 9.0, were incubated with BSA, washed, and then incubated for 3 h at room temperature with 10 μg purified antibody. After washing, the plates were incubated for 3 h with ^{125}I–rat anti–murine κ mAb (50,000 cpm/well).

Some *me*^{v} mAbs bound to a single cellular antigen: A-23-10 bound to glomerular basal membrane (Fig. 2); UN-5-8 bound to human skin with the same pattern as HB10, an mAb specific for basal keratinocytes (24) (A-23-10 was used as a negative control) (Fig. 2); and UN-55-5 bound to nuclear antigen (not shown).

Multispecific Antibodies. This second group contained 11 *me*^{v} mAbs which exhibited multiple antigen binding patterns (including both soluble and cellular antigens) (summarized in Table III). S2-6-10 and S2-17-6 both showed binding activity to MBP and IF (Fig. 3), and to either RBC or Thy, respectively. UN-59-9 bound to MBP, TG, and RBC, while UN-37-5 bound to TG, AcR, IgG2a and RBC (data not shown). Three antibodies, UN-40-3, UN-40-6, and UN-40-9, exhibited similar but not identical patterns of binding to a number of self-antigens including TG, TR, Sm, MBP, AcR, IF, and IgG2a (Fig. 4). All (11/11) of the multispecific antibodies exhibited binding to either RBCs or thymocytes or both. The data presented in Fig. 5 illustrate the three specific patterns of binding to either or both of these cell types. It should be noted that in all cases the antibodies bound at most 20–30% of these cell populations, suggesting cell subset specificities.

To further characterize the unusual antigen binding spectrum of the multispecific antibodies, we carried out competitive inhibition RIA analysis (using the soluble antigens only). At room temperature, aliquots of multispecific mAbs were incubated with a given self-antigen, and the antigen binding activity that remained was revealed in solid-phase RIA. In the case of S2-6-10 and S2-17-6, the binding to MBP was more strongly inhibited by IF than by MBP, while the binding to IF was only inhibitable by IF (Fig. 6). In contrast to these two, the binding to a particular autoantigen of the other five multispecific antibodies tested was inhibited to various degrees by all of the other autoantigens bound by a given antibody. A summary of these data is presented in Table IV.

The frequency of autoantibodies was significantly higher among *me*^{v} hybridomas compared with hybridomas obtained by fusion of nonstimulated C57BL/6J-*nu/nu* or C57BL/6J mice. Only 4 of 144 hybridomas obtained from nude and 5 of 36 Igκ-producing hybridomas obtained from C57BL/6J exhibited binding to self-antigens. The data depicted in Table V show that multispecific antibodies

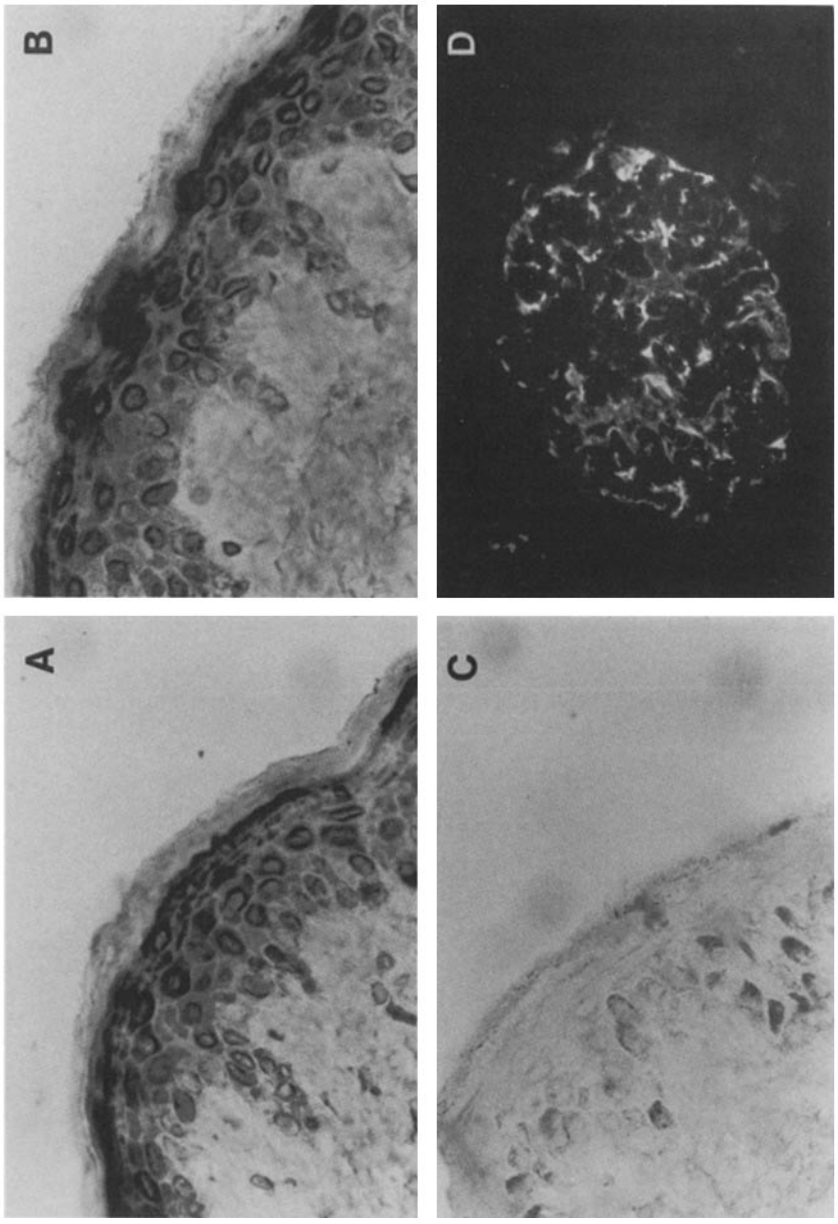

FIGURE 2. Human skin cryostat sections incubated with 0.5 ml of 10 μg/ml of UN-5-8 (*A*), HB10 (*B*), or A-23-10 (*C*) mAbs. After washing, these have been incubated with horseradish peroxidase–conjugated rabbit anti–mouse Ig antibody. (*D*) Rat kidney cryostat section incubated with 10 μg/ml A-23-10 antibody, washed, and incubated with fluoresceinated rabbit anti–mouse Ig antibody.

TABLE III

Summary Results: Immunochemical and Molecular Characteristics of Viable Motheaten mAbs

Designation	Isotype	V_H	V_k	Antigen specificity
S2-6-10	$\alpha\kappa$	7183	10	MBP, IF, Thy
S2-9-2	$\mu\kappa$	J558	NI	MBP
S2-10-9	$\gamma_3\kappa$	J558	1	MBP, RBC, Thy
S2-14-2	$\gamma_1\kappa$	J606	10	RBC, Thy
S2-17-6	$\mu\kappa$	7183	NI	MBP, IF, RBC
A23-10	$\mu\kappa$	S107	19	GBM
UN-5-8	$\mu\kappa$	S107	NI	Skin
UN-17-19	$\mu\kappa$	X24	4	IgG
UN-32-15	$\mu\kappa$	J558	10	IF
UN-34-11	$\mu\kappa$	ND	ND	RBC, Thy
UN-37-5	$\mu\kappa$	J606	4	TG, AcR, IgG, RBC
UN-40-3	$\mu\kappa$	7183/X24	1	IF, TG, AcR, IgG, RBC
UN-40-6	$\mu\kappa$	7183/X24	1	TG, Sm, TF, MBP, IgG, RBC, Thy
UN-40-9	$\mu\kappa$	7183/X24	1	IF, TG, Br-RBC, RBC, Thy
UN-42-5	$\mu\kappa$	J606	10	Thy, RBC
UN-55-5	$\mu\kappa$	S107	NI	Nuclear antigen
UN-59-9	$\mu\kappa$	J558	19	MBP, TG, RBC

MBP, myelin basic protein; IF, intrinsic factor; TG, thyroglobulin; RF, rheumatoid factor; AcR, acetylcholine receptor; TR, transferrin; IgG, gamma globulin; Br-RBC, bromelain-treated RBC membrane; GBM, glomerular basal membrane; RBC, red blood cells; Thy, thymocytes; NI, not identified; ND, not done.

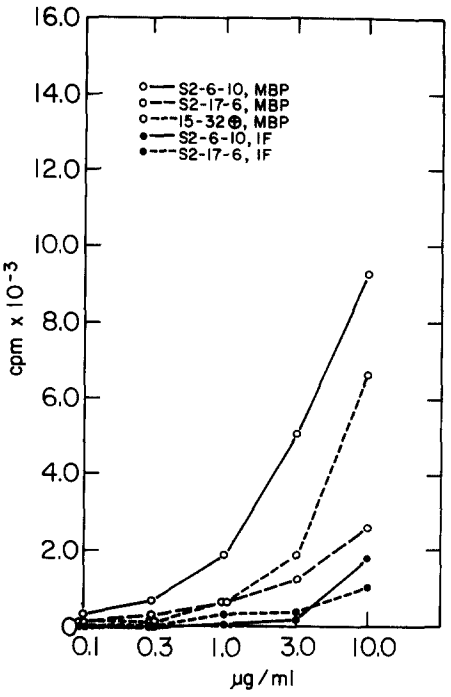

FIGURE 3. Dose-effect relationship of the binding activity of various concentrations (0.1–10 µg/ml) of two mAbs, S2-6-10 and S2-17-6, to microtiter plates coated with either IF or MBP. 15-32 is a monoclonal anti-MBP positive control.

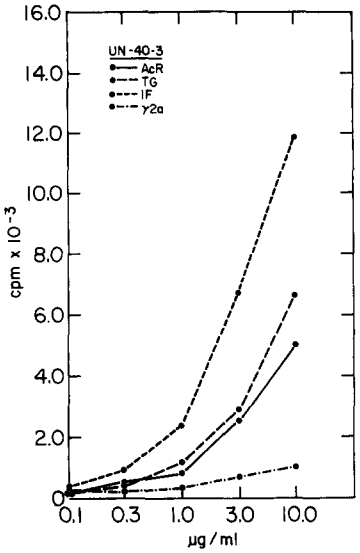

FIGURE 4. Dose-effect relationship of the binding activity of various concentrations (0.1–10 μg/ml) or mAb UN-40-3 to microtiter plates coated with AcR, TG, IF, or heat-aggregated IgG2a.

were also observed among autoantibodies obtained from unstimulated lymphocytes, but the antigen specificities displayed were different from *me*^{v} autoantibodies.

Northern Analysis of V_H and V_κ Gene Family Usage

An overrepresentation of 3′ V_H gene families among autoantibodies has been reported (25, 26), and a high usage of several V_κ gene families among murine and human RFs has been observed (27, 28). Therefore, we examined the V_H and V_κ gene family usage in *me*^{v} monoclonal autoantibodies to determine if any preferential usage had occurred. Three autoantibodies used V_H J606; one used V_H X24; four used V_H J558; three used V_H S107; two used V_H 7183; and three autoantibodies exhibited crosshybridization to V_H X24 and V_H 7183 (Fig. 7*A* and Table III). It has been previously observed (29) that under normal stringency hybridization conditions, crosshybridization may occur between V_H 7183 and V_HX24.

Among the 16 autoantibodies tested, V_κ4 and V_κ19 were used twice each, and V_κ1 and V_κ10 were each used four times (Fig. 7*B* and Table III). Since 12 of 16 *me*^{v} autoantibodies used genes from four V_κ subgroups (V_κ1, V_κ4, V_κ10 or V_κ19), the other four must use V_κ genes of the remaining 18 families. This usage is substantially different from that observed in 35 hybridomas obtained by fusion of resting (nonstimulated) C57BL/6J lymphocytes. The data depicted in Table VI indicate a more random usage of V_κ gene families among nonstimulated C57BL/6J-derived hybridomas (selected by Igκ secretion) than among the *me*^{v} autoantibodies reported here. Table III summarizes the antigen specificities, isotypes, and V gene family usage among the panel of 17 *me*^{v}-derived autoantibodies.

Discussion

The extreme severity of the autoimmune process in *me* and *me*^{v} mice (1, 3) and the fact that most or all of the B cells of these mice are of the Ly-1 B cell

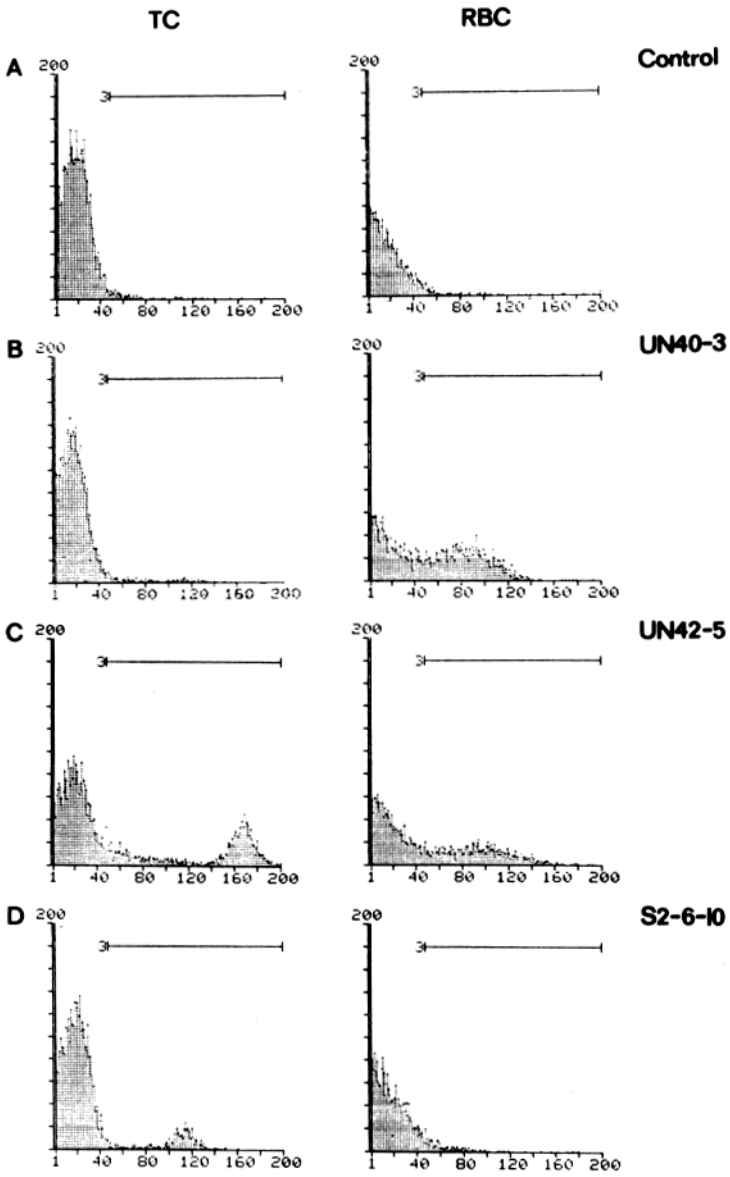

FIGURE 5. Three binding patterns of *me*^{v} mAbs to thymocytes and erythrocytes. RBCs or thymocytes from 1–2-mo-old C57BL/6J mice were reacted with culture supernatants from the indicated clones, counterstained with (1:10) FITC–goat anti–mouse Ig, and the scatter-gated (90° × forward) populations were examined for fluorescence on a cytofluorograph. In this experiment, the percentages of cells considered fluorescent (i.e., within gate 3 indicated were:

1st Antibody	Fluorescent cells	
	Thy	RBC
	%	
—	3	5
40-3	4	33
42-5	31	23
6-10	10	4

Staining is on a log scale. *x*-axis, fluorescence; *y*-axis, number of cells.

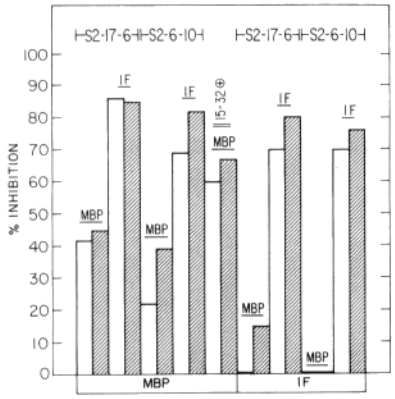

FIGURE 6. Percent inhibition of binding activity of mAb S2-17-6 or S2-6-10 to MBP- or IF-coated microtiter plates (as indicated at the bottom of the figure) in the presence of the inhibitor antigen (indicated above each pair of vertical bars). Open and dashed bars represent results obtained using 10 and 30 μg/ml of inhibitor, respectively. 15-32 is a monoclonal anti-MBP positive control.

TABLE IV

Binding Specificity to Soluble Autoantigens of Multiply-reactive Antibodies

Designation	Binding activity	Inhibition of binding (>50%)	
		To:	By:
S2-6-10	MBP, IF	MBP	IF
		IF	IF
S2-17-6	MBP, IF	MBP	IF
		IF	IF
UN-59-9	MBP, TG	MBP	MBP, TG
		TG	TG
UN-37-5	TG, AcR, G2a	TG	TG, G2a
		AcR	TG, G2a, AcR
		G2a	G2a
UN-40-3	IF, TG, AcR, G2a	IF	IF, TG
		TG	TG, G2a
		AcR	TG, G2a
		G2a	TG, G2a
UN-40-6	TG, Sm, TR, MBP, G2a, Sm	TG	TG, MBP, G2a, Sm
		Sm	Sm, MBP, G2a
		TR	G2a
		MBP	MBP, G2a
		G2a	MBP, TG, G2a, Sm
UN-40-9	IF, TG, G2a, Br-RBC	IF	IF, TG, G2a, Br-RBC
		TG	IF, TG, Br-RBC
		G2a	IF, G2a, Br-RBC
		Br-RBC	IF, TG, G2a, Br-RBC

Abbreviations as in Table III, and G2a, gamma globulin 2a.

lineage (4), make motheaten mice an interesting and important model for studying the genetic etiology of autoimmune disease.

The isotype distribution of the antibodies produced by hybridomas from 1- and 2-mo-old *me*^{v} mice (i.e., mostly IgM, with one each IgG3, IgA, and IgG1) was similar to that observed in the serum of *me*^{v} mice (4). The sole presence of IgM-secreting hybrids from the fusion of 1-mo-old *me*^{v} splenocytes correlates with the in vivo elevation of serum IgM levels occurring as early as 3 wk of age.

TABLE V

Specificity of Autoantibodies Obtained from C57BL/6JSmn-nu/nu and C57BL/6JSmn Unstimulated Spleen Lymphocytes

Origin of hybridomas	Frequency of autoantibodies	Designation	Specificity	Reference
C57BL/6JSmn-*nu/nu*	4/144	N100-1	IF, TG, DNA*	33
		N63-3	TG, IF	
		N65-1	RF	
		N64-1	TG	
C57BL/6JSmn	5/35	B6-105	IF, RF, CII, CIII,* TG	33a
		B6-112	IF, CII, CIII*	
		B6-21	CII, TG, RB	
		B6-22	TG, Thy	
		B6-46	TG	

Abbreviations: IF, intrinsic factor; TG, thyroglobulin; RF, rheumatoid factor; CII, collagen type II; CIII, collagen type III.

* This specificity was not tested for among *me*^{v} antibodies.

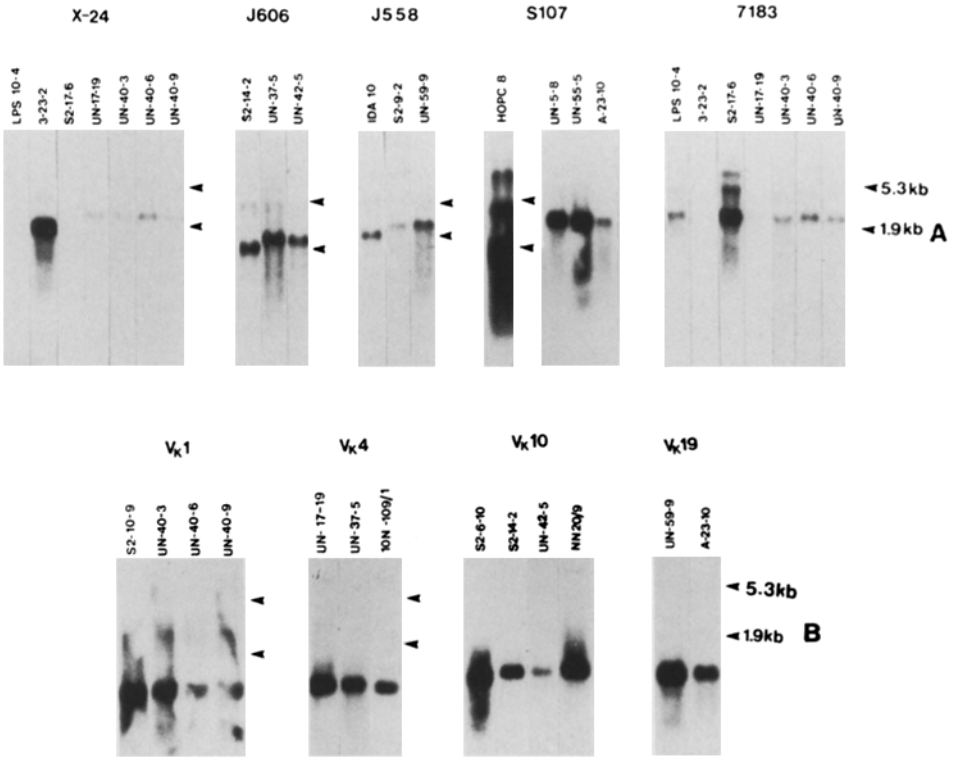

FIGURE 7. Northern blots of total cytoplasmic RNA probed with indicated V_H (*A*) or V_κ (*B*) probes. LPS10-4 is a V_H 7183 positive control; 3-23-2 is a V_HX24^+ control; IDA-10 is a $J558^+$ control; HOPC8 is an $S107^+$ control; 10N109/1 is a $V_\kappa4^+$ control; NN20/9 is a $V_\kappa10^+$ control.

TABLE VI

Frequency of V_κ Families Used by me^v Autoantibodies and by Antibodies Secreted by Hybridomas Obtained From Fusion of Nonstimulated C57BL/6JSmn Mouse Lymphocytes

V_κ Family	Motheaten	C57BL/6J
$V_\kappa 1$	4/16	1/35
$V_\kappa 2$	0/16	2/35
$V_\kappa 4$	2/16	3/35
$V_\kappa 8$	0/16	1/35
$V_\kappa 9$	0/16	1/35
$V_\kappa 10$	4/16	0/35
$V_\kappa 19$	2/16	2/35
$V_\kappa 21$	0/16	2/35
$V_\kappa 22$	0/16	1/35
$V_\kappa 24$	0/16	0/35
NI	4/16	25/35

Interestingly, no λ-bearing autoantibodies were found, even though up to one-third of the total *me*^{v}-derived serum IgM expresses the λ isotype (4).

Among our panel of *me*^{v}-derived antibodies, 33 were tested for antigen specificity and of these, 17 were self-reactive. Most of the reported in vivo autoantigen specificities of *me*^{v} mice (e.g., antibodies reactive with skin, kidney, nuclear antigens, thymocytes, or erythrocytes) were represented in this panel. These specificities may directly contribute to the documented pathophysiology of *me*^{v} mice.

Additionally, we identified hybridomas producing antibodies reactive with IF, TR, MBP, and TG. However, neither demyelinizing encephalitis nor thyroiditis has been observed in *me*^{v} mice. The life span of *me*^{v} mice (average of 9 wk) may be too short for disease processes due to these autoantibodies to be manifested.

Most (11/17) of the autoantibodies from *me*^{v} mice exhibited multiple binding properties. Autoantibodies able to bind to several self-antigens have been observed in humans (30) as well as in mice (31, 32). In murine systems, this type of antibody has been found among those produced by hybridomas prepared from unstimulated lymphocytes of adult C57BL/6J euthymic or athymic (nude) mice (33), and from adult mice subsequent to polyclonal stimulation (8).

Competitive inhibition binding assays suggest that some multispecific antibodies are more reactive for one antigen than to another. This was the case for S2-6-10 and S2-17-6, both of which bound to MBP and IF, and where the inhibition of binding to both of these antigens was significantly stronger with IF than with MBP. Five antibodies exhibited a variety of binding activities that were all inhibitable to various degrees by all of the recognized soluble antigens. It has been suggested that multispecific autoantibodies may have housekeeping function (34) and may not be involved in autoimmune pathogenesis. Alternatively, somatic mutation events occurring in the genes used may confer a stronger affinity for a given autoantigen and result in pathogenic autoantibodies.

The frequency of multispecific autoantibodies among our panel of *me*^{v}-derived antibodies (11/33) was significantly higher than those obtained from the non-autoimmune strains BALB/c (2/356 [8]), C57BL/6JSmn nude (2/144 [33]), and

C57BL/6JSmn (3/35 [33a; Table IV]). Of 18 autoantigen specificities screened, 11 were represented among 17 *me*^{v}-derived autoantibodies, indicating that a broad range of autoantigen specificities exists among *me*^{v} antibodies. Both the high frequency of multispecific autoantibodies and the broad range of autoantigens recognized may contribute to the pathogenesis of the several syndromes in *me*^{v} autoimmune disease.

The broad range of autoantigen specificities observed among the *me*^{v}-derived monoclonals may reflect polyclonal activation of *me*^{v} B cells that is not driven by autoantigens. Two findings are important in this regard. First, high levels of a novel B cell–produced B cell maturation factor (BMF) have been reported in *me*^{v} mice (35). Moreover, most of the *me*^{v} hybridomas reported here secreted detectable amounts of this factor (data not shown). Second, all or nearly all *me*^{v} B cells appear to be of the Ly-1 B lineage (4). These cells are responsible for most of the antithymocyte and anti-DNA autoantibody production in NZB mice, as well as a significant portion of the anti-Br–RBC response (6). Adoptive transfer experiments indicate that Ly-1 B cells are an independent lineage and possess an immature phenotype (increased sIgM and decreased sIgD) (reviewed in reference 36). It has been suggested that the predominance of the Ly-1$^+$ phenotype among *me*^{v} B cells may reflect an altered regulatory process during immune system development (4). Whether the elevated level of BMF is causally connected to the Ly-1 B cell predominance, or whether Ly-1 B cells are polyclonally activated by BMF, remain unanswered questions. For whatever reason, however, our data strongly suggest a high frequency of self-reactive B cells (17/33) in the Ly-1 B cell population in *me*^{v} mice.

In these *me*^{v} autoantibodies, we have observed a predominant usage (12/16) of four V_κ gene families whose usage was reported to be frequent in murine RFs (27) and other autoantibodies of various specificities (33a). In particular, the frequencies of two of these V_κ families ($V_\kappa 1$ and $V_\kappa 10$) in *me*^{v}-derived hybridomas were significantly higher than those observed in C57BL/6JSmn-derived hybridomas, and accounted for 8 of 16 *me*^{v}-derived but only 1 of 35 C57BL/6JSmn-derived hybridomas. The *me*^{v}-derived hybridomas did not, however, show the biased usage of 3′ V_H gene families, which was previously observed among autoantibodies spontaneously produced in animals prone to autoimmune disease (15, 16). Rather, the utilization of V_H families among autoantibodies produced by *me*^{v} mice was similar to that observed by antibodies obtained from LPS-stimulated lymphocytes from normal strains (37).

In summary, the high frequency of autoantibodies possessing multispecific binding properties, and the utilization of a wide variety of V_H but predominantly four V_κ gene families, appear to be due to an immunoregulatory defect in *me*^{v} mice operating at a more generalized level than the V_H and V_κ loci.

Summary

Several hundred hybridomas were obtained from 1–2-mo-old viable motheaten (*me*^{v}) mice. Among the Ig-secreting hybridomas tested, >50% (17/33) exhibited reactivity for autoantigens, supporting the idea that the Ly-1 B cells that predominate in *me*^{v} mice contain frequent precursors of autoantibody-forming cells.

Certain of the specificities of these autoantibodies correlated with the docu-

mented pathophysiology of *me*^{v} mice (antithymocyte, -erythrocyte, -skin, -kidney, and -IgG); others were specific for autoantigens not previously observed in motheaten mice but thought to be involved in other autoimmune diseases (e.g., intrinsic factor, transferrin, myelin basic protein, and thyroglobulin). About 2 of 3 (11/17) of the self-reactive antibodies exhibited multispecific binding activity for various autoantigens.

Analysis by Northern blotting of the V gene families used in *me*^{v} autoantibodies showed a random usage of V_H families and a biased usage of four V_κ gene families. Of 16 autoantibodies tested, 12 used a V_κ gene from the V_κ1, 4, 10, or 19 families. These patterns of V_κ gene usage differ from nonautoimmune control animals.

Overall, an immunoregulatory defect operating at a more generalized level than the V_H or V_κ loci, and due to a single gene mutation, appears to be responsible for the multiple immune abnormalities of *me*^{v} mice.

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