A Novel Class of Anti-DNA Antibodies Identified in BALB/c Mice

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Summary

We have characterized four IgG monoclonal antibodies (mAbs) derived from BALB/c mice that bind double-stranded DNA (dsDNA) with high affinity. The hydridomas were selected for expression of a member of the V_HS107 family. Three of the four cell lines use the V_H11 gene and one uses the V_H1 gene. These antibodies exhibit many characteristics of pathogenic anti-DNA antibodies. They are high affinity and not broadly crossreactive. Unlike the anti-DNA antibodies in autoimmune mice, they exhibit no somatic mutation in their V_H genes. These results demonstrate that somatic mutation of V_HS107 genes is not necessary for generating high affinity dsDNA binding. The fact that such antibodies have not previously been reported suggests that they are rare and that their expression may be downregulated in both nonautoimmune and autoimmune individuals.

Recent analyses of autoantibodies have established that there are two qualitatively different classes of autoantibodies. One class is the "natural" autoantibodies, made by autoantibody-producing B cell lines derived from both normal individuals and individuals with autoimmune disease; the other class can be derived only from autoimmune individuals. The physiologic function of the ubiquitous "natural" autoantibodies is not known and it is assumed that they have no pathogenic potential (1–5). "Natural" autoantibodies have been induced in normal and autoimmune strains both in vitro and in vivo by stimulation with bacterial LPS, a polyclonal B cell activator (6–9). Monoclonal autoantibodies derived from LPS-stimulated B cells are generally of the IgM isotype, are widely crossreactive with a variety of autoantigens and exogenous antigens, and are encoded by unmutated germline genes. Most of these antibodies are low affinity, but some may exhibit moderate binding to a particular autoantigen (10). However, because they are pentameric IgM, it is difficult to attribute this binding to true affinity. It has been suggested that these autoantibodies are the product of a particular B cell lineage that displays the CD5 (Ly1) marker on the membrane (11, 12).

In contrast, (NZB × NZW)F_1 and MRL/*lpr* autoimmune strains spontaneously produce various autoantibodies that differ in a number of parameters from natural autoantibodies. Autoimmune animals produce high affinity autoantibodies of the IgG isotype that are not widely crossreactive and are encoded by somatically mutated genes (13–16). Autoantibodies with these characteristics are a hallmark of both the human disease SLE and of the (NZB × NZW)F_1 and MRL/*lpr* mouse models of the human disease. Furthermore, these autoantibodies are believed to have pathogenic potential, since autoantibodies eluted from diseased kidneys of autoimmune mice exhibit the same characteristics: they are IgG, high affinity, and not broadly crossreactive (1, 17–21). Autoantibodies with these characteristics have not previously been found in nonautoimmune strains. Some investigators have speculated that these disease-associated autoantibodies may be the somatically mutated progeny of natural autoantibodies (22).

In this paper we describe four anti-DNA antibodies secreted by hybridomas derived from BALB/c mice. Three of the four hybridoma antibodies reported here were derived from animals immunized with an anti-I-J antibody (23), and a fourth was produced in an animal that received a single immunization of phosphorylcholine (PC)[1] coupled to a protein carrier. These anti-dsDNA hybridomas were selected for expression of a member of the S107 V_H family. The S107 gene family has four members, (V_H1, V_H3, V_H11, and V_H13) and is known to be important in the protective immune responses to pneumococcal bacteria (V_H1) and to influenza (V_H11) in BALB/c mice. Previous studies showed that a single amino acid substitution in the PC-binding, V_H1-encoded myeloma cell line S107, resulted in production of the U4 antibody, which no longer bound PC but had acquired reactivity with dsDNA (24). The analysis of the U4 mutant suggested that the S107 gene family might contribute to the in vivo produc-

[1] *Abbreviations used in this paper:* ARS, *p*-azo-phenylarsonate; HGG, human gammaglobulin; PC, phosphorylcholine.

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tion of anti-DNA antibodies and studies of serum and hybridoma antibodies have shown that the S107 family is indeed used in the anti-DNA response in both the (NZB × NZW)F_1 and MRL/*lpr* strains of mice (13–15, 25). The antibodies reported here have features of autoantibodies found in autoimmune disease; they are IgG, have high affinity for dsDNA and are not broadly crossreactive. They differ from the autoantibodies found in autoimmune disease because they are encoded by germline V_H genes containing no somatic mutations. Two are encoded by germline V_k genes also; the germline V_k genes for the other two antibodies are not known so it is not possible to determine whether the expressed V_k genes are unmutated, although this is likely to be the case. Autoantibodies with these characteristics in autoimmune disease display extensive somatic diversification.

The detailed analysis of these four antibodies leads us to posit a new third class autoantibody, a class of highly specific IgG anti-DNA antibodies that are encoded by nonmutated germline genes and that may not generally be expressed either in normals or in individuals with lupus-like autoimmune disease. The existence of these antibodies raises new questions about the nature of germline gene-encoded antibodies and the regulatory defect that may be present in autoimmunity.

Materials and Methods

Cell Lines. 8-wk-old female BALB/c mice were immunized intraperitoneally with either anti-I-J^d 50 μg in CFA or PC conjugated to human gammaglobulin (HGG), 100 μg in CFA. Mice were boosted intravenously with either 100 μg PC coupled to KLH, 100 μg *p*-azo-phenylarsonate (ARS)-KLH, or 100 μl of an antiserum to the T15 idiotype (Table 1). The antiidiotype was prepared as follows: rabbits were immunized with S107 protein and high titered antiserum was then absorbed on an irrelevant mouse IgA antibody (W1329)-Sepharose to render it variable region specific. Spleen cells from the immunized mice were fused to the nonproducing HAT-sensitive myeloma cell line X63Ag8.653 (26). Hybridomas were plated in 96-well tissue culture plates in HAT-containing medium and screened 2 wk later by RNA dot blot for expression of a T15 V_H gene.

RNA Dot Blot Analysis. Cells were lysed in 96-well plates and filtered through nitrocellulose with a 96-well manifold (Schleicher & Schuell, Keene, NH) and hybridized to a 210-bp nick-translated probe that detects all members of the S107 V_H gene family (27, 28).

mRNA Preparation and Sequencing. Total RNA was isolated from hybridoma cells by extraction with guanidinium thiocyanate. Samples were dissolved in 10 mM Tris-Cl, 1 mM EDTA, 400 mM NaCl heated to 68°C and loaded three times onto oligo(dT)-cellulose columns (Collaborative Research Inc., Bedford, MA). mRNA was eluted with TE (10 mM Tris-Cl, 1 mM EDTA) and precipitated with ethanol. The precipitate was dissolved in mRNA annealing buffer (250 mM KCl, 10 mM Tris-Cl, 1 mM EDTA) for sequencing. mRNA was annealed to radiolabeled Ig constant region oligonucleotide primers and dideoxy sequencing was performed according to Geliebter et al. (29).

Normalization of Cell Supernatants for Ig Concentration. Cells were grown in serum-free medium containing 5.0 μg/ml bovine insulin, 3.5 μg/ml human transferrin, 0.1% sodium selenite, and 0.1% ethanolamine, in a 1:1 mixture of DME (1,000 mg glucose per liter) and Ham's F12 nutrient mixture (Hazelton, St. Lenexa, KS). The Ig content of each culture supernatant was determined by ELISA. 96-well polystyrene E.I.A. plates (Costar Corp., Cambridge, MA) were coated for 2 h at 37°C with an anti-IgG isotypic reagent (Fisher Biotech, Orangeburg, NY) appropriate for each cell line. Plates were blocked with 1% BSA in PBS overnight at 4°C, and cell supernatants or dilutions of commercially purified Igs (Litton Bionetics, Charleston, SC) were added and the plates were incubated for 90 min at 37°C. Plates were washed three times with PBS, 0.05% Tween, and then incubated with alkaline phosphatase–conjugated anti-mouse IgG isotypic reagent for 90 min at 37°C. Plates were washed again and developed with alkaline phosphatase substrate (Sigma Chemical Co., St. Louis, MO). Plates were read in a Titertek ELISA reader (Flow Laboratories, McLean, VA) at 405 nm.

Millipore Filter Assay. Nick-translated DNA was made double-stranded by passage through a 0.45-μm nitrocellulose Millex syringe filter (Millipore Products Division, Bedford, MA). Cell supernatants normalized for Ig concentration were incubated with 10,000 cpm of ^{32}P-labeled, dsDNA in 96-well nitrocellulose-bottom Millititer-HA plates (Millipore Products Division) for 90 min at 37°C. The dsDNA/cell supernatant mixtures were then filtered through the nitrocellulose and washed three times with 1× SSC (0.15 M NaCl, 0.015 M citrate, pH 7.2) on a 96-well vacuum filtration apparatus (Millipore Products Division). The wells were punched out and counted on an LKB-RackBeta (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) scintillation counter.

DNA ELISA. Round-bottomed, polyvinyl plates (Falcon Labware, Oxnard, CA) were coated with 100 μl per well of poly-L-lysine (50 μg/ml), and incubated for 2 h at 37°C. Plates were shaken dry and rinsed with distilled water. 100 μl of calf thymus (CT) DNA either filtered (as above for dsDNA) or boiled for 10 min

Table 1. *Anti-dsDNA mAbs from BALB/c Mice*

Cell line	Immunization protocol	Isotype	V_H	D_H	J_H	V_K	J_K
2B11.1	αI-J^d, 5 wk Ars-KLH, 4 d	IgG2b	1	DFL16.2	3	8	1
C8.5	αI-J^d, 7 wk αT15, 4 d	IgG1	11	DFL16.1	1	V_k20	5
7D2.G12	αI-J^d, 4 wk PC-KLH, 3 d	IgG3	11	DFL16.1	1	12, 13	1
R4A.12	PC-HGG, 1 wk	IgG3	11	?	4	1	1

and quickly cooled on ice (ssDNA) was added at 4°C overnight. The plates were blocked with 1% BSA in PBS for 90 min, washed three times with PBS, 0.05% Tween 20, and then 50 μl of normalized serum-free supernatant or 50 μl of serum of 1:500 dilution in 1% BSA/PBS was applied. After a 90-min incubation and washing, a goat anti-mouse κ chain reagent or a sheep anti-mouse IgG reagent conjugated to horseradish peroxidase (Fisher Biotech, Orangeburg, NY) was applied and incubated for 90 min at 37°C. Plates were then washed again, ABTS peroxidase substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was applied, and color development was monitored at 405 nm.

Inhibition of dsDNA Binding. 100 μl per well of CT DNA (ds or ss) at various dilutions in PBS was preincubated with 15 μl of serum-free cell supernatant normalized to 40 μg/ml (final concentration 6.3 μg/ml) at 37°C for 1 h in a 96-well, nitrocellulose-bottomed plate. 10 μl containing 15,000 cpm of ^{32}P-labeled dsDNA was added per well and the incubation was continued for an additional hour at 37°C. The mixtures were filtered through the nitrocellulose on a 96-well vaccuum filtration apparatus and the wells were washed and counted as above.

ELISAs for Other Antigenic Specificities. Binding to thyroglobulin, mycobacteria 65-kD protein, PC-BSA, *p*-aminophosphorylcholine, and L-*o*-glycerophosphorylcholine was measured as follows. Antigen was diluted to 10 μg/ml PBS and 50 μl was added to each well of a 96-well polystyrene EIA plate (Costar Corp.) for 2 h at 37°C. The plates were blocked with 100 μl per well of 1% BSA in PBS at 4°C overnight. Plates were washed three times with BSA 0.05% Tween 20. Samples were added at concentrations ranging between 1 and 20 μg/ml (50 μl per well) at 37°C for 90 min, washed three times with PBS, 0.05% Tween 20 and then incubated with a peroxidase-linked anti-mouse κ chain reagent (Fisher Biotech) for 90 min at 37°C followed by three washes with PBS 0.05% Tween 20 and a final rinse with dH_2O. Plates were developed with hydrogen peroxide and ABTS (Kirkegaard and Perry Laboratories) and read at 405 nm.

For analysis of binding to phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine, antigens were diluted to 10 μg/ml in methanol and 50 μl per well of antigen was added to polystyrene plates. The plates were allowed to dry overnight at room temperature, then were blocked for 2 h at 37°C with 1% BSA in PBS. The remainder of the assay was performed as above.

For analysis of binding to myosin, mouse cardiac myosin was diluted to 2.5 μg/ml in a buffer containing 36.6 mM $NaHCO_3$, 12.8 mM Na_2CO_3, and 50 mM tetrasodium pyrophosphate. Polystyrene plates were coated with 100 μl of myosin solution per well for 1 h at 37°C. The buffer was then discarded and the plate was allowed to dry overnight (30). The plate was blocked with 1% BSA/PBS for 2 h at 37°C and the assay performed as above.

For analysis of binding to influenza, polystyrene plates were coated with 50 μl of 2.5 × 10^5 HAU/ml of influenza virus strain PR8. The plates was dried overnight at room temperature and given a 15-min wash with methanol followed by two PBS washes. The plates were then blocked as usual with 1% BSA/PBS and the assay was performed as above.

Results

BALB/c mice were immunized using two protocols intended to elicit anti-DNA antibodies encoded by members of the S107 heavy chain variable region gene family (V_H family). One strategy of immunization was designed to permit expression of anti-dsDNA antibodies by attempting to interfere with normal suppression of autoimmunity. This protocol involves the use of an anti-I-J^d antibody, WF18.2B15 (23). The I-J molecule, present on suppressor T cells, is believed to play a role in mediating suppressor cell activity since antibody to I-J can block induction of suppressor activity. Our laboratory has previously shown that polyclonal antiidiotype to anti-I-J can abrogate suppression in an in vitro assay system (23). In preparing monoclonal antiidiotypic reagents to anti-I-J^d, we observed high-titered anti-dsDNA antibodies as well as antiidiotype in BALB/c (H-2^d) mice immunized with anti-I-J^d. The anti-DNA response of several groups of mice immunized with anti-I-J^d was monitored by ELISA. Results from one group are illustrated in Fig. 1. The repetitive cycling of the anti-DNA response over a period of several months was reproduced in large numbers of mice. The high-titered anti-DNA activity in the anti-I-J^d immunized mice led us to use this model to generate anti-dsDNA mAbs in BALB/c mice. Subsequent analysis showed that many of these animals also had elevated S107 idiotype in their serum. In an effort to bias towards the production of S107 V_H-encoded anti-dsDNA antibodies, we boosted the mice with PC-KLH or anti-S107 idiotype. As a control, we boosted with the hapten ARS that has also been implicated in an anti-DNA response but elicits antibodies encoded by a different V_H gene family (5). The second strategy for the production of S107-encoded anti-DNA antibodies was to immunize BALB/c mice with PC in an attempt to isolate U4-like anti-dsDNA mAbs that might arise by in vivo somatic mutation of anti-PC antibodies encoded by an S107 V_H gene.

Spleen cells from the immunized mice were fused to the nonproducing myeloma cell line X63Ag8.653 by conventional methods. The hybridomas were screened first by RNA dot blot using a heavy chain probe that detects all members of the S107 V_H gene family. Supernatants from wells expressing an S107 heavy chain were analyzed for isotype. We were in-

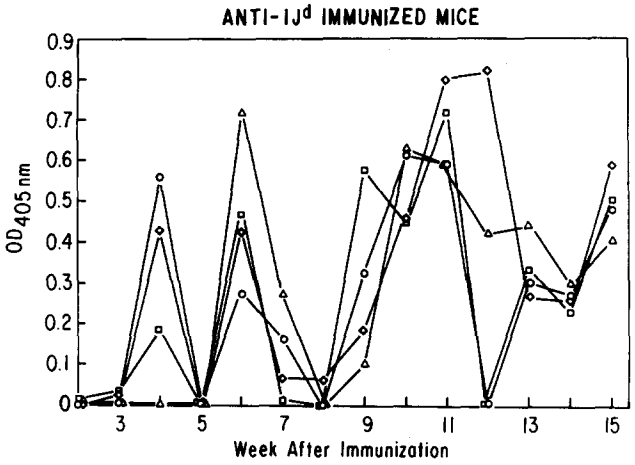

Figure 1. Mouse sera were diluted 1:500 and analyzed by dsDNA ELISA. The ELISA was developed with a peroxidase-conjugated anti-mouse IgG reagent. The mean dsDNA binding plus 2 SD of a panel of eight unimmunized age-matched BALB/c has been subtracted from the values that are shown.

Table 2. *Antigen Binding*

Cell line	dsDNA binding
	cpm
2B11.1	1,950
C8.5	1,211
7D2.G12	1,069
R4A.12	1,218
S107	304
U4	1,008

Hybridoma cell supernatants were analyzed by millipore filter assay at 2 μg/ml. The S107 parental (IgA) non-DNA binding cell line and the U4 mutant (IgA) DNA binding cell line are described elsewhere (24).

terested primarily in T15 antibodies of the IgG isotype. All T15 IgG antibodies were, therefore, analyzed for their ability to bind dsDNA by millipore filter assay. Four IgG-secreting hybridoma cell lines were isolated and are described in Table 1. The anti-DNA activity of the antibodies secreted by these cell lines was compared with that of the S107 (PC-binding) IgA antibody and the U4 (dsDNA binding) mutant IgA antibody (Table 2). Each antibody bind DNA as well as or better than the dimeric IgA U4 antibody and equivalently to a 1:1,000 dilution of serum from an autoimmune mouse (data not shown).

The heavy and light chain variable regions of these four antibodies were analyzed by mRNA dideoxy sequencing and the results are shown in Figs. 2 and 3. Fig. 2 *a* shows the heavy chain variable region genes compared to their corresponding germline genes. All of the antibodies use unmu-

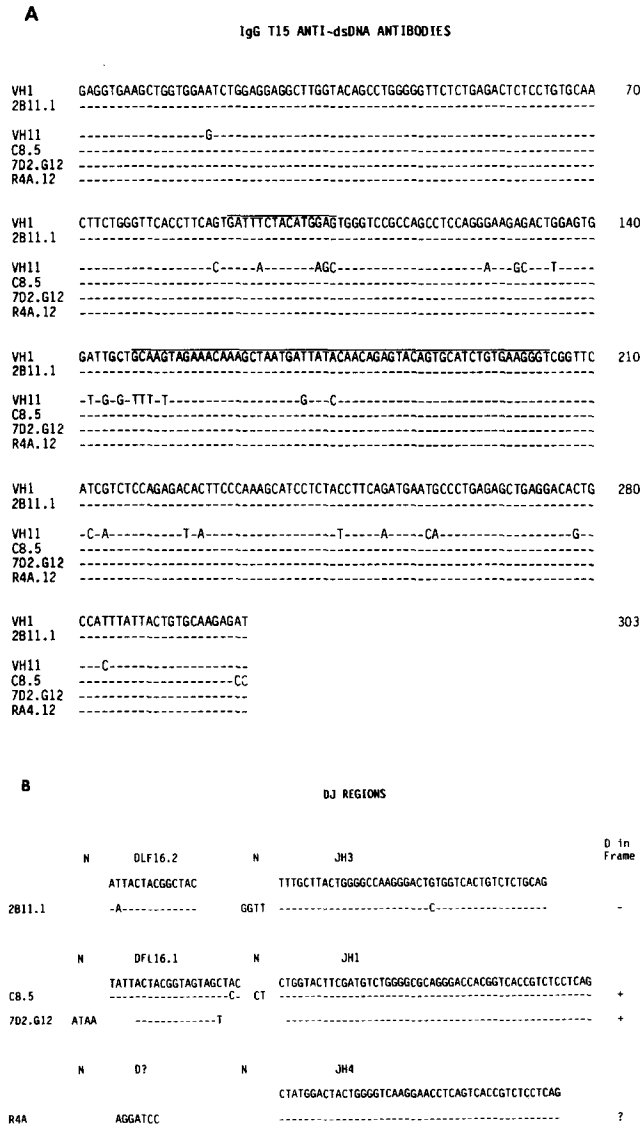

Figure 2. Heavy chain variable region sequences of BALB/c anti-dsDNA mAbs obtained by mRNA dideoxy sequencing compared with their appropriate V_H, D_H, and J_H genes. Overlining indicates the CDRs.

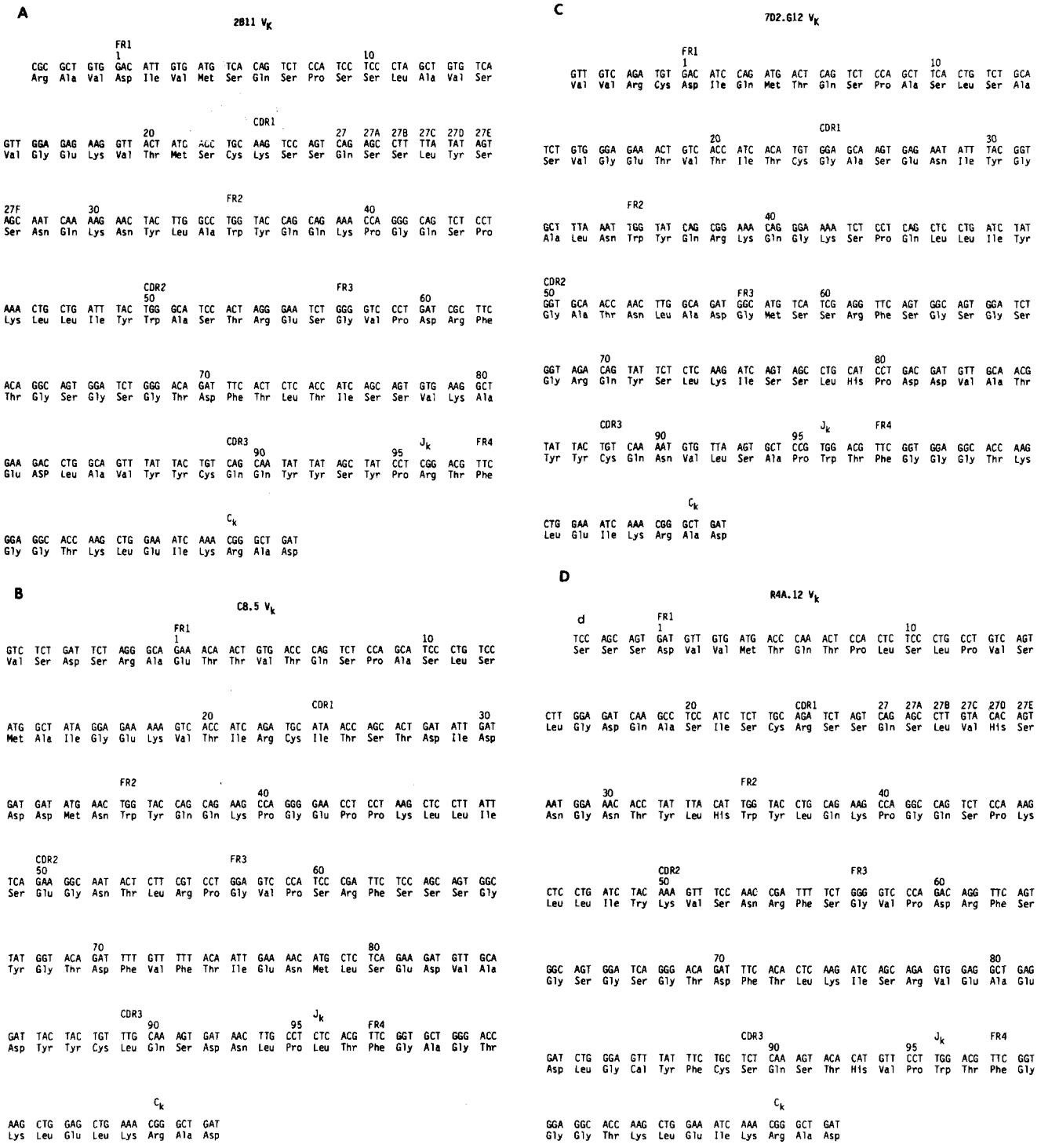

Figure 3. κ Light chain sequences of BALB/c anti-dsDNA mAbs obtained by mRNA dideoxy sequencing.

tated germline V_H genes except C8.5, which has a GAT to GCC substitution in V_H11, thereby generating an alanine residue instead of an aspartic acid at position 101 in the protein sequence. Because these substitutions in C8.5 occur at the D_HJ_H junction, it is probable that these changes arose by addition of N sequence and not by somatic point mutation. Fig. 2 *b* shows the D_HJ_H regions of all four antibodies as compared with the germline genes from which they are derived. Only the 2B11.1 antibody has a nucleotide substitution that is unquestionably due to somatic mutation. A substitution in the J_H3 region of CTG for GTG in amino acid number 108 as seen in Fig. 2 *c* causes a conservative amino

acid replacement of a leucine for a valine in the fourth framework region. The sequence of the 5′ end of the DFL16.2 region in the 2B11.1 antibody is AAT instead of ATT at amino acid position 96 which leads to an isoleucine to asparagine change. This may also represent N sequence rather than mutation. In this antibody the D region is out of frame relative to the most commonly used translation frame. In contrast, the C8.5 and 7D2.G12 DFL16.1 genes are read in the most commonly used frame. Both the C8.5 antibody and the 7D2.G12 antibody have changes at the 3′ end of their D regions. The substitution of a TCC for TAC at amino acid position 100B in C8.5 causes a tyrosine to serine change, and the AGC to AGT change at amino acid position 100A in 7D2.G12 is silent. These latter three changes are likely to have resulted from N sequence addition because of their proximity to the VD or DJ junctions. Finally, the R4A.12 antibody uses an extremely short D region that is not related to any of those previously described, therefore its usual reading frame can not be determined, nor can the degree of somatic mutation it exhibits be determined.

The light chain genes expressed by these cell lines are all different from each other, as shown in Fig. 3. The 2B11.1 antibody uses a V_k gene that is a member of the V_k8 family (Fig. 3 *a*). Its sequence is completely identical to a productively rearranged but unexpressed light chain gene of the plasmacytoma PC3609 from an NZB mouse whose antigen binding specificity is unknown (31, 32), and it is also 98% homologous to an antilysozyme antibody, Gloop 5 (33). While the 2B11.1 V_K gene is highly homologous to two rearranged V_k genes, it is only 88.3% homologous to a germline V_k gene, V-ser, cloned from liver DNA (34). Therefore, the 2B11.1 V_k is probably derived from an as yet unidentified germline gene and may itself, like the productively rearranged unexpressed but PC3609 V_k, constitute the unmutated sequence. The 7D2.G12 antibody uses a light chain V region that belongs to the V_k 12,13 family (Fig. 3 *c*). It exhibits greatest homology (84.2%) to a germline gene K2 cloned from the MOPC-149 myeloma and from BALB/c embryo DNA (35). It is likely again that the appropriate V_k germline gene has not yet been cloned and sequenced. The C8.5 cell line uses a V_k gene that is a member of the V_k20 gene family (Fig. 3 *b*) (36) and the R4A.12 V_k gene is completely identical to a V_k1 germline gene, K5.1, cloned from BALB/c embryo liver DNA (Fig. 3 *d*) (37).

The relative binding of these S107 V_H-encoded antibodies for dsDNA was analyzed by inhibition assays. The monoclonal antibody concentrations used in these assays were 0.63 μg per well (Fig. 4). All four antibodies were inhibited to 50% of maximum dsDNA binding by ds-calf thymus DNA concentrations between 0.2 and 1.0 micrograms per well. From these data, one can derive an apparent binding constant of 10^9 to 10^{10} for these antibodies using the equation of Nieto et al. (38).

The four anti-DNA antibodies were also analyzed by ELISAs to determine their antigenic cross-reactivity. Because the antibodies are encoded by the V_H1 and V_H11 gene members of the S107 V_H family, they were analyzed for binding to PC-KLH and influenza, and no binding to either antigen was seen. They were also analyzed for binding to mycobacterium 65kd protein, phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), L-α-Glycerophosphorylcholine, and *p*-aminophosphorycholine. No binding was seen to any of these exogenous antigens. Binding was analyzed to the autoantigens thyroglobulin and murine cardiac myosin and no binding was demonstrated (Table 3).

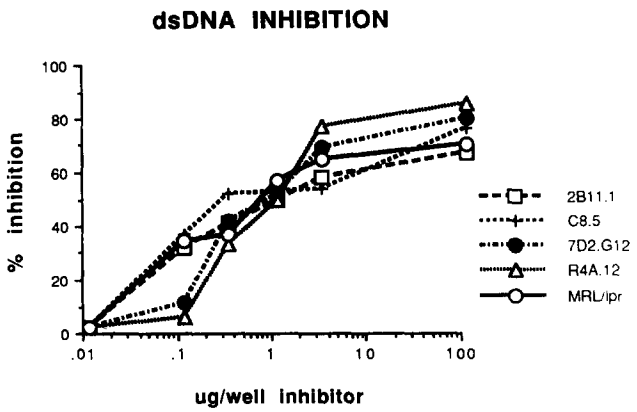

Figure 4. Cold CT-dsDNA (15 μ/ml) was preincubated with serum-free supernatant from hybridoma cell lines for 1 h at 37°C followed by addition of 15,000 cpm nick-translated dsDNA and incubation for 1 h at 37°C. Final concentration of mAb was 6.3 μg/ml. Serum from an MRL/*lpr* mouse was diluted 1:1,000 in serum-free supernatant as the DNA binding of this dilution of serum corresponds to the DNA binding of mAb at 6.3 μg/ml.

Discussion

We have isolated four anti-dsDNA mAbs from BALB/c mice that resemble the pathogenic type autoantibodies

Table 3. *Antigen Reactivity on ELISA*

	Reactivity on ELISA			
Antigens	2B11.1	C8.5	7D2,G12	R4A.12
ssDNA	–	–	+/– *	+/–
Thyroglobulin	–	–	–	–
Myosin	–	–	–	–
Cardiolipin	–	–	+/–	+/–
Influenza	–	–	–	–
Mycobacterium	–	–	–	–
Phosphatidylethanolamine	–	–	–	–
Phosphatidylinositol	–	–	–	–
Phosphatidylserine	–	–	–	–
L-α-Glycerophosphorylcholine	–	–	–	–
p-Aminophosphorylcholine	–	–	–	–

All ELISAs were performed using antibody at a concentration of 10 μg/ml. (+/–) Binding above the mean of irrelevant antibodies, but <2 SD above the mean.

described in autoimmune mouse strains, such as (NZB × NZW)F_1 and MRL/*lpr*, far more than "natural" autoantibodies found in normal mouse strains. Two strategies were used to induce the production of anti-DNA antibodies in normal mice. One strategy was intended to induce the production of these antibodies through somatic mutation of a response to the bacterial antigen PC. After immunization with PC-KLH we observed that the serological response to dsDNA undergoes a transient rise and fail simultaneously with the response to PC-KLH (data not shown). This coordinate response should occur if the antigens are potentially cross-reactive so that certain epitopes on PC-KLH can also stimulate secretion of anti-DNA antibodies. Alternatively, as suggested by the U4 mutant, somatic mutation of anti-PC antibodies could give rise to anti-DNA antibodies in a PC-immunized host.

The second strategy we used to induce anti-DNA antibodies in BALB/c mice was to immunize with anti-I-J^d antibody in an attempt to interfere with the normal pathways for regulating an immune response (39). In a similar system, Gibson et al, demonstrated that autoantibodies could be induced in the offspring of B10.A(3R)(H-2^b) mothers and B10.A(5R)(H-2^k) fathers when the pregnant females were immunized with paternal lymphoid cells (40). This immunization induced the production of anti-I-J^k antibodies in the gestating mothers, which crossed the placenta to the offspring and presumably led to the depletion or inactivation of suppressor T cells. When the offspring were immunized with rat red blood cells, they developed a crossreactive response to mouse red blood cells that is not normally seen in naive syngeneic animals. We believe that the long-term cycling (over 30 wk; data not shown) of the level of anti-DNA antibodies that we see in anti-I-J^d immunized BALB/c mice might be due to a similar perturbation in the ability of these animals to suppress an autoimmune response. It is not possible, however, to determine whether a given antibody arose as a result of a particular immunization protocol. More extensive serological and molecular analysis must be undertaken in order to ascertain the role that these immunizations might play in eliciting these antibodies, or whether they might occur in unimmunized animals as well.

Although anti-DNA activity is most commonly measured by ELISA, this method detects low affinity crossreactive antibodies as well as the high affinity, monospecific antibodies that are seen in autoimmune disease (41). We, therefore, chose to measure the binding of our antibodies to dsDNA by a liquid phase assay (millipore filter assay). The millipore filter assay is used as a clinical assay because it is believed to measure the high affinity anti-dsDNA antibodies that bind in solution and titers of anti-DNA antibody measured with this assay correlate with disease activity. All four antibodies display high binding of dsDNA in solution. A low concentration (0.2–1.0 μg) of unlabeled dsDNA inhibitor causes a 50% inhibition of binding. These antibodies bind dsDNA with similar avidity to the IgG mAbs that have been isolated from autoimmune strains. Most autoantibodies from nonautoimmune animals bind antigen only at very high antibody concentrations. While it is not possible to obtain an affinity constant for anti-dsDNA antibodies, these antibodies display apparent antigen binding constants of 10^9 to 10^{10}, which is, in fact, greater then the binding constants of "natural" germline, gene-encoded pentameric IgM antibodies for DNA. In addition to their high affinity, these antibodies are highly specific for nucleic acid antigens, and unlike IgM "natural" autoantibodies exhibit little or no crossreactivity to a panel of autoantigens and to exogenous antigens.

We analyzed only IgG antibodies as we expected to study the somatic mutations of germline genes of the S107 gene family that can lead to anti-DNA activity. The four autoantibodies described here have V_H sequences that are germline-encoded with limited junctional diversity. Only one antibody, 2B11.1, has a clearly identifiable somatic point mutation in the middle of the J_H3 gene in framework 4. Three of the four antibodies express the V_H11 gene that encodes most of the S107 V_H-expressing anti-DNA antibodies identified in autoimmune mice. Moreover, 6G6, a V_H11-encoded antibody from a CBA/J mouse (42), was shown to have specificity for dsDNA (43). This antibody is IgM and has only one nucleotide difference from the CBA/J germline V_H11 gene at the 3′ end of the antibody, resulting in an aspartic acid to alanine substitution. Therefore, it is possible that the V_H11 germline gene may predispose an antibody to bind DNA, even in an unmutated state.

Three of the four antibodies use a member of the DFL16 family, the significance of which is unclear. No such predominance is seen in the VDJ sequences of anti-DNA antibodies that have been reported from autoimmune mice or from normal mice. However, since these antibodies describe a novel class of autoantibodies that are high affinity for DNA despite being germline gene encoded, and it has been shown that CDR3 is important for DNA binding, it is possible that the usage of the DFL16 family may be significant. It will be necessary to examine more antibodies of this class to understand the importance of specific D region families for DNA binding activity. However, it has been suggested that anti-DNA antibodies often contain in their D regions positively charged amino acids such as lysine, arginine, and histidine or the polar but uncharged amino acids glutamine, glycine, and asparagine which can form covalent bonds to nucleic acid bases (15, 44, 45). An absolute requirement of a given number of these residues for DNA binding to occur has not been established, however, and the presence of one such amino acid in the CDR3 of each of the antibodies may be sufficient for dsDNA binding. Also it has been proposed that the D regions in autoantibodies are often read in an unusual frame (46). Two D regions in these antibodies are unremarkable in this respect; however, a third is read in an uncommon frame and the fourth derives from an unidentified D segment. The D region from R4A.12 antibody is very short and cannot be assigned to any of the previously described D regions. Extremely short D regions have been found in antidextran antibodies, and have also been described in anti-RNA antibodies (15). Because these D's are so short it is often difficult to assign them to a family, or to distinguish D segment from N sequence.

It is more difficult to ascertain whether somatic mutation has occurred in the V_k genes. The R4A.12 V_k gene clearly

expresses an unmutated sequence, while the other three antibodies probably use germline genes that have not yet been cloned and sequenced. Because there is a rearranged V_k sequence that is 100% identical to the 2B11.1 V_k, we believe that the 2B11.1 V_k gene represents a germline gene sequence that has not as yet been cloned. The sequence that is identical to the 2B11.1 V_k is an unexpressed double recombination product from an NZB plasmacytoma, PC3609. The expressed light chain from this IgG2b secreting cell line is a member of the V_K14 family, while the unexpressed light chain is a member of the V_K8 family. Because this rearrangement is not expressed, and has not undergone selection by antigen, the likelihood that it represents an unmutated sequence is increased. The C8.5 and the 7D2.G12 cell lines both use V_k genes that are members of families whose germline sequences have not been completely analyzed, so we cannot know whether these hybridoma V_k sequences represent germline sequences or not. Previous studies, however, have shown that in general somatic mutations in the heavy and light chain variable regions tend to accumulate concurrently. Thus, when the heavy chain variable regions do not exhibit mutations, the light chain variable regions are also unmutated.

We have described a set of anti-DNA antibodies exhibiting unusual properties that do not permit their classification either as "natural" autoantibodies or as autoantibodies associated with autoimmune disease. These antibodies are IgG, bind DNA with high avidity, do not crossreact with non-nucleic acid antigens, and are the products of unmutated Ig genes. These binding and isotypic properties are, in general, associated with the somatically mutated antibodies that are part of autoantibody responses seen in autoimmune disease; however, the apparent absence of somatic mutation that is seen in these antibodies is a property characteristic of "natural" autoantibodies. We are currently investigating the pathogenic potential of these antibodies in BALB/c mice.

These observations generate several questions regarding the regulation of autoantibodies in nonautoimmune and autoimmune animals. We have previously suggested that anti-dsDNA antibodies with high avidity for antigen arise in autoimmune individuals and autoimmune mice by somatic mutation of germline Ig genes. Data from many laboratories have confirmed this hypothesis; to date, all anti-dsDNA antibodies from autoimmune animals or patients with SLE reflect the accumulation of somatic mutations. The present data indicate that antibodies that have some of the features associated with pathogenic autoantibodies need not only arise through the accumulation of somatic mutations. Contrary to the observations made previously about germline, gene-encoded autoantibodies, we have shown that such autoantibodies may be neither low affinity nor broadly crossreactive. While it had been suggested that germline gene-encoded anti-dsDNA antibodies are not found because germline genes do not encode such high avidity IgG antibodies, our data suggest that the more likely explanation is that in both normal and autoimmune individuals B cells expressing such antibodies are deleted or rendered anergic when they arise as IgM antibodies in an immature B cell. In autoimmune mice high affinity IgG anti-dsDNA antibodies are routinely found; however, they are always the products of somatic mutation. The nonmutated high binding antibodies described here have not been found, presumably because their regulation is intact.

This might imply two pathways of regulation; one for less mature B cells without somatic mutation and one for more mature B cells that have accumulated somatic mutations. Matzinger and Guerder have formulated a similar hypothesis regarding the differential regulation of immature and mature T cells (47). We hypothesize that in autoimmune disease the defect may exist solely in the regulation of mature B cells whose autospecificity is acquired by somatic mutation. For future studies it will be important to determine the frequency of these monospecific high avidity anti-dsDNA autoantibodies in the preimmune repertoire and to understand their cellular origin and regulation in both nonautoimmune and autoimmune mice.

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