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Two murine natural polyreactive autoantibodies are encoded by nonmutated germ-line genes

(preimmune repertoire/polyspecificity/anti-DNA antibody/variable-region sequence/germ-line gene expression)

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ABSTRACT **Two monoclonal IgM natural autoantibodies (E7 and D23) obtained from the fusion of normal, nonimmunized, BALB/c mouse spleen cells and nonsecreting myeloma cells were selected on the basis of their polyreactivity with auto- and xenoantigens and chemical haptens. Nucleotide sequence analysis of the variable and constant regions of the heavy and light chains showed the following. (*i*) The antibodies arise from different genetic elements with very low or no homology—E7 from a heavy-chain variable region (V_H) of family 36-60 and κ light-chain variable region ($V_κ$) from group 19—whereas D23 derives from a V_H of family Q52 and $V_κ$ derives from group 8. (*ii*) E7 and D23 are probably of germ-line origin, as suggested by high homology with V_H genes from the unrearranged genome. Compared with the germ-line V_H 1210.7 gene, E7 has a single nucleotide difference leading to a silent mutation at position 15, whereas D23 seems to be encoded by germ-line V_H 101 with one nucleotide difference causing replacement of Ser-84 by Ala. (*iii*) The genetic $V_κ$ and V_H elements for E7 and D23 also give rise to different responses to phenyloxazolone, dinitrophenyl, 5-(dimethylamino)naphthalene-1-sulfonyl, arsonate, phosphocholine, and influenza virus hemagglutinin. Antibodies from normal and autoimmune mice with rheumatoid factor-like activity are also homologous to E7 and D23. These results indicate that polyreactive autoantibodies are encoded by germ-line genes and that, starting with the preimmune poly- and autoreactive repertoire, mutated forms of antibodies recognizing exogenous antigens can be obtained and selected.**

Natural autoantibodies (NAAb) in sera of healthy humans, unstimulated normal rabbits, mice, rats, and certain fish species of very distant phylogenetic orders have been reported (1). Some NAAb are directed against easily accessible autoconstituents (e.g., soluble plasma proteins, hormones, surface components of senescent red blood cells, fetal cells, and tumor cells) (2). Other NAAb react with hidden self antigens (e.g., cytoskeletal and other nuclear antigens or cryptic membrane epitopes exposed after proteolytic enzyme treatment) (3).

Specificity studies demonstrated that some NAAb, whether polyclonal and purified on immunoadsorbent or monoclonal, were polyreactive, recognizing two or more self and non-self antigens. For example, murine monoclonal natural IgM from normal nonimmunized mice cross-reacted with different structurally unrelated antigens such as DNA, myosin, actin, tubulin, and spectrin (4).

Autoantibodies are thought to be involved in the pathogenesis of some autoimmune diseases and especially in systemic lupus erythematosus (SLE), which is characterized by B-cell hyperactivity resulting in high levels of circulating autoantibodies and immune complexes whose deposition in various organs leads to tissue damage and disease (5). SLE autoantibodies, like NAAb, react with DNA, synthetic polynucleotides, phospholipids, cytoskeletal proteins, the constant (C) portion of IgG, and cell surface antigens (6). Therefore, knowledge of the presence, specificity, regulation, physiological significance, and molecular features of polyreactive NAAb is of primary importance.

In this report we present the complete cDNA sequences corresponding to the variable (V) and C regions of the heavy (H) and light (L) chains of the two previously reported (4) natural monoclonal autoantibodies D23 and E7.† The results indicate that these autoantibodies are encoded by unmutated germ-line genes and that autorecognition by antibodies is not *per se* an aberrant event but rather an important physiological function. Furthermore, mutated forms of the genetic elements, active in their germ-line forms in preimmune D23 and E7, are also encountered in certain murine immune responses.

MATERIALS AND METHODS

Hybridomas. Preparation of hybridoma clones D23 and E7 and characterization of the synthesized antibodies have been described in detail (4, 7).

Hybridoma cells were transplanted subcutaneously into BALB/c mice, and the resulting tumors were harvested and frozen in liquid nitrogen until use.

cDNA Cloning and Nucleic Acid Sequence Analyses. Poly(A)$^+$ RNA extracted from the frozen tumors by urea/LiCl (8) was purified by oligo(dT)-cellulose column chromatography (9), copied into cDNA (10), and cloned in the plasmid pAT153. Transformed *Escherichia coli* (strain MC1061) colonies were screened with ^{32}P-labeled C-region probes for murine μ- and κ-chain sequences (kind gifts from F. Rougeon, Institut Pasteur, Paris). Plasmid DNA from positive clones was prepared as described (11) and digested with a series of restriction enzymes. Appropriate fragments were subcloned in M13 DNA (12) and sequenced by the dideoxynucleotide method (13).

RESULTS

Two hybridoma clones producing IgM (κ light chains), D23 and E7, were obtained from two separate fusions with splenocytes of 12-week-old unprimed BALB/c mice and nonsecreting myeloma lines and were selected because of the polyreactivity of the antibodies secreted (4). Their reactivities are given in Table 1. Although both D23 and E7 bound

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Abbreviations: NAAb, natural autoantibodies; V, variable; C, constant; D, diversity; J, joining; H, heavy; L, light; mAb, monoclonal antibody; DNP, 2,4-dinitrophenyl; TNP, 2,4,6-trinitrophenyl; CDR, complementarity determining region.

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†The sequences reported in this paper are being deposited in the GenBank data base (accession no. J04565).

Table 1. Binding of E7 and D23 mAbs to various antigens

Antigen	mAb E7	mAb D23
Actin	++++	++
Tubulin	+++	++
Myosin	++++	+
DNA	++	++++
Renin	++++	++++
Neurofilament	−	+
α-Fetoprotein	++++	++++
Ovalbumin	−	−
TNP-ovalbumin	+++	++
PC-ovalbumin	++	+
ARS-ovalbumin	+	+
OX-ovalbumin	+++	++

All antigens except bovine neurofilaments were extracted from mouse organs. 2,4,6-Trinitrophenyl (TNP), phosphocholine (PC), *p*-azophenylarsonate (ARS), and 2-phenyl-5-oxazolone (OX) were coupled to ovalbumin by standard methods (7, 14–16). Concentrations of purified monoclonal antibodies (mAbs) were adjusted to 100 ng/ml, and binding to coated plates was revealed by β-galactosidase-labeled anti-mouse immunoglobulin. Conversion of optical densities at 412 nm: −, <0.040; +, 0.040–0.100; ++, 0.100–0.300; +++, 0.300–0.500; ++++, >0.500.

to several of these antigens, their affinities were different (7). cDNA corresponding to the V and C regions of the H and L chains were synthesized and sequenced. The nucleic acid sequences of the 5′ untranslated, leader peptide, and variable regions of the H chains (Fig. 1) and the L chains (Fig. 2) in comparison with reported sequences of germ-line genes are shown. Similarities with reported mAbs and myeloma proteins are summarized in Table 2.

H-Chain cDNA Sequences. The H-chain joining (J) region (J_H) of E7 corresponds to germ-line J_H4 (19) beginning with the third codon. Within the 21-nucleotide-long diversity (D) segment of E7, 9 nucleotides showed sequence identity with the core sequence of germ-line DSP2.7 (18), whereas 8 5′ end nucleotides and 4 3′ terminal nucleotides were probably noncoded junctional ones (Fig. 1*A*). Alternatively, 13 nucleotides could be derived from the germ-line DSP2.7 with one single substitution (adenosine for thymidine in position 4 of the germ-line DSP2.7), while 4 noncoded 5′ end nucleotides and 4 3′ end nucleotides completed the D segment. The E7 V_H corresponds to the germ-line V_H 1210.7 (15) (group 3, family 36-60) except for the third nucleotide of codon 15 (TCT for TCG in the germ line), which did not lead to an amino acid substitution. If compared with sequences of other induced mAbs, identity is found between TF5-139, an anti-2,4-dinitrophenyl (DNP) mAb that represents a predominant clonotype in 3-day-old BALB/c neonates (22), and DF8-611.1, an anti-5-(dimethylamino)naphthalene-1-sulfonyl IgM (23) (Table 2 and results not shown). Actually TF5-139 shows identity with E7 in all V_H, leader peptide, and 5′ untranslated regions. A single nucleotide difference is shown by another neonatal BALB/c mAb with anti-DNP activity (TF2-36) (22), by two anti-phenyloxazolone mAbs isolated during the secondary response (NQ10.212.8 and NQ10.4.6.1) (16), and by an anti-DNP mAb bearing idiotype 460 (M460) (58). The E7 V_H sequence also matched that of the D35 anti-DNP mAb (58) bearing idiotype 460 (280 of 282 nucleotides), that of a 36-60 anti-arsonate mAb (15) (276 of 282 nucleotides), and that of the H-37-85 anti-influenza virus hemagglutinin mAb (24) (273 of 282 nucleotides).

As shown in Fig. 1*B*, D23 displays sequence identity with the germ-line J_H2 (19) and with 10 nucleotides of the core sequence of DFL16.2 (18), while 5 noncoded 5′ terminal nucleotides and 4 3′ end nucleotides complete the D segment. Compared with the germ-line BALB/c V_H 101 (group 2, family Q52) (17), D23 shows 4 nucleotide differences, but

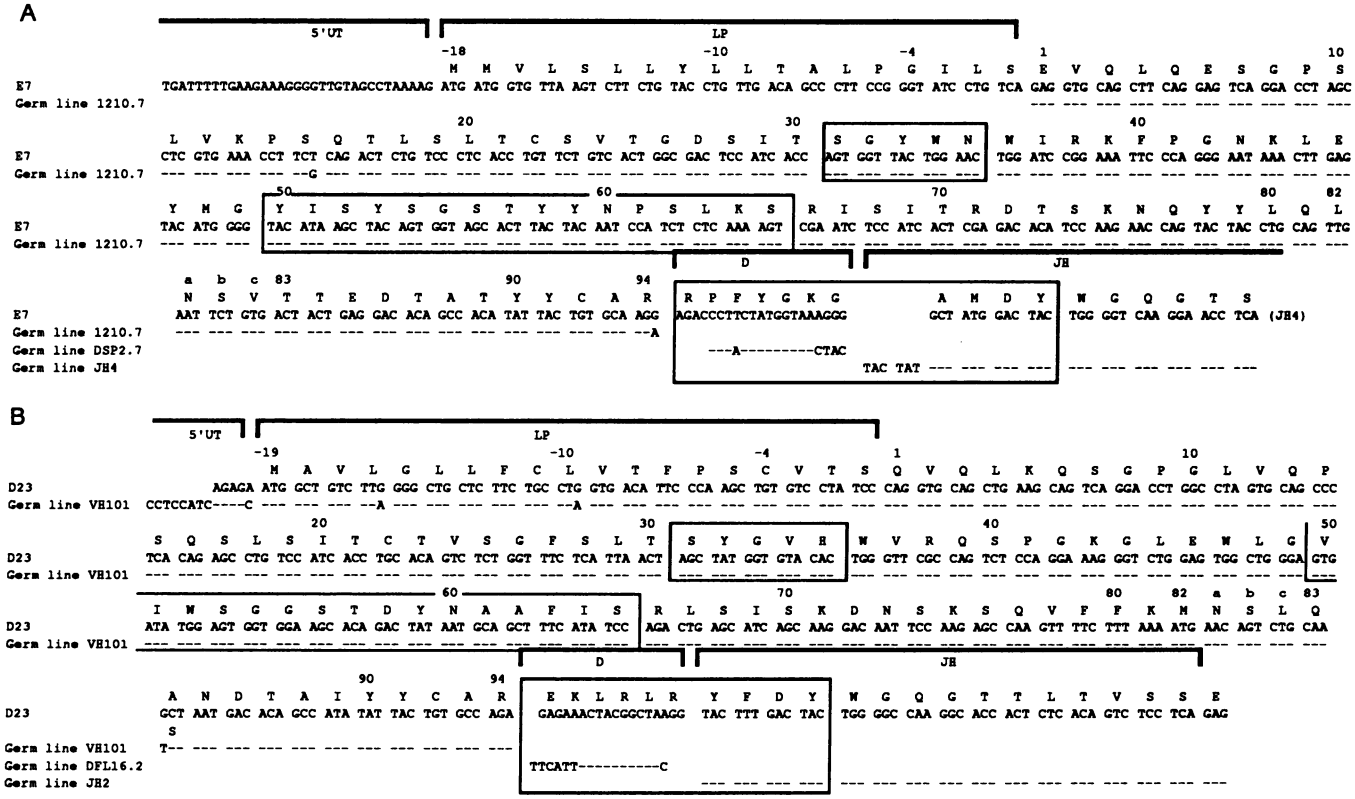

FIG. 1. Nucleotide and deduced amino acid sequences of V_H, D, and J_H regions encoding E7 (*A*) and D23 (*B*), compared with those reported for the germ-line genes V_H 1210.7 (16), V_H 101 (17), D (18), and J_H (19). Dashed lines indicate identity with E7 (*A*) or D23 (*B*). CDRs are boxed. Amino acid numbering is according to Kabat *et al.* (20). 5′ UT, 5′ untranslated region; LP, leader peptide.

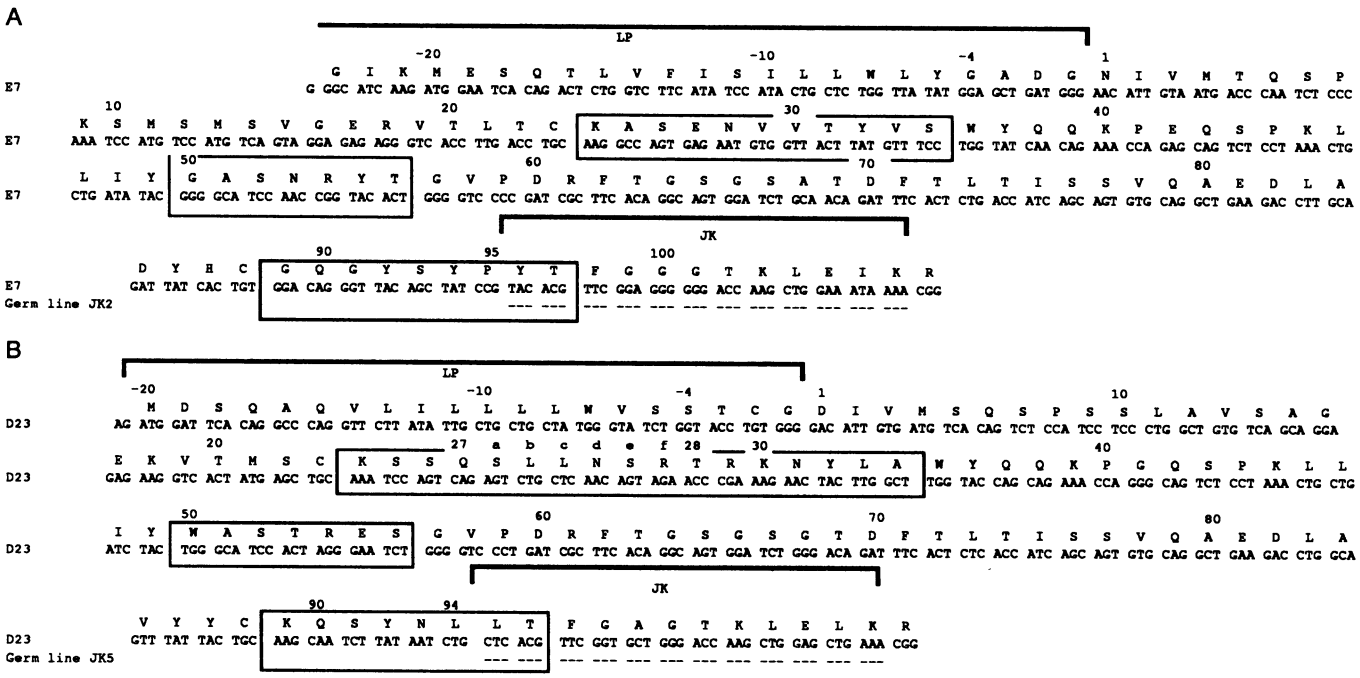

FIG. 2. Nucleotide and deduced amino acid sequences of V_κ and J_κ regions encoding E7 (*A*) and D23 (*B*), compared with those reported for germ-line genes (21). Dashed lines indicate identity with E7 (*A*) or D23 (*B*). CDRs are boxed. Amino acid numbering is according to Kabat *et al.* (20). LP, leader peptide.

only 1 (alanine for serine in position 84) is in the V_H coding region. An anti-phenyloxazolone mAb (NQ2.45.10.4) (29) isolated from the early primary response has the identical sequence as D23 V_H, whereas 2 nucleotide differences occur in a BALB/c IgM mAb (JV10) (26) with rheumatoid factor activity (Table 2).

Table 2. Induced mAbs expressing the same or similar V regions as preimmune E7 and D23

NAAb region	mAb with similar V region			
	Specificity	Designation	Ref.	*n**
E7 V_H	DNP	TF5-139	22	0
	DNS	DF8-611.1	23	0
	OX	NQ10.2.12.8	16	0†
	ARS	1210.7	15	0‡
	OX	NQ10.4.6.1	16	1†
	DNP	TF2-36	22	1
	DNP	M460	58	1
	DNP	D35	58	2
	HA	H37-85	24	4
	ARS	36-60	15	5
E7 V_κ	?	MOPC21	25	0
	IgG1	RF34	26	1
	TNP	SP6	27	21
	IgG1	RF51	26	22
	?	MPC11	28	22
D23 V_H	OX	NQ2.45.10.4	29	0†
	IgG1	JV10	26	1
	?	MC101	17	2
D23 V_κ	PC	HPCG15	14	0§
	ARS	45.165.CRI	30	1§
	PC	MCPC603	31	15‡
	IgG1	JV10	26	16
	DNP	TF5-139	22	16

The E7 V_H corresponds to the germ-line V_H 1210.7 (15), and D23 V_H corresponds to the germ-line V_H 101 (17). DNS, 5-(dimethylamino)naphthalene-1-sulfonyl; OX, 2-phenyl-5-oxazolone; ARS, *p*-azophenylarsonate; HA, influenza virus hemagglutinin; PC, phosphatidylcholine.

*The number of amino acid differences from E7 or D23.

†Partial sequence.

‡Protein sequence.

§Partial protein sequence.

mAbs E7 and D23 have identical C_μ coding and 3′ untranslated regions (results not shown) and differ from a genomic BALB/c IgM sequence (32) in only two nucleotides (position 187 is guanosine instead of adenosine and 1655 is cytidine instead of thymidine in both E7 and D23). Both differences failed to lead to an amino acid change. Another difference was found in the 3′ untranslated region at position 1991, where thymidine became cytidine (32).

L-Chain cDNA Sequences. The J_κ of E7 matched the germ-line $J_\kappa 2$ (21) (Fig. 2*A*). The V_κ E7 (group 19) showed only one nucleotide difference when compared with MOPC21 (25) and with BALB/c IgM RF34 (26), obtained after lipopolysaccharide stimulation, which has rheumatoid factor activity and specificity for IgG1 (Table 2).

Sequence of the J_κ of D23 corresponds totally to that of the germ-line $J_\kappa 5$ (21) (Fig. 2*B*). The V_κ sequence of D23 (group 8) showed 90% homology (differences at 29 nucleotides) when compared with that of anti-IgG1 rheumatoid factor JV10 (26) and 89% homology (35 differences) with the neonatal anti-DNP mAb TF5-139 (22) (Table 2). The predicted amino acid sequence of D23 L chain differed from the complete protein sequence of anti-phosphocholine mAb MCPC603 (31) by 15 amino acids (86% homology), whereas identity was found in the first 38 amino acids of another anti-phosphocholine mAb (HPCG15) (14), and only 1 difference was observed in the 48-amino acid partial protein sequences of anti-arsonate mAb 45.165 (30) (Table 2). The C_κ and complete 3′ untranslated regions (data not shown) of E7 and D23 were identical to the germ-line BALB/c C_κ (33).

DISCUSSION

We report here the nucleotide sequence of two preimmune natural polyreactive autoantibodies, E7 and D23, which react with antigens such as DNA, myosin, actin, tubulin, spectrin, and TNP. E7 and D23 appear to be encoded by different elements. Since until now the germ-line genes corresponding

to the V_κ genes used were not known, a direct evaluation of the somatic mutation level of the L chains was impossible. The V_H, D, J_H and J_κ genetic elements expressed by E7 and D23, however, seem to be derived directly from their genomic counterparts. V_H of E7 corresponds to the germ-line V_H 1210.7 (15) (group 3, family 36-60), with one nucleotide difference, whereas V_H of D23 corresponds to the germ-line V_H 101 (17) (group 2, family Q52), with one nucleotide difference, variations that may be due either to the presence in the families 36-60 and Q52 of two other V_H genes or to a polymorphism between BALB/c sublines. In fact, the same difference between the E7 V_H and V_H 1210.7 is found in all previously described mAbs expressing a V_H homologous to V_H 1210.7. Thus, two mAbs have exactly the same V_H sequence (22, 23), and two mAbs have exactly the same 5′ untranslated region and leader peptide sequences as E7 (22). Similar findings were also made for D23 V_H (26, 29).

Comparison of genes from lupus-prone strains and their nonautoimmune ancestors by means of restriction fragment length polymorphism and/or sequence analysis revealed no differences in immunoglobulin V_H loci, switch region, and immunoglobulin enhancer region sequences (34, 35), although recently polymorphism was found for some V_κ groups (36). Moreover, with few exceptions (37), autoantibodies seem to arise from the same genetic repertoire that encodes antibodies directed against exogenous antigens (34). The slight restriction for most 3′ V_H families (7183 and Q52) found for several autoantibodies (38, 39) seems to correspond to a preferential rearrangement in normal pre-B cells (40) and to reflect the V_H gene distribution in the total repertoire of normal mice (41). Our present results confirm the observation that autoantibodies and antibodies to exogenous antigens can be encoded by the same germ-line genes. Indeed, mutated forms of the germ-line V-region genes that encode E7 and D23 are encountered in different murine immune antibodies reacting with phenyloxazolone, DNP, arsonate, 5-(dimethylamino)naphthalene-1-sulfonyl, and phosphocholine (Table 2).

Somatic mutation can be an important mechanism in the generation of autoantibodies. Thus a single mutation in an anti-phosphocholine mAb V_H gene may give rise to antibody reacting with DNA (42). Similarly, sequence analysis of mAbs reacting with DNA or possessing rheumatoid factor activity showed that, in a single autoimmune MRL/*lpr*/*lpr* mouse, most antibodies were produced by a few clones; the high percentage of replacement mutations in the complementarity determining regions (CDRs) suggested that a positive selection had been exerted on these clones (43, 44). However, our results and those obtained by other authors indicate that autoantibodies can be encoded by unmutated germ-line genes. Thus the same sequence that encodes the anti-DNA H130 antibody in MRL/*lpr*/*lpr* mice is also found in the BALB/c genome (45). Furthermore, some preimmune A/J IgM expressed by the same but unmutated V_H that dominates the anti-arsonate response seems to react with single-stranded DNA and cytoskeletal proteins (46). These findings, together with the high frequency of natural autoantibodies observed in normal mice, suggest that the recognition of autoantigens is an important mechanism in the establishment of the preimmune repertoire.

Several hypotheses can be advanced to explain polyreactivity. The first is that the antibody site is polyvalent and is able to bind various haptens (47–49). From the three crystallographic structures of the Fab–protein complex known (50–52), such polyvalence cannot be excluded, since only 15–17 amino acids out of ≈60 in the CDR loops are directly involved in interaction with the antigen. Polyvalence can be extended even further if it is considered that the potential interaction surface includes the entire variable portions of Fab. Thus, two contacting amino acids from the D1.3 anti-lysozyme mAb (50) as well as one from the Hy HEL-5 anti-lysozyme (52) are located in the framework regions (FR). Also, because the only sequence homologies between 10 anti-IgG1 rheumatoid factor antibodies were found on FR2 and FR3 of the V_κ used, Shlomchik *et al.* (53) proposed that these regions are involved in an unorthodox interaction with IgG1 and that these antibodies could have at least two functional combining sites. Notably E7 and D23 are also homologous only within V_κ FR2 and V_κ FR3 (Fig. 2).

A second possibility for polyreactivity is that cross-reacting antigens share similar but not necessarily identical epitopes (4). Implication of charged groups in cross-reactions was suggested by Heidelberger (54) some years ago. More recently, Kabat *et al.* (55), by studying a monoclonal macroglobulin reacting with $\alpha(2\rightarrow8)$-linked poly(*N*-acetylneuraminic acid) and with denatured DNA, proposed that "similarities and differences in charge distribution can be responsible for immunological crossreactions among what have been generally considered diverse and structurally unrelated substances". Similarly, polyvalence of antibodies could be due to a particular distribution on the variable portions of the Fab surface of charged amino acids, which leads to the possibility of forming salt bridges. If the analysis is limited to the CDR loops, D23 has a net positive charge of +6, whereas that of E7 is +3. This could explain the higher affinity of D23 for DNA. In addition, the guanidinium group of the five arginines in D23 could stabilize the complex by forming hydrogen bonds with either phosphate and cytosine (only in single-stranded DNA) or with guanine (in both single-stranded and double-stranded DNA) (56); the latter interpretation is in accordance with the observation that D23 possesses a higher affinity for single-stranded DNA than for double-stranded DNA (7). In the E7 CDRs, a particularly high representation of aromatic amino acids is found, especially tyrosine with hydroxyl groups able to form hydrogen bonds. However, such a distribution of amino acids can also be observed in other mAbs reported to be monospecific (20). Consequently, either postimmune antibodies are also able to react with autoantigens, as E7 and D23 do, or additional characteristics must be found by examining more sequences of polyreactive autoantibodies.

A third alternative, which is not contradictory with the others, is that, like allosteric proteins, antibodies and antigens exist in different conformations and cooperate in the establishment of better interaction. Strickland *et al.* (57) reported that a mutant of T15 antibody that has lost the original idiotope reexpressed it after binding to another anti-idiotype; it was suggested that the second anti-idiotype induced a conformational change in the T15 mutant. Although available crystallographic data indicate that the tertiary structure of V_H and V_L domains on Fab can be considered constant, Colman *et al.* (51) reported that, in the NC41 influenza virus neuraminidase complex, changes occurred in the structure of the antigen and probably in the quaternary structure of the Fab. Comparison with other Fab structures indicated that the NC41 showed significant differences in the relative positions of its V_H and V_L, which resulted in an altered (up to 4 Å) position of the CDRs. We consider it not impossible that this type of sliding between V_H and V_L domains, which involves particular amino acids at their interfaces, may take place in germ-line-encoded antibodies rather than in the mutated postimmune ones.

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