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CDR3 regions in the preimmune V_H B cell repertoire of *lpr* mice

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SUMMARY

Previous studies have suggested that the CDR3 genetic element of the heavy chain variable region of autoantibodies is important in determining reactivity against self antigens, particularly against DNA. The *lpr* mutation was recently found to encode for a defective form of the fas protein, a molecule important for the transmission of the apoptotic signal into cells. Our aim was to determine whether CDR3 elements similar to those described for autoantibody-producing hybridomas derived from lupus-prone strains could be found in the preimmune repertoire of B cells in mice with the *lpr* mutation. The analysis of the junctions of the V_H-$C\mu$ functional rearrangements derived by polymerase chain reaction (PCR) amplification of RNA obtained from splenic small, resting cells stimulated with lipopolysaccharide (LPS) from male *lpr* mice showed that a large proportion of them expressed D genes in the unusual reading frames 2 and 3. Two of the *lpr* joints were formed by D–D fusions. Similarly, nearly half of the *lpr* sequences had arginines, an amino acid which promotes binding to dsDNA and is seldom observed in normal junctions. Our results show that the preimmune repertoire of *lpr* animals has abnormal CDR3 elements which may result from a failure at different levels of selection. The antigen-dependent selection of such elements that leads to the expansion of specific, high-affinity anti-dsDNA antibody-producing clones might depend on other genetic factors not found in the C57Bl/6-*lpr* strains but in the MRL-*lpr*.

Keywords systemic lupus erythematosus autoantibodies CDR3 diversity

INTRODUCTION

The presence of antibodies reactive against dsDNA is a general feature of murine strains that develop a lupus-like disease. Some of these strains, like the MRL-*lpr*/*lpr* mouse, develop, as a consequence, severe glomerulonephritis, due to immune complex deposition [1,2]. The high binding to DNA appears to be conferred mainly by the unusual presence of positively charged amino acids, as arginine (R) and lysine (K) within the CDRs of the heavy and light chains of the immunoglobulin molecule [3,4]. However, particularly important has been the presence of arginines within the CDR3 [5]. Most of the arginines present in the CDRs have been proposed to result from somatic hypermutation during antigen-dependent selection in the periphery [3–5]. However, the CDR3, formed by the joining process during recombination of the V_H, D and J_H segments in the heavy chain and V_L and J_L in the light chain, may undergo selection events independently of antigen and T cells. The presence of arginines within the heavy chain CDR3 of high-affinity, IgG anti-DNA antibodies has been shown to result from the translation of the D elements in unusual reading frames, inverted D genes or the fusion of D genes. However, polyreactive IgM antibody-producing hybridomas from autoimmune mice, selected for their histone binding, may also show similar unusual features [6].

Mice with the *lpr* mutation have a defect in the expression of the apoptosis-mediating molecule Fas [7]. The failure in inducing cell death during certain steps of selection may lead to the presence of antibody molecules that otherwise would have disappeared from the periphery.

In order to ascertain if the *lpr* mutation might be important in promoting the appearance of unusual CDR3 elements which might predispose to the presence of anti-dsDNA antibodies, we have chosen the C57Bl/6 strain homozygous for the *lpr* mutation. Such mice, in contrast to the MRL-*lpr* strain, do not develop arthritis or glomerulonephritis, but do have lymphoproliferation, and have low or negative reactivity against ds-DNA by the *Crithridia licillae* method or by ELISA [1,2]. Lipopolysaccharide (LPS)-stimulated, small resting cells from C57Bl/6-*lpr* mice, shown to lack any bias in V_H gene expression [8], were used for this purpose.

MATERIALS AND METHODS

Mice

C57BL/6-*lpr*/*lpr* (B6MRL-*lpr*, IgH^b) mice were originally obtained from the Jackson Laboratories (Bar Harbor, ME),

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and later bred in our own animal facilities. Male mice were used at 5 months of age and showed no splenomegaly at the time of the study, in contrast to their female littermates. Before being killed, mice were bled from the retrooribital plexus.

Separation of B cell populations

The spleens were handled separately. Total spleen cells were subjected to Percoll density gradient centrifugation (Pharmacia, Uppsala, Sweden) as previously described [9]. High density (small, resting) cells from each mouse were obtained. These were cultured at 10^6 cells/ml with 25 μg of LPS/ml for 3 days. At this time, cells were subjected to another Percoll separation to eliminate dead cells and the B cell LPS blasts were recovered.

Preparation of RNA, cDNA and polymerase chain reaction amplification

Total RNA was extracted by the use of guanidine thiocyanate as described by Chirgwin *et al.* [10]. The obtained RNA was treated with DNAse (Boehringer, Mannheim, Germany) in order to destroy genomic DNA, followed by heating to 92°C for 10 min to eliminate the enzyme. Preparation of the first strand cDNA was done using the $C\mu$ primer: 5′ GCT CCT GCA GGA GAC GAG 3′. Reverse transcriptase (SuperScript) AMV was used (Gibco BRL, Gaithersburg, MD). Nested polymerase chain reaction (PCR) was done as follows. The first amplification was done using the $C\mu$ primer, and a back primer recognizing nucleotides 2–18 of all of the J558 family of V_H genes. The primer was designed based on the sequences obtained from Gu *et al.* [11]: 5′ AGG TCC AGC TGC AGC CA 3′. The conditions were: 5 min denaturation at 99°C, hot start with addition of Taq DNA polymerase (Gibco BRL) at 85°C and 30 cycles with 2 min 94°C, 2 min 58°C and 1 min 72°C. The second PCR amplification was done using a back primer recognizing nucleotides 264–285 of all V_H J558 family genes [11]: 5′ TGA GGA CTC TGC AGT CTA TTA C 3′ and the $C\mu$ primer. The conditions of the PCR were the same as mentioned above, with the exception that 25 cycles were used.

The expected PCR product containing the CDR3 regions is of 118 bp. This fragment was cloned [12] and sequenced using a semi-automated sequencer (Applied Biosystems, Perkin-Elmer, Foster City, CA).

V_H J558 probe hybridization

PCR products derived from the first PCR amplification were cleaned using a geneclean II kit (Bio 101, La Jolla, CA) and digested with *Pst I* (Gibco BRL). Digestion with this enzyme gives three V_H fragments (380, 260 and 200 bp) which define specific subfamilies of the V_H J558 family [8,11]. Such fragments can be easily recognized by hybridizing with a specific probe, and a pattern present in normal, LPS-stimulated cells represents the random expression of the V_H J558 subfamilies [8], as expected [13]. Hybridization was done using a 196-bp nucleotide fragment as specific probe for the V_H B512 gene of the anti-dextran B512 hybridoma cell line F_4H_2 [14]. This gene belongs to the 205.12 subfamily of J558 genes (with 99·3% homology to the germ-line 205.12 gene) [12]. The probe was labelled with ^{32}P-dCTP (Amersham International plc., Aylesbury, UK) and hybridized using a random priming kit (Boehringer).

Pre- and hybridizations were done at 42°C, as described [15]. After washing three times under medium–high stringency conditions in 0·5 × SSC and 0·1% SDS at 65°C for 20 min, films were exposed for various times and developed (Kodak, Rochester, NY). To assess the relative proportions of the three major V_H fragments, the LKB 2202 ultrascan laser densitometer (Kabi-Pharmacia, Uppsala, Sweden) was used.

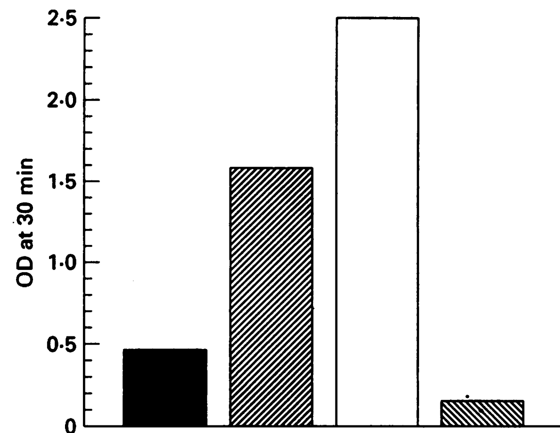

Fig. 1. Presence of IgM anti-dsDNA antibodies in the sera of male (■) and female (▨) C57Bl/6-*lpr* mice, MRL-*lpr* mice (□) and normal animals (▧).

ELISA for IgM anti-dsDNA antibodies

Immulon II plates (Dynatech Laboratories Inc., Chantilly, VA) were coated with 1 μg/ml calf thymus DNA (Serva, Heidelberg, Germany), where PBS pH7 was used as coating buffer, described previously [9]. Sera from individual mice were serially diluted. Sera from MRL-*lpr* mice were used as a pool of various female animals. The assay was developed using a goat anti-mouse IgM conjugated with alkaline phosphatase (Southern Biotechnologies, Birmingham, AL) and nitrophenyl phosphate was used as substrate (Sigma, St Louis, MO). The results are expressed as the OD measurements using a 405 nm filter on an Anthos filter photometer (Anthos Labtech Instruments, Salzburg, Austria) 30 min after substrate addition.

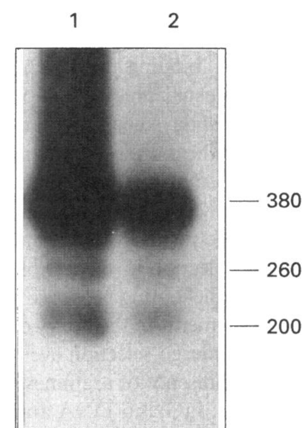

Fig. 2. Autoradiograph showing the three major fragments obtained after *Pst I* digestion and hybridization with a J558-specific probe. Lane 1, lipopolysaccharide (LPS)-stimulated cells derived from *lpr* mice; lane 2, those derived from normal mice. This pattern of expression represents a random distribution of the V_H J558 subfamilies [8].

RESULTS AND DISCUSSION

Serum levels of IgM anti-dsDNA antibodies

Levels of the IgM anti-dsDNA antibodies were measured by ELISA in the serum of male C57Bl/6-*lpr* mice and compared with the female littermates, an MRL-*lpr* pool and a pool from normal C57Bl/6 mice. As observed in Fig. 1, the male C57Bl/6-*lpr* animals had low levels of these antibodies compared with the female and MRL-*lpr* animals. In addition, IgG anti-dsDNA antibodies were undectable in the male animals, in contrast to their female littermates or the MRL-*lpr* (not shown).

LPS-stimulated cells from lpr *mice have a random V_H J558 subfamily gene expression*

In order to exclude the possibility of a bias in the expression of V_H genes in the cell population studied, we determined V_H J558 subfamily expression by a *Pst-I*-based restriction fragment length polymorphism (RFLP), as described in Materials and Methods. As shown in Fig. 2, the pattern of V_H fragments in the normal and *lpr* population studied was similar, suggesting a random V_H gene expression in the preimmune repertoire of *lpr* mice. On the other hand, a bias in V_H J558 subfamily expression has been demonstrated in the large, *in vivo* activated B cell population of *lpr* mice, where only the two major fragments of 380 and 200 are observed (not shown) [8].

*General features of the CDR3 sequences found in LPS-stimulated cells of C57Bl/6-*lpr/lpr *mice*

Nineteen functional CDR3 sequences derived from small, resting, LPS-stimulated cells amplified using the J558 and Cμ specific primers from individual male C57Bl/6-*lpr/lpr* mice were analysed and are shown in Fig. 3a.

Ten out of 19 sequences had their D elements translated in reading frames 2 and 3, while the sequences derived from normal mice had all D elements in reading frame 1 (Fig. 3b). Sequences S5.1 and S5.5 most probably represent the fusion of D genes, all of which are found in reading frames 2 and 3. In S5.1, reading frame 3 of the DFL 16.1 gene gives rise to an arginine codon. The same is observed in sequence S5-12. Sequences S5.9, S9.5 and S9.8 have arginine codons formed both by an unusual reading frame and a possible N addition. Arginines formed by unusual reading frames have been described only in cell lines producing anti-dsDNA or anti-histone autoantibodies derived from autoimmune-prone strains [5]. Arginines formed solely by N additions were found in three of the *lpr* sequences, whose D elements are in

Clone	V_H																		J_H	D genes	Reading frame	Length (aa)
(a)	94	95	96	97	98	99	100	A	B	C	D	E	H	I	J	K	101	102				
S5.1	AGA	TTA	CTA	CGG	ATT	GTT												TAC	3	FL16.1/SP2.2	RF3/RF2	(7)
	arg	leu	leu	arg	ile	val												tyr				
S5.2	AGG	GAC	AGC	TCA	GGT											TCC	TAT	GCT	4	FL16.1	RF1	(7)
	arg	asp	ser	ser	gly											ser	tyr	ala				
S5.5	AGA	TCC	GCT	ACG	GTA	GCC									TAC	TTT	GAC	TAC	2	FL16.1/FL16.1	RF2/RF2	(10)
	arg	ser	ala	thr	val	ala									tyr	phe	asp	tyr				
S5.6	AGA	GGA	TAC	TAC	GGT	AGT	GGC						TAC	TGG	TAC	TTC	GAT	GTC	1	FL16.1	RF1	(13)
	arg	gly	tyr	tyr	gly	ser	gly						tyr	gly	tyr	phe	asp	val				
S5.7	AGA	GAC	TAC	TAT	AGT	AAC	TAC	GGG							TAC	TTC	GAT	GTC	1	SP2.5	RF1	(12)
	arg	asp	tyr	tyr	ser	asn	tyr	gly							tyr	phe	asp	val				
S5.8	AGA	AGT	ATT	ACT	ACG	GTA							gAC	TGG	TAC	TTC	GAT	GTC	1	FL16.1	RF3	(12)
	arg	ser	ile	thr	thr	val							asp	gly	tyr	phe	asp	val				
S5.9	AGA	CAg	CTG	GGA	CGT												GAC	TAC	2	Q52	RF2	(7)
	arg	gln	leu	gly	arg												asp	tyr				
S5.10	AGA	CGG	GGC	CTT	CTG	GAC	AGC	TCA	GGC	CCC					TGG	TTT	GCT	TAC	3	FL16.1	RF1	(14)
	arg	arg	gly	leu	leu	asp	ser	ser	gly	pro					trp	phe	ala	tyr				
S5.11	AGA	TAC	AGC	TCA	GGC	TCG	TAC								TAC	TTT	GAC	TAC	2	FL16.1	RF1	(11)
	arg	tyr	ser	ser	gly	ser	tyr								tyr	phe	asp	tyr				
S5.12	AAG	CGG	TTA	TGG	CCC	CCC												TAC	2	FL16.2	RF3	(7)
	arg	arg	leu	trp	pro	pro												tyr				
S9.1	AGA	AGA	GAA	GAT	AGT	AAC	TAC	GTG	GGA						GCT	ATG	GAC	TAC	4	SP2.X	RF1	(12)
	arg	arg	glu	asp	ser	asn	tyr	val	gly						ala	met	asp	tyr	.			
S9.2	AGA	TCA	GCA	GCT	CAG	GCT	ACC	GGC						TAT	GCT	ATG	GAC	TAC	4	FL16.2	RF2	(13)
	arg	ser	ala	ala	gln	ala	thr	gly						tyr	ala	met	asp	tyr				
S9.3	AGA	TGG	GGG	GTA	GTA	GCT	CCT	CAC							TAC	TTT	GAC	TAC	2	FL16.1	RF3	(12)
	arg	trp	gly	val	val	ala	pro	his							tyr	phe	asp	tyr				
S9.4	AGA	GGG	TGG	CTC													GAC	TAC	2	SP2.3-8	RF3	(6)
	arg	gly	trp	leu													asp	tyr				
S9.5	AGA	CCC	GGA	CGA	GAC									TAT	GCT	ATG	GAC	TAC	4	Q52	RF2	(10)
	arg	pro	gly	arg	asp									tyr	ala	met	asp	tyr				
S9.6	AGA	CGG	TTC	CCC	TCT	TAC	TAC	GGT	AGT	AGC	TCC			tAC	TAC	TTT	GAC	TAC	2	FL16.1	RF1	(15)
	arg	arg	phe	pro	ser	ser	ser	gly	ser	ser	ser			tyr	tyr	phe	asp	tyr				
S9.7	AGA	TCC	TAC	TAT	GGT	AAC	TAC	GTG	TAC						TAC	TTT	GAC	TAC	2	SP2.5	RF1	(13)
	arg	ser	tyr	tyr	gly	asn	tyr	val	tyr						tyr	phe	asp	tyr				
S9.8	AGA	AGG	GAC										gAC	TAT	GCT	ATG	GAC	TAC	4	Q52	RF3	(9)
	arg	arg	asp										asp	tyr	ala	met	asp	tyr				
S9.9	AGA	CCG	GGG	GGT	AGT	AGC	TAC	GTT	TCT							TTT	GAC	TAC	2	Fl16.1	RF1	(12)
	arg	pro	gly	gly	ser	ser	tyr	val	ser							phe	asp	tyr				
(b)																						
S20.6	TCC	TAC	GGT	AGT	AGT	CCT												cAC	2	FL16.1	RF1	(7)
	ser	tyr	gly	ser	ser	pro												his				
S20.7	AGG	CCG	TAC	TAC	GGT	AGT	AaC	TcC	CTT								GCT	TAC	3	FL16.1	RF1	(11)
	arg	pro	tyr	tyr	gly	ser	asn	ser	leu								ala	tyr				
S20.9	AGA	GTC	GAC	TAT	GAT	TAC	GAC	CCC							TGG	TTT	GCT	TAC	3	SP2.9	RF1	(12)
	arg	val	asp	tyr	asp	tyr	asp	pro							trp	phe	ala	tyr				
S20.10	AGA	CAT	AGT	AaC	TAC	GTC										TTT	GCT	TAC	3	FL16.1	RF1	(9)
	arg	his	ser	asn	tyr	val										phe	als	tyr				
S20.12	CCA	TTC	TAT	GAT	GGT										TGt	TTT	GCT	TAC	3	SP2.9	RF1	(9)
	pro	phe	tyr	asp	gly										cys	phe	ala	tyr				

Fig. 3. Nucleotide and amino acid sequences of the V_HDJ_H rearrangements derived from the preimmune repertoire of C57Bl/6-*lpr/lpr* mice. Amino acid codons are numbered according to Kabat *et al.* [16]. The D and J_H genes used are indicated for each clone [16]. The germ-line segments of the D genes are underlined. The reading frame in which the D genes are read is also shown. Arginines are shown in bold, and possible mutations are represented as lower case letters in the nucleotide sequence. (a) The sequences derived from *lpr* mice. (b) Those from normal animals.

Table 1. Presence of arginines within heavy chain CDR3 elements, in sequences taken from the literature

Sequences	No.	Arginine frequency (%)	Reference
Antigen-specific hybrid*	79	22 (28)	[16]
Adult cDNA nl	63	9 (14)	[17]
Adult J558 nl	24	7 (29)	[18]
Autoantibody†	74	29 (39)	[16]
Adult cDNA *lpr*	19	8 (42)	This paper

*This refers to all heavy chain antibody sequences from normal mice that encode for antibodies binding to immunizing antigens or natural antibodies found in Kabat *et al.* Arginines at codon 94 were excluded.

† Includes all autoantibodies (also non-DNA binding) described in lupus-prone animals.

reading frame 1 (S5.10, S9.1 and S9.6). On the other hand, no arginines were found in the normal sequences (Fig. 3b).

A search through heavy chain sequences found in the literature [16–18] (Table 1) shows that the frequency of arginines in autoantibodies is nearly 40%. This included not only anti-DNA autoantibodies, but also antibodies with other autoreactivities [16]. Most IgG anti-dsDNA antibodies have two or three or more arginines within the CDR3, and those have been specifically selected for DNA binding. On the other hand, sequences derived from antibodies against common protein antigens after immunization with arginines (28%) have no more than one arginine at the CDR3 [16]. Other sequences from spleen RNA from normal mice reveal only 14–29% arginines (Table 1) [17,18]. We show here a frèquency of 42%, close to that found for autoantibodies [16]. Although the number of normal sequences that we have is low, it is clear that every second *lpr* sequence analysed here is unusual: it either has an uncommon reading frame or encodes for an arginine.

In both the *lpr* and normal sequences, most of the D genes used are DFL 16.1, although some of the *lpr* use also DFI16.2. Three of the *lpr* sequences use DQ52. The use of J_H appears to be random in the *lpr* population.

A certain over-representation of J_H3 usage was observed in the normal mouse-derived sequences, possibly due to the cloning procedure. *Pst I* restriction enzyme was used for cloning, and J_H3 has a *Pst I* site. This may have conferred the preferential insertion of these sequences into the K18 vector. However, it is unlikely that this J_H bias had an effect on the type of CDR3 sequenced regarding D gene usage.

We have previously shown that there is no difference in the production of IgM anti-dsDNA antibodies by LPS-stimulated cells from normal and lupus-prone mice [9]. In addition, the absolute number of small, resting, LPS-reactive B cells tends to decrease with age in the *lpr* animals, while the *in vivo* activated, large cells increase and take over in the spleen [8]. This may argue against the possibility that within the LPS-reactive cells there might be increased numbers of memory cells, and in order to avoid this possibility we have amplified only the IgM rearranged V_H genes.

We describe in this study the presence in the preimmune B cell repertoire of C57Bl/6-*lpr*/*lpr* mice of a high frequency of CDR3 elements with unusual features, typically found in anti-dsDNA antibodies and other autoantibodies [6] from lupus-prone strains. We postulate that the presence of unusual CDR3 elements in anti-dsDNA antibodies could be the result not only of somatic hypermutation, but possibly also of a defect in the selection mechanisms acting independently of, and earlier than antigen selection, promoted by the absence of a functional Fas molecule. This, however, does not exclude the possibility that most of the arginines present in the CDR3 region of described sequences [3–5] from autoantibody molecules spontaneously generated in autoimmune animals are due indeed to somatic hypermutation during the antigen-driven process. Nevertheless, as described by Radic *et al.* [5], the presence of a single arginine within the CDR3, irrespective of the position (excluding 94), is enough to confer anti-DNA binding. The presence of uncommon CDR3s could be a predisposing factor for the development of autoantibody reactivities in *lpr* mice, but peripheral selection and expansion of particular clones may depend on other genetic factors. In consequence, only under the appropriate background do extremely high levels of anti-DNA antibodies develop, as occurs in the MRL-*lpr* strain, but not in the C57Bl/6-*lpr* mouse [1,2].

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