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Chain Dominance Study of Monoclonal *MRL-lpr/lpr* Anti-DNA Autoantibodies by Ultraviolet Cross-linking Experiment

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=국문초록=

자외선 친화표지법을 이용한 *MRL-lpr/lpr* 항 DNA 단일클론 자가항체의 사슬우위성에 관한 연구

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전신성홍반성낭창은 항DNA 자가항체 등의 자가반응성 항체를 생성하는 것을 그 특징으로 하는 자가면역질환으로서, 환자에서는 DNA/항DNA 항체 면역복합체가 조직에 축적이 되어 조직이 상해를 입는 것으로 알려져 있다. 항DNA 항체의 상대적 생성량과 전신성홍반성낭창 환자의 조직 상해도나 질병의 활성도는 깊은 연관성이 있기 때문에, 항DNA 항체가 DNA와 다른 교차항원과 어떻게 상호작용을 하는지를 밝히는 것은 흥미로운 일이다.

자외선을 이용한 친화표지법(affinity labeling)은 두 물질에서 매우 가깝게 접촉하는 잔기들을 공유적으로 연결시키는 방법인데, 항체의 중쇄나 경쇄와 항원 DNA의 상호작용을 이 방법으로 직접적으로 측정할 수 있다. 자외선 친화표지법으로 생쥐의 항DNA 자가항체 H241의 경우에는 DNA 항원에 의해 중쇄와 경쇄가 모두 표지되었으며, 항DNA 자가항체 2C10의 경우는 중쇄 만이 표지됨이 보고된 바 있으며, 이 방법으로 밝혀진 DNA 결합 시의 2C10 항체의 중쇄 우위성은 2C10의 중쇄와 경쇄의 가변부위를 클로닝하여 박테리아에서 표현시키는 실험을 통하여 확인된 바 있다. 항 DNA 자가항체가 DNA에 결합 시 중쇄가 우위성의 경향을 나타내며, 어떤 경우에는 중쇄 만으로도 충분히 결합할 수 있다는 사실은 이미 몇몇 논문에 보고된 바 있다.

이 논문에서는 *MRL-lpr/lpr* 생쥐로부터 만들어진 두 개의 IgG 항 DNA 자가항체 2B8 과 3D8을 oligonucleotide와 자외선으로 친화표지함으로써, 이들 항체가 DNA에 결합 함에 있어서 중쇄와 경쇄의 상대적인 참여도를 밝혔다. 이 두 개의 항체는 모두 중쇄가 경쇄에 비해 DNA 결합시에 상대적으로 우위성을 나타냄이 밝혀졌다.

Key Words: anti-DNA Autoantibody, chain dominance, UV cross-linking

INTRODUCTION

SLE (systemic lupus erythematosus) is an autoimmune disease characterized by the production of self-reactive antibodies including anti-DNA autoantibodies[1-4]. In SLE patients, DNA/anti-DNA immune complexes are depo-

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sited in the tissues mediating tissue damage[5-7]. The production of relatively high concentrations of IgG antibodies to both native and denatured DNA is associated with active episodes of SLE[8-10].

Because some of the anti-DNA antibodies contribute to development of lesions and clinical disease, it is important to know how they interact with DNA and, in some cases, with other cross-reacting antigens[11,12]. Analysis of the gene segments that code for V regions of anti-DNA antibodies[13-19] have revealed recurrent sequence motifs, relationships to germline sequences, and the effects of directed or naturally occurring mutations on DNA binding. Crystallographic analyses have been reported for an anti-DNA autoantibody[20] and for immunization-induced antibodies to poly(dG) · poly(dC) and triplex DNA[21,22], providing a basis for modeling antibody-DNA interactions[23]. Antibodies may present a groove into which ssDNA can fit or a surface that accommodates the wider dsDNA or triplex[20-22,24,25]. Crystal-derived structures of a lupus mouse IgG autoantibody to ssDNA (BV04-01) and its immune complex with trinucleotide[24] reveal how both H and L chain CDR residues of that antibody interact with phosphates, sugars and bases of the trinucleotide $(dT)_3$. These structural data, together with functional analysis of H and L chain contributions to binding in several antibodies[26-31], are building a picture of the different ways in which anti-DNA antibodies can be assembled and recognize DNA.

Structural features required for interaction with helical native DNA are of particular interest because of the clinical associations of antibodies with this activity, and the fact that many anti-native DNA autoantibodies have V regions with differences from germline sequences[18]. The roles of H and L chains of mouse autoantibodies to native DNA have been studied[27,30,32-34]. The first direct measurement[32] of interactions of H and/or L chains with DNA in immune complexes has been provided by the covalent cross-linking experiments by UV irradiation, which is a "zero-length" cross-linker of residues that are in close contact[35,36]. With the UV cross-linking experiments, it has been reported that oligonucleotide antigens were cross-linked to both the H and L chains of a mouse IgG antibody to native DNA, H241, but only to the H chain of another mouse IgG antibody to native DNA, 2C10[32]. H chain dominance in the binding of DNA by antibody 2C10 have been confirmed by cloning and sequencing V regions of the H and L chains and by expressing their V regions in a bacterial system[33]. Other examples of H chain dominance or sufficiency for DNA binding by autoantibodies have been reported[27,30,32-34].

We have undertaken the UV cross-linking studies, which have been used to determine convincingly variations in H and L chain binding to DNA[32]. Two IgG anti-native DNA mAbs, 2B8[37] and 3D8[38], originated from *MRL-lpr/lpr* mice were used to test involvement of the H and L chains of the antibodies in binding to DNA.

MATERIALS AND METHODS

Oligonucleotides. Oligonucleotides were synthesized and purified by the solid-phase phosphoramidite method with automated synthesizer (Bioneer, Korea). Oligonucleotides 1138, d(ATATAGCGCGCGCTATAT); and 1500, d(GCGCGATATATTATCGCGC) are self-complementary, and each forms a double-helix molecule. Oligonucleotide $d(GA)_{10}$ and $d(TC)_{10}$ were annealed to form a 20-bp duplex. The concentrations of oligonucleotides were determined by measuring the absorbance at 260 nm.

End labeling of oligonucleotides. Oligonucleotides were radiolabeled at their 5' ends (to sp. act. of 3×10^7 to 5×10^7 cpm/μg) with T_4

polynucleotide kinase (Pharmacia/LKB, Piscataway, NJ) and γ[^{32}P]ATP. The reaction was carried out at 37℃ for 1 h with a buffer containing 70 mM Tris (pH 7.6), 10 mM $MgCl_2$, and 5 mM dithiothreitol and was stopped by the addition of EDTA (to 20 mM) in ice. The labeled oligonucleotides were purified from free [^{32}P]ATP with a QIAquick Nucleotide Removal Kit (Qiagen, Germany). Purified samples were boiled for 5 min and allowed to cool slowly to room temperature.

mAb. *MRL-lpr/lpr* murine hybridomas 2B8[37] and 3D8[38] were grown in Dulbecco's modified Eagle's medium supplemented with L-glutamine, penicillin, streptomycin, gentamycin, nonessential amino acids, 10 mM HEPES buffer and 10% fetal calf serum(FCS). The cells were grown in 750-ml microcarrier stirrer bottles and maintained at 37℃ in humidified 5% CO_2 incubators. The cell lines were subcloned by limiting dilution and tested for both antibody production and antibody binding to DNA by an ELISA[19]. The 2B8 and 3D8 antibodies were purified from tissue culture fluid with a single-strand DNA-agarose affinity column(GibcoBRL, U.S.A.).

Gel retardation assay. Mixtures of 250 ng of DNA and 10μg of antibody in 10μl of TBM buffer were incubated for 1 h at room temperature. Glycerol (30%; 3 μl) was added to each mixture, and the samples were analyzed by electrophoresis in a 12% polyacrylamide gel with a 3.5% stacking gel. The gel was prepared in TBM buffer. After electrophoresis, the wet gel enclosed within a vinyl Seal-a-Meal bag was exposed to X-OMAT AR film at room temperature for autoradiography.

UV cross-linking. Antibody (20μg) was mixed with ^{32}P-labeled oligonucleotide (0.5μg) in TME buffer in a total volume of 50 μl in a well of a polystyrene microtiter plate. The plate, resulting in an ice water bath, was irradiated with short-wavelength (peak, 254 nm) UV light with a Mineralight lamp (model UVGL-25; UVP inc., SanGabriel CA) at a distance of 4 cm for 10 min. The irradiated samples were boiled in SDS dissociation buffer (0.0625 M Tris hydrochloride (pH 6.8), 5% sodium dodecyl sulfate(SDS), 5% 2-mercaptoethanol(2-ME), 20% glycerol, 0.015% bromophenol blue) for 3 min and analyzed by discontinuous SDS-PAGE as described by Laemmli [21]. After electrophoresis, the gel was fixed in 5% methnol-7.5% acetic acid and stained with freshly prepared 0.1% Coomassie brilliant blue solution in 50% TCA for 1 h. The gel was destained with 5% methanol-7.5% acetic acid, dried, and exposed to X-OMAT AR film for autoradiography at -70℃.

Densitometric scanning. Lightly exposed autoradiograms were scanned by Kaiser RS1 Image Analyzer or MCID Microcomputer Imaging Device. One dimensional densitometric analysis was performed by Vilber Lourmat Bio 1D program version 6.32.

RESULTS

Binding of oligonucleotides by mAb

2B8, an IgG2a mAb from an *MRL-lpr/lpr* mouse, binds native DNA in preference to denatured DNA[37].

As a preliminary step, the binding of oligonucleotides by antibody 2B8 was tested in an electrophoretic assay. Antibody 2B8 showed a broad sequence-specificity. Ten micrograms of antibody retarded the migration of radiolabeled oligonucleotide all 1138, 1500 and $(GA)_{10} \cdot (TC)_{10}$ in a 12% nondenaturing polyacrylamide gel (Fig. 1). Some dissociation from antibody 2B8 occurred during electrophoresis, as reflected in the smear of labeled oligonucleotide $(GA)_{10} \cdot (TC)_{10}$ between the bound and free forms.

Antibody 3D8 is an *MRL-lpr/lpr* IgG3 mAb that prefers native DNA to denatured DNA[38]. In competitive ELISA, it has been known to bind to poly(dA-dT), poly(dG-dC), poly(dA-

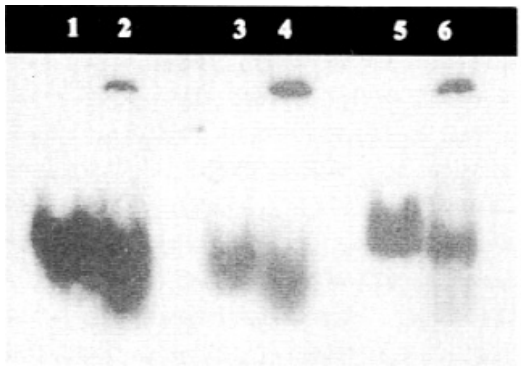

Figure 1. Gel retardation assay for the binding of ^{32}P-labeled oligonucleotides by IgG mAb 2B8 in TBM buffer. Migration of 0.05 μg of 1138, 1500 and $(GA)_{10} \cdot (TC)_{10}$ with no antibody (lane 1, 3 and 5, respectively), and with 10 μg of antibody 2B8 (lane 2, 4 and 6, respectively).

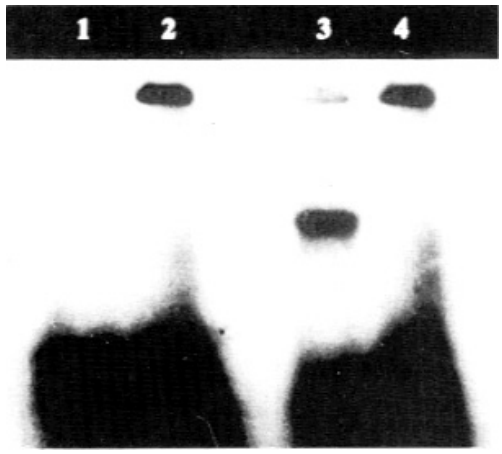

Figure 2. Gel retardation assay for the binding of ^{32}P-labeled oligonucleotides by IgG mAb 3D8 in TBM buffer. Migration of 0.05 μg of oligonucleotide 1138 alone (lane 1) and with 10 μg of antibody 3D8 (lane 2). Migration of 0.05 μg of oligonucleotide 1500 alone (lane 3) and with 10 μg of antibody 3D8 (lane 4).

dC) · (dG-dT) and poly(dA) · (dT)[38]. In gel retardation assay, it bound to both 1138 and 1500 well (Fig. 2).

UV cross-linking of oligonucleotide-antibody mixtures

A mixture of antibody 2B8 or 3D8 and oligonucleotide 1138 in TME buffer was exposed to UV-light (short wave-length) for 10

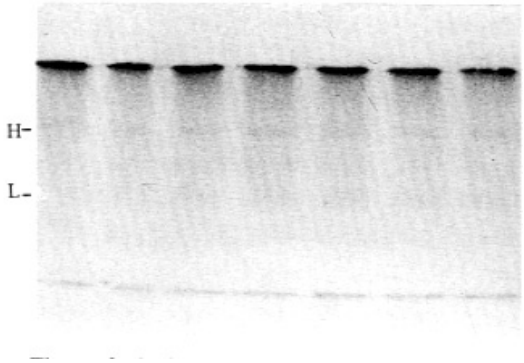

Figure 3. SDS-PAGE analysis of UV cross-linked antibody 2B8-oligonucleotide 1138 complexes. Mixtures of ^{32}P-labeled 1138 and antibody 2B8 placed in the several wells of polystyrene plate were UV-irradiated at the same time. Each samples containing 0.5 μg of labeled 1138 and 20 μg of 2B8 from each well were electrophoresed in 12% polyacrylamide gels under denaturing and reducing conditions. The gel was stained with Coomassie brilliant blue, and dried. The dried gel was exposed to X-OMAT film for autoradiography.

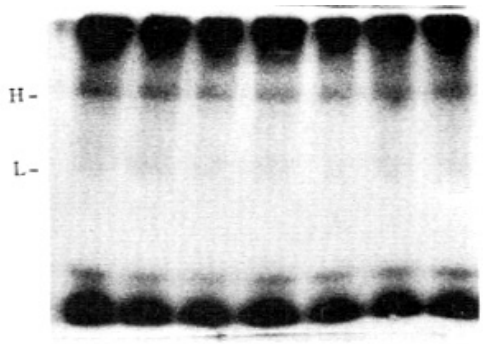

Figure 4. SDS-PAGE analysis of UV cross-linked antibody 3D8-oligonucleotide 1138 complexes. Mixtures of ^{32}P-labeled 1138 and antibody 3D8 placed in the several wells of polystyrene plate were UV-irradiated at the same time. Each samples containing 0.5 μg of labeled 1138 and 20 μg of 3D8 from each well were electrophoresed in 12% polyacrylamide gels under denaturing and reducing conditions. The gel was stained with Coomassie brilliant blue, and dried. The dried gel was exposed to X-OMAT film for autoradiography.

min, denatured at 100℃ in SDS with 2-ME, and analyzed by PAGE (Fig. 3 and 4). It has been shown that when the mixture was irradiated for more than 10 min, significant amount of aggregates other than labeled H and L chains was formed[32]. Under denaturing con-

Table 1. Relative cross-linking of H and L chains of antibodies 2B8 and 3D8

	2B8		3D8	
	H chain	L chain	H chain	L chain
	59.8	40.2	74.6	25.4
	63.6	36.4	71.3	28.7
	64.3	35.7	72.2	27.7
	53.9	46.1	74.7	25.3
	59.6	40.4	77.5	22.5
	59.0	41.0	74.2	25.8
	64.8	35.6	68.5	31.5
			65.8	34.2
Mean[a]	60.7	39.3	72.4	27.6
S.D.	3.9	3.9	3.8	3.8

[a]Replicate UV-exposed mixtures of labeled oligonucleotide and antibody 2B8 or 3D8 were electrophoresed in polyacrylamide gels under denaturing and reducing conditions. The amount of cross-linking was determined by one-dimensional densitometric scanning of lightly exposed autoradiograms.

ditions, the labeled oligonucleotide remains associated with the protein only if it is covalently cross-linked to the protein. In control experiments, there was no labeling of normal IgG (data not shown).

Cross-linking was repeatedly performed with 2B8-1138 mixtures (Fig. 3). When the irradiated 2B8-oligonucleotide 1138 was denatured and electrophoresed in the presence of 2-ME, the radiolabeled antibody chains migrated more slowly than did the free H and L chains (Coomassie brilliant blue-stained bands; H and L in Fig. 3) because the oligonucleotide added approximately 6000 mass units for each DNA strand that remained cross-linked under denaturing conditions. Cross-linking was quantified by densitometric scanning of lightly exposed autoradiograms in a linear density range. 61% of the H chains and 39% of the L chains of 2B8 were labeled by oligonucleotide 1138 (Table 1).

Cross-linking of 3D8-1138 mixtures were repeatedly cross-linked in TME buffer, and was quantified by densitometric scanning. The H chains of 3D8 was also cross-linked with 1138 more strongly than the L chains. 72% of the H chains and 28% of the L chains of 3D8 were labeled by oligonucleotide 1138 (Table 1).

DISCUSSION

Crystallographic analysis of complexes of antibody with protein or small hapten reveals many contacts of antigen with amino acid side chains of immunoglobulin[39]. In the absence of crystallographic data, affinity-labeling can identify some of the close antibody-hapten contacts[40,41]. It has been known that different antihapten antibodies vary in the relative labeling of the two chains by affinity reagents. In one case, 95% of one affinity reagent labeled the H chains and 95% of another affinity reagent labeled the L chains of the same antibody[40], reflecting the involvement of both chains in the binding site, but with each chain contacting distinct regions of the Ag.

It has been reported that two anti-native DNA autoantibodies from *MRL-lpr/lpr* mice, H241 and 2C10, which are differ in their recognition of base sequences in DNA varied in the chains cross-linked to oligonucleotides such that UV irradiation could cross-link oligonucleotide antigens both to the H and L chains and only to the H chains of 2C10[32].

We studied the chain dominance of two anti-native DNA autoantibodies 2B8 and 3D8 in binding with DNA in immune complexes by UV cross-linking method. The H chains of both anti-native DNA autoantibodies were dominantly labeled by helical oligonucleotide antigens. Specially 3D8 showed the tendency of H chain dominance in binding antigen more clearly. H chain dominance has been reported in several examples of both autoantibodies and immunization-induced antibodies to nucleic acids. Even though diverse gene segment families occur in anti-DNA autoantibodies, certain VH segments recur frequently and mediate DNA binding when associated with different

L chains[15,42-44]; and recurrent features of H chain sequence, particularly in CDR3, are associated with DNA-binding activity[13,15]. More directly, the H chain of an NZB/NZW mouse autoantibody, HED-10, was able to bind DNA even in the absence of L chain, and it conferred DNA-binding activity on bacterially expressed scFv with different L chains[30]. The H gene V region of an anti-native DNA antibody, 3H9, together with various different L chains produced in transfectomas, maintained anti-DNA activity even when the L chain came from a cell that did not make anti-DNA antibody[27]. Bacterially expressed H chain alone of anti-Z-DNA antibody Z22 was able to bind Z-DNA specifically, with the same affinity as the parent Fab[45] and a human VHY-encoded H chain bound DNA[34]. Bacterially expressed H chain alone of anti-native DNA autoantibody, 2C10, bound native DNA as well as or better than the H-plus-L chain sc Fv[33].

Apart from anti-DNA antibodies, Ig cDNA libraries from various species have identified other H chains which, by themselves, have significant affinity for protein antigen[46], and in camels, dimers of only H chains comprise a large fraction of serum antibody, with an extensive antigen-binding repertoire[47].

A potential interest in sufficiency of H chain for autoantigen binding is related to the origins of autoantibody-producing cells, which comprise a significant part of the repertoire in the neonatal stage of immunological system development[48]. Developing B cells that express H chains with potential for high affinity autoreactivity may be eliminated or inactivated[49,50], or may escape tolerance mechanisms by combination with L chains that prevent autoreactivity[49,51,52]. However, autoreactivity of modest affinity may participate in positive selection of B cells at an early stage of differentiation. Positive selection may begin before L chain rearrangement, when the H chain is expressed on the cell surface in combination with surrogate L chain polypeptides[53,54]. Further positive selection of H and L chain combinations by autoantigens, including ssDNA, may act after L chain rearrangement also, leading to a prominent representation of autoreactivity in the fetal and neonatal repertoire[48,55].

Further studies such as analyses of the variable-region cDNAs of 2B8 and 3D8 by sequencing and expression will be helpful to explain these results obtained by UV cross-linking experiment. Crystallographic analysis will be required to define the precise three-dimensional structure of DNA-anti-DNA immune complexes. Examples of crystallized anti-DNA antibodies have been reported[56,57], so far they are antibodies that react with ssDNA and oligo(dT) and not with B-helical DNA. For the present, affinity labeling is helpful in identifying the varying involvement of antibody chains in the binding of DNA.

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