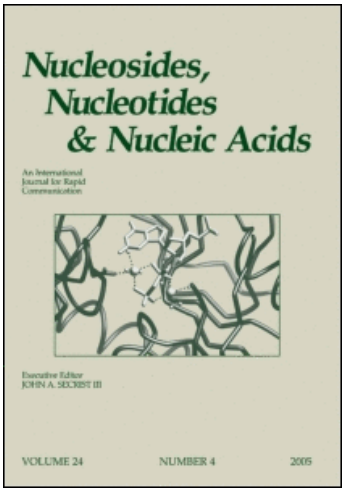

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

DNA-Binding Properties of the Antibody Specific for the Dewar Photoproduct of Thymidylyl-(3''-5'')-Thymidine

Hiroshi Morioka[a]; Mikiko Kurihara[a]; Hiroyuki Kobayashi[a]; Kousuke Satou[a]; Yasuo Komatsu[a]; Makiyo Uchida[a]; Eiko Ohtsuka[a]; Takuya Torizawa[b]; Koichi Kato[b]; Ichio Shimada[b]; Tsukasa Matsunaga[c]; Osamu Nikaido[c]
[a] Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita-ku, Sapporo, Japan [b] Graduate School of Pharmaceutical Sciences, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo, Japan [c] Faculty of Pharmaceutical Sciences, Kanazawa University, Kakuma, Kanazawa, Japan

To cite this Article Morioka, Hiroshi , Kurihara, Mikiko , Kobayashi, Hiroyuki , Satou, Kousuke , Komatsu, Yasuo , Uchida, Makiyo , Ohtsuka, Eiko , Torizawa, Takuya , Kato, Koichi , Shimada, Ichio , Matsunaga, Tsukasa and Nikaido, Osamu(2006) 'DNA-Binding Properties of the Antibody Specific for the Dewar Photoproduct of Thymidylyl-(3''-5'')-Thymidine', Nucleosides, Nucleotides and Nucleic Acids, 25: 4, 667 — 679
To link to this Article: DOI: 10.1080/15257770600686469
URL: http://dx.doi.org/10.1080/15257770600686469

Nucleosides, Nucleotides, and Nucleic Acids, 25:667–679, 2006

ISSN: 1525-7770 print / 1532-2335 online
DOI: 10.1080/15257770600686469

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DNA-BINDING PROPERTIES OF THE ANTIBODY SPECIFIC FOR THE DEWAR PHOTOPRODUCT OF THYMIDYLYL-(3′-5′)-THYMIDINE

Hiroshi Morioka, Mikiko Kurihara, Hiroyuki Kobayashi, Kousuke Satou, Yasuo Komatsu, Makiyo Uchida, and Eiko Ohtsuka □ *Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita-ku, Sapporo, Japan*

Takuya Torizawa, Koichi Kato, and Ichio Shimada □ *Graduate School of Pharmaceutical Sciences, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo, Japan*

Tsukasa Matsunaga and Osamu Nikaido □ *Faculty of Pharmaceutical Sciences, Kanazawa University, Kakuma, Kanazawa, Japan*

□ *A monoclonal antibody (DEM-1) specific for the Dewar photoproduct is used for detection and quantification of photolesions in DNA. To help understand the molecular recognition of damaged DNA by the antibody protein, we have cloned and sequenced the variable region genes of DEM-1. We have also prepared Fab fragments of DEM-1 (DEM1Fab), and synthesized two kinds of 3′-biotinylated oligonucleotides of different lengths containing a central Dewar photoproduct of TpT to analyze the effects of the antigen size on the binding rates by means of surface plasmon resonance (SPR). Results obtained from SPR analyses suggest that DEM1Fab may recognize tetranucleotide unit as the epitope.*

Keywords UV-damaged DNA; Dewar photoproduct; Monoclonal antibody

Received 31 January 2006; accepted 13 February 2006.

This research was supported by a Grant-in-Aid for Specially Promoted Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

This article is dedicated to Prof. Eiko Ohtsuka on the occasion of her 70th birthday.

Present address for Mikiko Kurihara: Central Research Lab., Kaken Pharmaceutical Co., Ltd., 14, Shinomiya, Minamigawara-cho, Yamshina-ku, Kyoto 607-8042, Japan.

Present address for Hiroyuki Kobayashi: Tsukuba Research Labs., Takeda Pharmaceutical Co., Ltd., 10, Wadai, Tsukuba 300-4293, Japan.

Present address for Yasuo Komatsu: Research Institute of Genome-based Biofactory, National Institute of Advanced Industrial Science and Technology, 2-17-2-1, Tsukisamu-Higashi, Toyohira-ku, Sapporo 062-8517, Japan.

Present address for Takuya Torizawa: Fuji Gotemba Research Labs., Chugai Pharmaceutical Co., Ltd., 1-135 Komakado, Gotemba, Shizuoka 412-8513, Japan.

Present address for Koichi Kato: Graduate School of Pharmaceutical Sciences, Nagoya City University, 3-1 Tanabe-dori, Mizuho-ku, Nagoya 467-8603, Japan.

Address correspondence to Hiroshi Morioka, Graduate School of Pharmaceutical Sciences, Hokkaido University, N12, W6, Kita-ku, Sapporo 060-0812, Japan. E-mail: morioka@pharm.hokudai.ac.jp

INTRODUCTION

Irradiation of DNA with ultraviolet (UV) light produces a variety of photoproducts, which are considered to induce mutations, neoplastic cellular transformation, and cell death.[1,2] At adjacent pyrimidine sites, two major types of photoproducts (i.e., *cis-syn* cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6–4) pyrimidone photoproducts [(6–4) photoproducts]), are formed (Figure 1). (6–4) Photoproduct is converted to an isomeric form, namely Dewar photoproduct by 313 nm UV light efficiently (Figure 1).[3] Although *cis-syn* CPDs of TpT (T[c,s]T) are found not to be very mutagenic, (6–4) photoproducts of TpT (T[6–4]T) are highly mutagenic and yield a specific mutation in SOS-induced *Escherichia coli* cells.[4,5] Dewar photoproducts of TpT (T[Dewar]T) are less mutagenic and induce a broader range of mutations than T[6–4]T.[4,5]

It has been revealed that DNA damage in organisms is corrected by a variety of cellular repair mechanisms.[2,6,7] In order to investigate the DNA repair systems, detection and quantification of DNA photolesions have so far been performed by use of antibodies against the individual photoproducts.[8–15] Previous studies from our laboratories have focused on a series of monoclonal antibodies isolated from a single mouse immunized with UV-irradiated

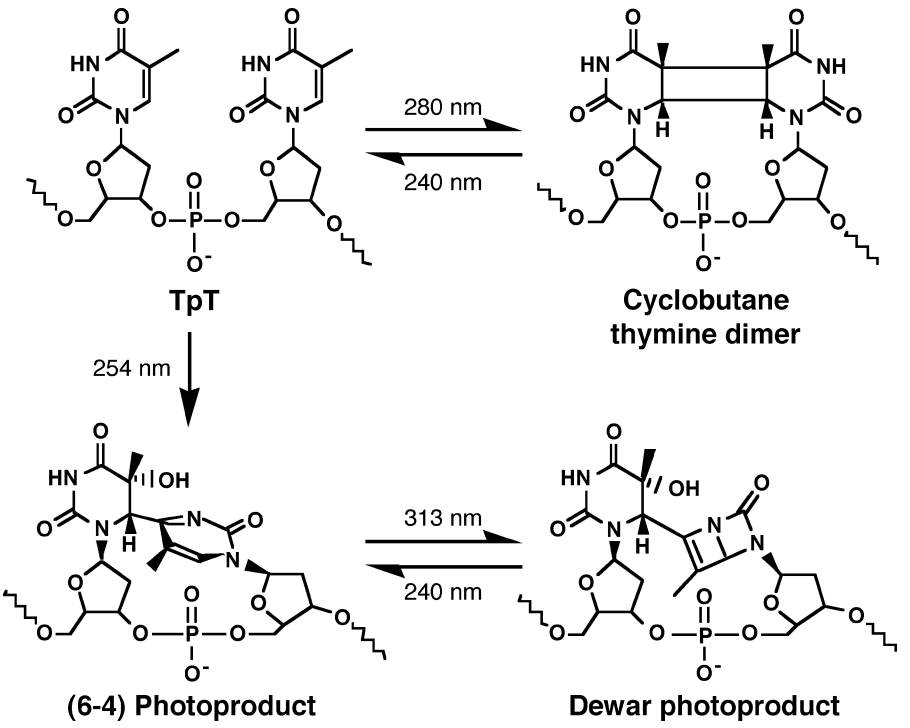

FIGURE 1 Structures of major TpT photoproducts.

calf thymus DNA.[13] Two of the monoclonal antibodies specifically bound d(T[c,s]T) (designated TDM-2 and TDM-3) and four recognized d(T[6-4]T) (designated 64M-2, 64M-3, 64M-4 and 64M-5).[13] Variable regions from the four antibodies recognizing (6-4) photoproducts have been cloned and sequenced and computer models for each have been constructed.[16] To facilitate further studies, a single-chain Fv (scFv) derivative was created from the monoclonal antibody with the highest affinity for d(T[6-4]T) (64M-5).[16] Furthermore, a variety of techniques have been used to examine the details of protein-DNA interactions in the series of anti-DNA photolesions antibodies. It was observed experimentally that the affinity of the 64M-5 for oligonucleotides containing a central (6-4) photoproduct increased with increasing lengths up to a hexanucleotide.[17,18] Mutagenesis studies of CDR loop amino acids and NMR spectroscopy using stable isotopes suggested that 64M-5 undergoes conformational changes on antigen binding, and it appeared that these conformational changes lead the higher affinity of 64M-5.[19,20] The functional importance of the strongly cationic patch consisting the four lysine residues on the 64M-5 VH surface and the Trp33H side-chain, which plays a key role in accommodation of 3′-pyrimidone base of T[6-4]T in combining site of 64M-5, were investigated by site-directed mutagenesis of 64M5scFv.[21,22] The X-ray crystal structure of 64M-2 Fab fragment in complex with d(T[6-4]T) dimer was solved, and the structural recognition of (6-4) photoproduct was elucidated.[23] Analyses of antigen recognition mechanisms of TDM-2 were also conducted by both competitive ELISA and stable isotope-assisted NMR spectroscopy using various antigen analogs.[24,25]

In this article, we describe cloning and sequencing of the variable region genes of a monoclonal antibody (DEM-1)[15] that recognizes Dewar photoproduct specifically. In order to understand the molecular recognition of T[Dewar]T by DEM-1, surface plasmon resonance (SPR) analyses were carried out using Fab fragments of DEM-1 and synthetic oligonucleotides containing T[Dewar]T. On the basis of the data, we will discuss DNA-binding properties of DEM-1.

MATERIALS AND METHODS

Enzymes and Other Reagents

Restriction and DNA modifying enzymes were purchased from Takara Bio, New England Biolabs (Beverly, MA), Bethesda Research Labs (Grand Island, NY), Stratagene (La Jolla, CA), or Toyobo (Osaka, Japan). Reagents were obtained from Wako Pure Chemical Industries (Osaka, Japan), Nacalai Tesque (Kyoto, Japan), or Sigma Chemical (St. Louis, MO). DNA phosphoramidite reagents were obtained from Perkin-Elmer Applied Biosystems (Foster City, CA). Routine cloning was performed according to Sambrook et al.[26]

Synthesis of Oligonucleotides

Solid-phase oligonucleotide synthesis was carried out with an Applied Biosystems Model 394 DNA/RNA synthesizer, using standard β-cyanoethyl chemistry according to the manufacturer's protocol. Oligonucleotides used for molecular biology techniques were purified by denaturing polyacrylamide gels. 3′-Biotinylated oligonucleotides for surface plasmon resonance analyses were synthesized on biotinylated CPG columns obtained from Clontech (3′ Biotin-ON CPG, Palo Alto, CA), and purified by reversed-phase HPLC (μ-Bondapak C18, Waters (Malboro, MA) or Inertosil ODS3, GL Science (Tokyo, Japan)) as previously described.[17]

Preparation of the 3′-Biotinylated Oligonucleotides Containing a T[Dewar]T Photoproduct

3′-Biotinylated oligonucleotides containing the (6-4) photoproduct or the Dewar photoproduct of TpT were synthesized using procedures similar to those previously described.[17,27] The (6-4) tetramer [d(AT[6-4]TA)-bio] and the (6-4) hexamer [d(AAT[6-4]TAA)-bio] were produced by UV irradiation of d(ATTA)-bio and d(AATTAA)-bio in a UV cross-linker with five 8-watt germicidal lamps (Funakoshi, FS800, Tokyo, Japan). The total 254 nm UV dose used to synthesize the (6-4) tetramer was ca. 70 kJ/m^2, 50 kJ/m^2 being used for the (6-4) hexamer. The (6-4) tetramer and the (6-4) hexamer were isolated directly from the UV-irradiated reaction mixtures by reversed-phase HPLC (μ-Bondapak C18 or μ-Bondasphere C18, Waters). The yields of oligonucleotides containing the (6-4) photoproduct were typically 2.6–3.6%. The (6-4) tetramer [d(AT[6-4]TA)-bio] was also synthesized using the dinucleotide building block of T[6-4]T in a similar manner as previously described.[28–30] The (6-4) tetramer and the (6-4) hexamer were quantitatively converted to the corresponding Dewar oligomers by irradiation with Pyrex-filtered light from a 100-watt medium-pressure mercury arc lamp (Ushio, UM-102, Tokyo, Japan). The reaction was periodically monitored by μ-Bondasphere C18 HPLC and the Dewar tetramer [d(AT[Dewar]TA)-bio] and the Dewar hexamer [d(AAT[Dewar]TAA)-bio] were obtained.

Cloning and Sequencing of VH and VL Region Genes of DEM-1 Antibody

The preparation of variable region genes from DEM-1 (IgG1, λ) hybridoma (A-3-3-3, 5.0×10^8 cells) was performed using procedures similar to those described previously.[16] The 5′-phosphorylated primers used for PCR amplification were VHB [5′-d(AGG T(G/C)(A/C) A(A/G)C TGC AG(G/C) AGT C(A/T)G G)-3′], located at the VH N-terminal region; MOCG12-F [5′-d(CTC AAT TTT CTT GTC CAC CTT GGT GC)-3′], which

annealed at the 3′ end of the heavy-chain constant region (CH1); LambdaV1 [5′-d((G/C)A(G/C) GCT GTT GTG ACT CA(A/G) GAA TCT G)-3′], located at the VL N-terminal region; LambdaC1[5′-d(GAG CTC (C/T)TC AGA GGA AGG TGG AAA CA)-3′], which annealed at the 3′ end of light-chain constant region (CL).[16,31,32] The PCR products were inserted into *Eco*RV site of pBluescript II KS(+) vector, and were sequenced using an automated sequencer (Applied Biosystems model 310, Foster City, CA). The VL N-terminal sequence was also determined by direct amino acid sequencing of VL chain of DEM-1 antibody, whereas the VH N-terminal sequence was not determined because of N-terminal blocking.

Detection of Cysteine Residues with 4-(Aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (ABD-F)

Thiol groups of antibody proteins were detected by ABD-F method as described previously.[33,34] Briefly, proteins were labeled at 60°C for 10 min with 250 μM ABD-F in 0.1 M borate buffer (pH 8.0) containing 20 mM EDTA and 6 M urea. Fluorescence excitation and emission spectra of ABD-S-adducts were measured using a Shimadzu RF-5300PC spectrofluorometer. The emission spectra were recorded over the wavelength range from 400 nm to 600 nm with an excitation wavelength of 380 nm. Excitation and emission bandpasses were 5 and 10 nm, respectively.

Preparation of DEM-1 Fab Fragment by Papain Digestion

The purified DEM-1 antibody was reduced by 10 mM DTT and carboxymethylated by 22 mM iodoacetamide, and then digested by papain as previously described.[18,20] The reduced and alkylated DEM-1 was dissolved at a concentration of 5 mg/ml in a digestion buffer (75 mM sodium phosphate (pH 7.0), 75 mM NaCl, 2 mM EDTA, and 5 mM NaN_3), and then incubated for 2 h at 37°C in the presence of papain, at an enzyme to substrate ratio of 1:500 (w/w), and 11 μM L-cysteine. The reaction was terminated by the addition of 33 mM *N*-ethylmaleimide. The digestion products were loaded onto a Mono Q anion exchange column (Amersham Biosciences, Piscataway, NJ) equilibrated with 20 mM Tris-HCl (pH 8.0). The Fab fragment (DEM1Fab-SR) was eluted at the flow rate of 1.0 ml/min with linear gradient of 0–0.2 M NaCl. The purity of the preparation was confirmed by SDS-polyacrylamide gel electrophoresis.

Preparation of DEM-1 Fab Fragment by Metalloendopeptidase Digestion

The purified DEM-1 antibody was reduced by 10 mM DTT and then digested by metalloendopeptidase in a similar fashion as previously described.[35] The reaction was terminated by the addition of 10 mM EDTA.

The Fab fragment (DEM1Fab-SH) was purified using a Mono Q anion exchange column (Amersham Biosciences, Piscataway, NJ).

Surface Plasmon Resonance Measurement of DEM-1 Fab Fragments Binding to the Dewar Oligomers

Binding of DEM-1 Fab fragments to oligonucleotides containing the Dewar photoproduct was measured by surface plasmon resonance (SPR) measurements using a Biacore 2000 instrument (Biacore, Uppsula, Sweden), as described previously.[17,19] All experiments were performed at 25°C. Sensor chip SA (Biacore) surfaces with streptavidin pre-immobilized to the dextran were used. Injections of biotinylated oligonucleotides [0.01 pmol/μl in HBS (10 mM HEPES, 0.15 M NaCl, 3.4 mM EDTA, and 0.005% Tween 20, pH 7.4)] were repeated until the SPR signal was increased by 50 resonance units (RU) above the original baseline. Purified DEM-1 Fab fragments were diluted in HBS buffer, and then they were injected over the immobilized oligonucleotides at a flow rate of 100 μl/min over a concentration range from 60 to 180 nM. Sensorgrams were recorded and normalized to a baseline of 0 RU. Equivalent volumes of diluted Fab fragments were also injected over a non-oligonucleotide surface to serve as blank sensorgrams to allow subtraction of the bulk refractive index background. The surface of the sensor chip was regenerated to remove bound Fab fragments by injection of 100 mM HCl for 30 s. Kinetic rate constants were calculated using BIAevaluation 3.01 software (Biacore) with a single-site binding model (A + B = AB). The ratio of the rate constants allowed the apparent equilibrium constant to be calculated, $K_{D,app} = k_{diss}/k_{ass}$.

RESULTS AND DISCUSSION

Preparation of DNA Fragments Containing the Dewar Photoproduct of TpT

The two 3′-biotinylated oligonucleotides containing a central (6-4) photoproduct were synthesized by irradiation of d(ATTA)-bio or d(AATTAA)-bio with 254 nm UV light. These photoproducts were purified by a reverse-phase HPLC, and characterized by UV absorption maxima at 325 nm (data not shown).[36] The tetramer, d(AT[6-4]TA)-bio, and the hexamer, d(AAT[6-4]TAA)-bio, were quantitatively converted to their Dewar valence isomer, d(AT[Dewar]TA)-bio and d(AAT[Dewar]TAA)-bio, by irradiation at wavelengths greater than 320 nm, respectively. Preparation of the tetramer, d(AT[Dewar]TA)-bio was confirmed by coinjection with d(AT[6-4]TA)-bio onto a reverse-phase HPLC (Figure 2), and could be monitored by the disappearance of UV absorption maxima at 325 nm (data not shown).

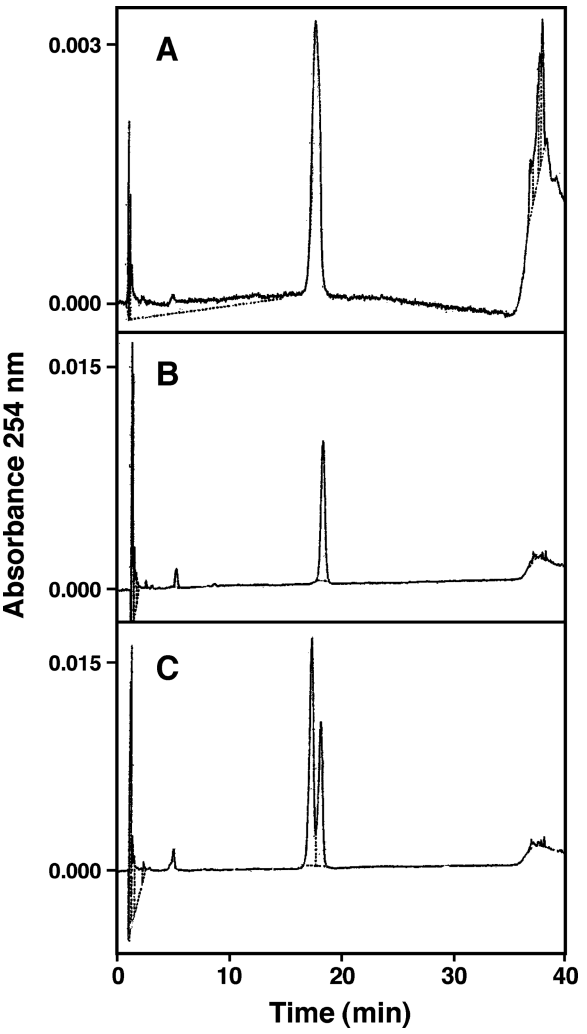

FIGURE 2 HPLC elution profiles of the purified d(AT[6-4]TA)-bio (A), the purified d(AT[Dewar]TA)-bio (B), the purified d(AT[Dewar]TA)-bio coinjected with d(AT[6-4]TA)-bio (C). A μ-Bondasphere C18 300 Å column (3.9 mm × 150 mm) was used with a linear gradient of 6–13% CH_3CN for 30 min in 0.1 M TEAA (pH7.0) at the flow rate of 1.0 ml/min.

Cloning and Sequencing VH and VL Region Genes of DEM-1 Antibody

We used the RT-PCR method to amplify cDNAs encoding the VH and VL regions of DEM-1 antibody from the corresponding hybridoma mRNA preparation. The DEM-1 antibody was found to be of the IgG1 (λ) class using an isotype determination kit.[15] Therefore, we used a set of primers as previously described[32] for amplification of VH fragment. Based on the amino acid sequence of the conserved region, PCR primers were designed for

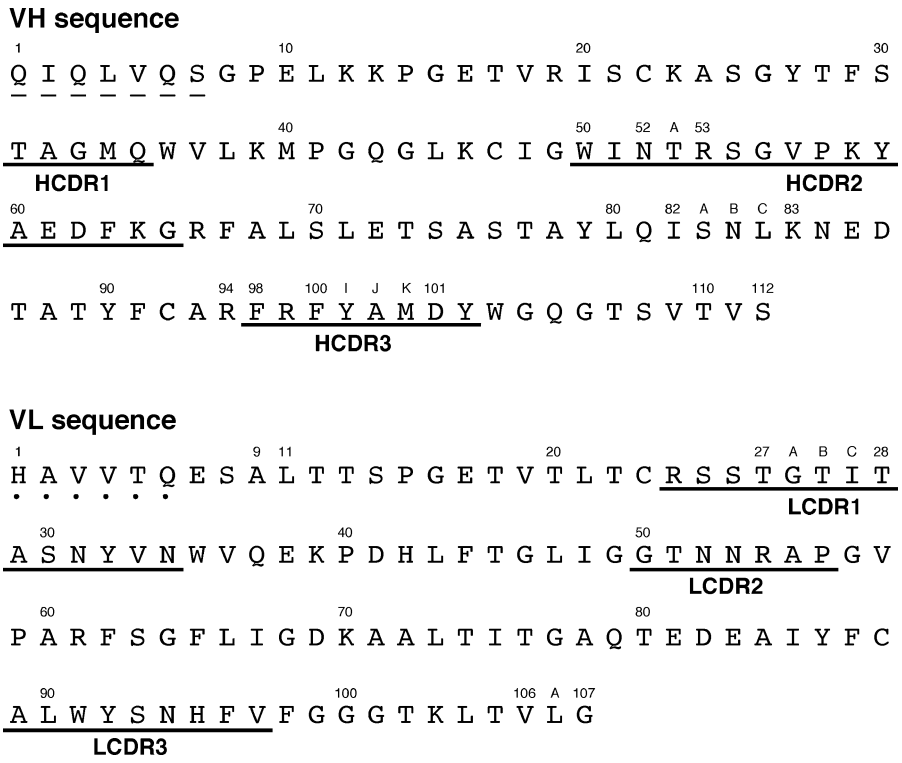

FIGURE 3 Amino acid sequences of the variable regions of DEM-1. The complementarity-determining regions (CDRs) of both the VH and VL portions are underlined. N-terminal sequence of the VH portion is derived from the PCR primer used to amplify the variable region gene (shown with dashed line), and N-terminal region of the VL portion is directly determined by peptide sequencing from DEM-1 antibody (shown with dots).

amplification of lambda light chain.[31] To improve the ligation efficiency of PCR products into blunt-ended vector [*Eco*RV site of pBluescript II KS(+)], the 5′-phosphorylated primers were used for PCR cloning, and Klenow enzyme treatment was carried out after PCR. The nucleotide sequences were determined by automated DNA sequencing. The deduced amino acid sequences of VH and VL regions of DEM-1 are shown in Figure 3. Moreover, we have tried to determine N-terminal amino acid sequences of both VH and VL portions directly from the DEM-1 monoclonal antibody. The N-terminal sequence of VL (indicated by dots) could be determined by direct peptide sequencing. In contrast, the N-terminal sequence of VH could not be determined, because α-amino group of N-terminus might be chemically modified. Note that the amino acid sequence at the N-terminal portion of VH region is derived from the primer used in PCR amplification (indicated by dashed line). The complementarity-determining regions (CDRs) of both the VH and VL portions are underlined. In the VH region of DEM-1, unusual cysteine residue was found out at one position (Cys47H). The VL portion of DEM-1

has high sequence similarity to a typical λ chain in the available databases.[31] Interestingly, the VH region of anti-DNA antibody (6O) from the MRL/lpr mouse model for systemic lupus erythematosus (SLE) has approximately 85% amino acid identity in the VH region with DEM-1 antibody.[37] It appears that the VH regions of both 6O and DEM-1 antibodies belong to the same VGAM3.8 VH family. The amino acid sequences of the VGAM3.8 VH family have been seen in the monoclonal antibodies of diverse specificities (e.g., anti-progesterone, anti-colon carcinoma, anti-neuraminidase, anti-DNA), although the sequences are highly conserved and polymorphisms in the VH region appear to be limited.[38] Differences in the VH regions seem to be mostly confined to CDRs. Therefore, the CDR sequences may be significant for recognition of DNA strands, and differences in the CDR sequences between 6O and DEM-1 antibodies may also be essential for discrimination of base structures.

Verification of the Thiol Residue in VH Region

It is well known that mutations in a gene are generated by RT-PCR cloning, therefore, we have verified an existence of cysteine residue in VH region of DEM-1 using a fluorogenic reagent, ABD-F for thiol group (Figure 4A). ABD-F reacts specifically and quantitatively with thiols of cysteine residues to produce a flurophore, but neither with the S-S group of cystine nor with the N-terminal and lysine ε-amino groups. As shown in Figure 4B, DEM-1 antibody and cysteine were labeled by ABD-F, and

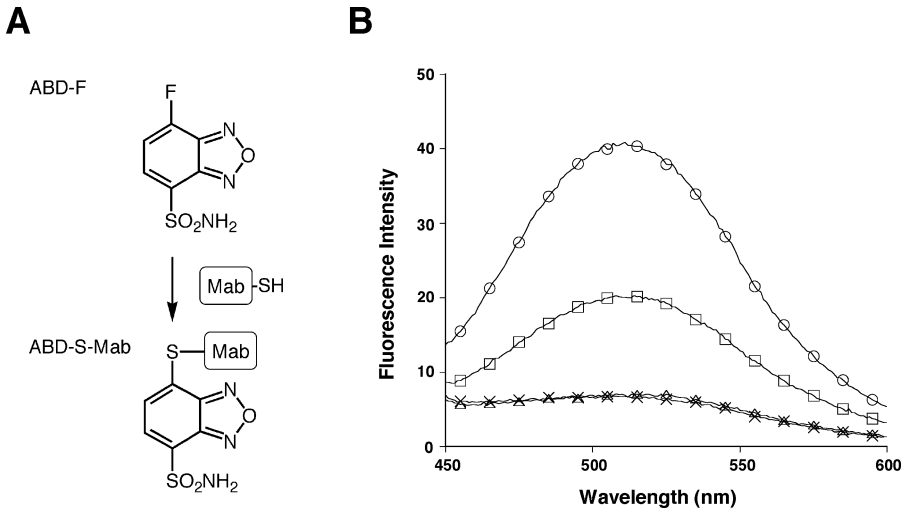

FIGURE 4 (A) Reaction of ABD-F with thiol group of antibody molecule. (B) Emission spectra of ABD-S-adducts with excitation at 380 nm: (○) 0.1 μM DEM-1; (△) 0.1 μM 64M-5; (□) 1 μM cysteine; (×) buffer [0.1 M borate buffer (pH 8.0) containing 20 mM EDTA and 6 M urea].

fluorescence excitation and emission spectra of ABD-S-adducts were observed. However, 64M-5 antibody as a negative control was not labeled. Moreover, ABD-F labeling of DEM-1 protein without 6 M urea was not observed (data not shown). These results indicate that the thiol groups are probably located in the inside of the antibody molecule.

Preparation of DEM-1 Fab Fragments

For a detail investigation of DNA-binding properties of DEM-1 by SPR-based methodology, we have prepared DEM-1 Fab fragments, which contain one antigen-binding site. In the case of preparation of DEM-1 Fab fragment by papain digestion, the thiol group of Cys47H proved to be alkylated by *N*-ethylmaleimide, which terminates the reaction of papain digestion. Such a modification predicted that the structure of the antigen-binding site might be partially denatured and the binding affinity would be decreased. Therefore, we have also prepared DEM-1 Fab fragment by use of metalloendopeptidase. We have named the former Fab fragment as DEM1Fab-SR and the latter Fab fragment as DEM1 Fab-SH.

Antigen-Binding Constants of DEM-1 Fab Fragments

The binding rate constants for both DEM1Fab-SR and DEM1Fab-SH were determined by surface plasmon resonance (a Biacore biosensor). 3′-Biotinylated tetramer (d4-mer) or hexamer (d6-mer) containing a central undamaged TpT, T[6-4]T, or T[Dewar]T were immobilized on the Sensor chip SA surfaces via streptavidin-biotin conjugation. Their amounts were intended to maintain the lowest density of immobilized antigen analogs, in order to reduce the potential of antibody rebinding during the dissociation phase. To examine the effects of oligonucleotide length on the recognition by each Fab fragment, and to compare the affinities of DEM1Fab-SR and DEM1Fab-SH to the DNA lesions, sensor chip surfaces with two kinds of oligonucleotides containing T[Dewar]T (d4-and d6-mer-bio) were prepared. No binding was detected for sensor chips covered with streptavidin alone, coupled with the undamaged oligonucleotide or oligonucleotide containing T[6-4]T (data not shown).

To measure the binding kinetics of each Fab fragment as to the T[Dewar]T-oligonucleotides, sensorgrams were analyzed using a BIAevaluation 3.01 program. The association and dissociation rate constants of DEM1Fab-SR and DEM1Fab-SH are shown in Table 1. The association rate constants for each Fab fragment binding to two oligonucleotides containing T[Dewar]T are virtually identical, suggesting that both Fab fragments can recognize the d4-mer unit of the Dewar photoproduct, d(AT[Dewar]TA). The association rate constants for DEM1Fab-SH as to T[Dewar]T are only 1.7-fold larger than those for DEM1Fab-SR. The dissociation rate constants

TABLE 1 Kinetic Constants for the Binding of DEM-1 Fab Fragments to Oligonucleotides Containing the Dewar Photoproduct of TpT

	k_{ass} (M^{-1} s^{-1})	k_{diss} (s^{-1})	K D (M)
d4-mer-Dewar[a]			
DEM1Fab-SR	$3.7 \pm 0.5 \times 10^5$	$9.6 \pm 0.3 \times 10^{-2}$	$2.7 \pm 0.4 \times 10^{-7}$
DEM1Fab-SH	$6.4 \pm 0.4 \times 10^5$	$7.3 \pm 0.2 \times 10^{-2}$	$1.1 \pm 0.1 \times 10^{-7}$
d6-mer-Dewar[b]			
DEM1Fab-SR	$4.0 \pm 0.3 \times 10^5$	$9.3 \pm 0.2 \times 10^{-2}$	$2.3 \pm 0.2 \times 10^{-7}$
DEM1Fab-SH	$6.6 \pm 0.3 \times 10^5$	$6.9 \pm 0.1 \times 10^{-2}$	$1.0 \pm 0.1 \times 10^{-7}$

[a] d(AT[Dewar]TA).

[b] d(AAT[Dewar]TAA).

DEM1Fab-SR (Fab fragment prepared by papain digestion). DEM1Fab-SH (Fab fragment prepared by metalloendopeptidase digestion).

The apparent association rate constants (k_{ass}) and dissociation rate constants (k_{diss}) were averaged for five independent experiments. These rate constants were calculated from the collected data using BIAevaluation 3.01 software (Biacore).

of both DEM1Fab-SR and DEM1Fab-SH as to the d4-mer-bio containing T[Dewar]T were similar to those of the d6-mer-bio containing T[Dewar]T. These data indicate that both Fab fragments may recognize the d4-mer unit of T[Dewar]T as the epitope and that the binding affinity as to the antigen analogs is not affected by the oligonucleotide length. The dissociation constants (K D) of DEM1Fab-SR are about 2.4-fold larger than those for DEM1Fab-SH, suggesting that the structure of antigen-binding site may be affected by alkylated modification of Cys47H.

As compared to the binding rate constants for a high-affinity Fab fragment specific for damaged DNA, 64M5Fab ($k_{ass} = 2.7$–2.9×10^5 M^{-1} s^{-1}, $k_{diss} = 8.6$–0.91×10^{-4} s^{-1}),[17] the association rate constants for both DEM-1 Fab fragments are virtually identical, however, the dissociation rate constants of both DEM-1 Fab fragments are large by two or three orders of magnitude. DEM-1 Fab fragment exhibits a high binding affinity, while the complex between antibody and antigen is not so stable. In the case of 64M-5, it was suggested that (a) interactions between the antigen-binding site and the regions flanking the photoproduct and (b) conformational multiplicities of antigen-binding site contribute to higher affinity for oligonucleotide containing (6-4) photoproduct.[17,18,20] DEM-1 antibody does not seem to have such molecular recognition mechanisms as 64M-5.

In summary, the results of the present study indicated that (a) DEM-1 molecule specifically recognizes a Dewar photoproduct in DNA and binds to a certain span of an oligonucleotide containing the photolesion; and (b) the regions flanking the Dewar photoproduct are not involved in stabilization of the binding to the antibody. The genes for the variable regions of DEM-1 have been cloned for further mutagenesis to investigate the detailed interactions of the present antibody with photo-damaged DNA.

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