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Specificities and Rates of Binding of Anti-(6-4) Photoproduct Antibody Fragments to Synthetic Thymine Photoproducts[1]

Hiroyuki Kobayashi,* Hiroshi Morioka,* Takuya Torizawa,† Koichi Kato,† Ichio Shimada,† Osamu Nikaido,‡ and Eiko Ohtsuka*,[2]

*Faculty of Pharmaceutical Sciences, Hokkaido University, Kita-ku, Sapporo 060; †Faculty of Pharmaceutical Sciences, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113; and ‡Faculty of Pharmaceutical Sciences, Kanazawa University, Kanazawa 920

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Pyrimidine (6-4) pyrimidone photoproducts are some of the major DNA photolesions induced by ultraviolet (UV) light. A monoclonal antibody (64M5) specific to a (6-4) photoproduct has been established and the corresponding single-chain antibody (64M5scFv) has been prepared. In this study, we characterized the ligand selectivities of 64M5 and 64M5scFv using synthetic octadeoxynucleotides containing either a central *cis-syn* cyclobutane thymine dimer (T[c,s]T), the (6-4) photoproduct of TpT (T[6-4]T), or its Dewar isomer (T[Dewar]T) by means of enzyme-linked immunosorbent assays (ELISA). Both 64M5 and 64M5scFv recognized T[6-4]T, but not the other photoproducts. We synthesized several biotinylated oligonucleotides of different lengths containing (T[6-4]T) to analyze the effects of the antigen size on the binding rates of an antigen binding fragment (64M5Fab) and 64M5scFv by means of surface plasmon resonance. The association rate constants for oligonucleotides of different sizes containing T[6-4]T as to 64M5Fab were found to be almost the same ($1.9\text{-}5.6\times10^{5}$ $M^{-1}\cdot s^{-1}$), while the dissociation rate constant for the largest oligonucleotide (d8mer, 8.0×10^{-5} s^{-1}) was significantly smaller than that for the d2mer (4.2×10^{-2} s^{-1}). These results indicate that 64M5Fab recognized the d2mer as the epitope and that the binding affinity for T[6-4]T depended on the flanking oligonucleotides. The dissociation rate constants for 64M5scFv as to the antigen analogs were almost the same as those for the various T[6-4]T-oligonucleotides as to 64M5Fab, suggesting that the conformations of these antibody binding regions are pretty similar to each other.

Key words: antigen binding fragment, binding affinity, single-chain antibody, surface plasmon resonance, UV-damaged DNA.

The ultraviolet (UV) component of sunlight has toxic, mutagenic, and carcinogenic effects on living organisms (*1*). The major UV photoproducts of DNA occur at dipyrimidine sites, which include a *cis-syn* cyclobutane thymine dimer (T[c,s]T), a (6-4) photoproduct (T[6-4]T), and a Dewar isomer (T[Dewar]T) (Fig. 1). UV-induced mutations appear to occur more frequently at T[6-4]T sites than T[c,s]T ones (*2*). The majority of the biological effects of UV-light were found to be due to T[6-4]T, and not to T[c,s]T, in a xeroderma pigmentosum revertant (*3*). An *in vitro* study of these lesions suggested that T[6-4]T is less tolerated than T[c,s]T in cellular genomes during replication (*4*). These data suggest that T[6-4]T may play a major role in biological lesions produced by UV-light.

A system for the detection and quantification of these photoproducts induced in cells is required to evaluate their biological significance. There are several natural proteins that interact with T[6-4]T (*5-7*). In order to understand the molecular recognition of photo lesions by these proteins, characterization of monoclonal antibodies specific for T[6-4]T might be helpful. A number of polyclonal (*8, 9*) and monoclonal antibodies (*10-13*) against various classes of DNA photoproducts have been developed. These antibodies are also attractive molecules for molecular recognition studies, since they show high substrate binding affinity and specificity. Recently, Mori *et al.* established monoclonal antibodies highly specific for T[6-4]T (*12*). Of them, 64M5 to binds to its target most tightly. In order to investigate the molecular recognition of the antibody and to facilitate future protein engineering studies, we have

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[2] To whom correspondence should be addressed. Tel: +81-11-706-3975, Fax: +81-11-706-4989, E-mail: ohtsuka@pharm.hokudai.ac.jp

Abbreviations: Mab, monoclonal antibody; Fab, antigen binding fragment(s); scFv, single-chain antibody; CDR, complementarity-determining region(s); VH, heavy-chain variable domain; VL, light-chain variable domain; SPR, surface plasmon resonance; RU, resonance unit; TT, TpT; T[6-4]T, (6-4) photoproduct of TpT; T[c,s]T, *cis-syn* cyclobutane thymine dimer; T[Dewar]T, Dewar isomer of TpT; ELISA, enzyme-linked immunosorbent assay(s); k_{ass}, association rate constant; k_{diss}, dissociation rate constant; bio, biotin; TEAA, triethylammonium acetate; FBS, fetal bovine serum; PBS, phosphate-buffered saline.

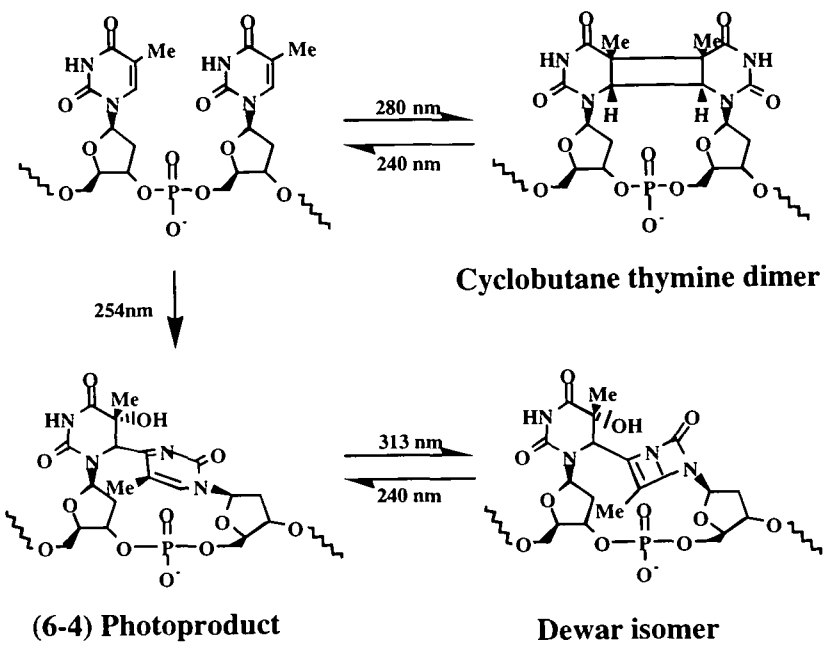

Fig. 1. **Major TpT photoproducts.**

prepared a single-chain derivative of the antibody (scFv) (Morioka *et al.*, submitted for publication).

In this study, we have characterized the 64M5 antibody and a single-chain Fv (64M5scFv) using oligonucleotides containing T[6-4]T at a single site. Several oligonucleotides of different lengths containing one T[c,s]T, T[6-4]T, or T[Dewar]T photoproduct were synthesized and examined as to the binding specificity of intact 64M5 and 64M5scFv by means of enzyme-linked immunosorbent assays (ELISA) and surface plasmon resonance (SPR) with a biosensor (BIAcore, Pharmacia). We found that 64M5-scFv retains the binding property of the parent antibody, although with slightly less affinity as compared with 64M5Fab. These results indicate that the single-chain Fv of 64M5 may become a useful tool for *in vivo* analyses of the molecular recognition of DNA lesions.

MATERIALS AND METHODS

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. The DNA phosphoramidites used in the synthesis were purchased from Perkin Elmer. The oligomers (Figs. 2A and 4) were cleaved from the support and deprotected by treatment with 4 ml of 28% ammonium hydroxide for 6 h at 55°C. 3′-Biotinylated oligonucleotides were prepared by the same protocol on biotinylated CPG columns (1.0 μmol) obtained from Glen Research (BioTEG CPG, Biotin[1]) and Clontech (3′Biotin-ON CPG, Biotin[2]). The dimethoxytritylated oligonucleotides were applied to a C-18 silica gel column, and the products were eluted with a gradient of 5-35% CH_3CN in 0.1 M triethylammonium acetate (TEAA, pH 7.0). Appropriate fractions were concentrated to dryness. Acetic acid (80%, 2 ml) was added to the residue. After 20 min, the solution was evaporated *in vacuo* and coevaporated with H_2O three times. The residue was dissolved in water and then washed with ethyl acetate. The aqueous layer was evaporated to dryness, and the residue was dissolved in 1 ml of H_2O and then passed through a Millipore filter (0.45 mm pore size). Purification was performed by reversed-phase HPLC with a linear gradient of CH_3CN in 0.1 M TEAA. The sample was desalted on a Sephadex G-25 column (Pharmacia Biotech).

UV-Irradiation and Preparation of d(CAAT[6-4]TAAG)—The d8mer, d(CAATTAAG) (2.0 μmol), was dissolved in 5 ml of water, degassed, placed in a 9.6 cm^2 dish on ice under argon in a Ziplock bag, and then irradiated with a Funakoshi FS-800 UV-crosslinker equipped with five 8 W germicidal lamps (total UV dose, *ca.* 200 kJ/m^2). The irradiated solutions were fractionated by preparative μ-Bondapak C-18 (7.8×300 mm)(Waters) HPLC with a 30 min 8.0-14.0% gradient of CH_3CN in 0.1 M TEAA, at the flow rate of 2.0 ml/min. Fractions exhibiting an absorption maximum near 325 nm were collected [characteristic absorption band of the (6-4) photoproduct adduct], concentrated, and then desalted on a Sephadex G-25 column. These fractions were concentrated and repurified by μ-Bondasphere C-18 (3.9×150 mm) (Waters) HPLC with a 20 min 0-8.0% gradient of CH_3CN in 0.1 M TEAA, at the flow rate of 1.0 ml/min. Fractions exhibiting an absorption maximum near 325 nm, eluted at 20 min, were collected, concentrated, and then desalted on a Sephadex G-25 column. The overall yield of d(CAAT[6-4]TAAG) was typically 2.6-3.6%. The major fraction eluted at 16 min, identified as d(CAAT[c,s]TAAG), was collected, concentrated, and desalted.

The 3′-biotinylated oligonucleotides containing a T[6-4]T photoproduct were synthesized using biotinylated CPG columns (1.0 μmol) obtained from Glen Research (Biotin[1]) and Clontech (Biotin[2]), and were prepared by the same procedure as described above. The total UV dose uesd to construct the d2, d6, and d8mer-bio was *ca.* 50 kJ/m^2, 70 kJ/m^2 being used for the d4mer-bio.

Preparation of d(CAAT[Dewar]TAAG)—Approxi-

mately 1.0 nmol of d(CAAT[6-4]TAAG) was dissolved in 500 μl of H_2O, transferred to a 1 cm^2 cell, and then degassed. The sample was irradiated with IWAKI CODE 7740 GLASS filtered light from a 100-watt medium pressure mercury arc lamp at a distance of 30 mm from the lamp center. The reaction was periodically monitored by μ-Bondasphere C-18 HPLC for a total of 60 min, and d(CAAT[Dewar]TAAG) was obtained in an estimated yield of 99%.

Expression and Purification of the 64M5scFv Protein—A clone encoding 64M5scFv was prepared as described (Morioka *et al.*, submitted for publication). Transformed cells were grown and the active scFv was isolated by several chromatography steps.

Preparation and Purification of a Fab Fragment—Hybridoma cells that produce 64M5 (*12*) were grown in NYSF 404 (Nissui) supplemented with 2% heat-inactivated fetal bovine serum (FBS) (JRH Biosciences) at 37°C under a humidified atmosphere of 5% CO_2/95% air. After cell growth, the supernatant was concentrated with a Millipore Minitan ultrafiltration system and then applied to an Affi-Gel protein A column (BIO-RAD). The purified 64M5 antibody (10 mg/ml) was reduced with 10 mM DTT at room temperature for 1 h in 1.5 M Tris-HCl (pH 8.5), and 2 mM EDTA. For alkylation, 22 mM iodoacetamide was added to the reaction mixture, which was then incubated in the dark for 20 min at room temperature. The reaction was stopped by the addition of 10 mM DTT, and then the reaction mixture was dialyzed against 10 mM sodium phosphate buffer (pH 7.3), 0.15 M NaCl, and 3 mM NaN_3. The reduced and alkylated 64M5 was dissolved at a concentration of 5 mg/ml in a digestion buffer comprising 75 mM sodium phosphate (pH 7.0), 75 mM NaCl, 2 mM EDTA, and 5 mM NaN_3, and then incubated at 37°C for 2 h in the presence of papain, at an enzyme to substrate ratio of 1:500 (w/w), and 11 mM L-cysteine. The reaction was terminated by the addition of 33 mM *N*-ethylmaleimide. The digestion products were loaded onto a Pharmacia Mono Q HPLC column equilibrated with 20 mM Tris-HCl (pH 8.0), and then separately eluted at the flow rate of 1.0 ml/min with increasing NaCl concentrations, from 0 to 400 mM. The purity of the Fab preparation was confirmed by SDS-polyacrylamide gel electrophoresis.

Enzyme-Linked Immunosorbent Assays (ELISA)—Direct binding of 64M5 and 64M5scFv to an antigen was measured by an ELISA method. ELISA was performed as follows: polyvinylchloride flat-bottom microtiter plates (Nunc, MaxiSorp) precoated with 1% protamine sulfate (50 μl/well, Sigma) were incubated with various oligonucleotides containing a photoproduct (0.05-1.0 pmol/50 μl/well) in 10 mM phosphate-buffered saline (PBS, pH 7.4) at 37°C for 16 h. After drying, the plates were washed 5 times with 100 μl of PBS containing 0.05% Tween 20 (PBS-T). The plates were incubated with 2% skim milk in PBS (200 μl/well) at 37°C for 90 min to prevent non-specific binding of the antibody, washed 5 times with 200 μl of PBS-T, and then filled with either 64M5 or 64M5scFv (200 ng/100 μl/well) in the 2% skim milk solution. In the case of 64M5scFv, after 60 min incubation, the plates were washed 5 times with 150 μl of PBS-T and then filled with 100 μl of a 1:1,000 dilution of mouse anti *c-myc* IgG (Oncogene Science) in the 2% skim milk solution. For both 64M5 and 64M5scFv, after 60 min incubation, the plates were washed 5 times with 150 μl of PBS-T and then filled with 100 μl of a 1:1,000 dilution of horseradish peroxidase-linked goat anti-mouse IgG (BIO-RAD) in the 2% skim milk solution. After 60 min incubation, the plates were washed 3 times with 150 μl of PBS-T and then washed twice with citrate-phosphate buffer (pH 5.0). The substrate solution (100 μl) consisting of 0.04% *o*-phenylenediamine and 0.007% H_2O_2 in citrate-phosphate buffer was added to each well. Following 15 min incubation at room temperature, 50 μl of 2 M H_2SO_4 was added to stop the reaction, and then the absorbance at 492 nm was measured with an immunoreader (Model 2550 EIA READER, BIO-RAD).

BIAcore Measurement of 64M5Fab and 64M5scFv Binding to the (6-4) Photoproduct—The effects of the sizes of the oligonucleotides containing T[6-4]T on 64M5Fab and 64M5scFv binding were determined by surface plasmon resonance (SPR) measurements with a BIAcore instrument. All experiments were performed at 25°C. Sensor chip SA (Pharmacia Biosensor) surfaces with streptavidin pre-immobilized to the dextran were used. Oligonucleotides were then captured on the chips by injection of 5-10 μl of biotinylated oligonucleotides diluted to 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) at the flow rate of 20 μl/min. Injections were repeated until the SPR signal had increased by 35-100 resonance units (RU) above the original baseline.

Prior to SPR analysis, 64M5Fab and 64M5scFv were diluted in HBS buffer. The antibodies were injected over the immobilized oligonucleotides at the flow rate of 60-100 μl/min, over a concentration range from 3.0 to 200 nM. Sensorgrams were recorded and normalized as to a base line of 0 RU. Equivalent volumes of these diluted antibodies were also injected over a non-oligonucleotide surface to obtain blank sensorgrams for subtraction of the bulk refractive index background. The association was monitored by measuring the rate of binding to an antigen at different protein concentrations. The dissociation of these antibodies from the antigen surface was monitored after the end of the association phase. The remaining bound antibodies were completely removed by an injection of 50 μl of 100 mM HCl.

Kinetic rate constants were calculated from the collected data using BIA evaluation 2.1 software (Pharmacia). A single-site binding model (A+B=AB) was used for the analysis of the interactions of these antibodies with oligonucleotides of various lengths containing T[6-4]T.

RESULTS

Preparation of Damaged DNA Fragments—The oligonucleotides (Fig. 2A) containing a central *cis-syn* cyclobutane thymine dimer or a (6-4) photoproduct used in the ELISA were obtained by irradiation of d(CAATTAAG) with 254 nm light. These photoproducts were isolated by HPLC on a C-18 column (Fig. 2B). The product eluted at 16 min was identified as d(CAAT[c,s]TAAG) from its susceptibility to T4 endonuclease V (*14*), under the conditions described previously (*15*) (data not shown). The product eluted at 20 min, d(CAAT[6-4]TAAG), was characterized by the UV absorption at 325 nm (*16*) (Fig. 3). The octamer, d(CAAT[6-4]TAAG), was isomerized to d(CAAT[Dewar]TAAG) in an essentially quantitative yield (>99%) on UV irradia-

tion at wavelengths greater than 320 nm. The octamer, d(CAAT[Dewar]TAAG), was analyzed by HPLC on a C-18 column. It exhibited no UV absorption in the long wavelength region characteristic of T[6-4]T. Four 3′-biotinylated oligonucleotides containing a single (6-4) photoproduct (Fig. 4) were also synthesized for kinetic analyses of 64M5Fab and 64M5scFv using a biosensor. Two linkers, Biotin[1] and Biotin[2], were inserted between each oligonucleotide and the support during the synthesis. The longer linker (Biotin[1]) was required for the dimer to link to the sensor chip, because the biotin-avidin complex with the short linker (Biotin[2]) interfered with the interaction between the antibody fragments and the antigen analogs.

Analysis of the Specificities of 64M5 and 64M5scFv by ELISA—To determine whether or not the single-chain antibody (64M5scFv) could maintain the specificity of the intact antibody (64M5) (*12*), the specificities of 64M5 and 64M5scFv were analyzed by means of a direct ELISA using synthetic octanucleotides containing the T[c,s]T, T[6-4]T, or T[Dewar]T photoproduct as the antigen analog. In the case of 64M5scFv, we used an anti *c-myc* antibody as the second antibody in the ELISA. As shown in Fig. 5, both 64M5 and 64M5scFv appeared to be highly specific for the single- and double-stranded DNA fragments containing a central (6-4) photoproduct, but the other photoproducts and the undamaged TT were not recognized.

Affinities of 64M5Fab and 64M5scFv for the Immobilized (6-4) Photoproduct—The binding rate constants for 64M5scFv were determined using a BIAcore biosensor and were compared to those for 64M5Fab. Either undamaged TT- or T[6-4]T-containing oligonucleotides were immobilized on the Sensor chip SA surfaces *via* streptavidin-biotin conjugation. Acidic conditions (100 mM HCl, 20 min flow) were used to regenerate the sensor chip, since the Sensor chip SA surfaces were stable as to the degradation of antigen analogs, as determined in preliminary experiments. Therefore, successive experiments could be performed with a small quantity of T[6-4]T-containing oligonucleotides immobilized on a sensor chip. 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 antibody, and to compare the affinities of 64M5scFv and 64M5Fab to the DNA lesions, Sensor chip surfaces with four oligonucleotides containing T[6-4]T (d2, d4, d6, and d8mer-bio) were prepared. Typical sensorgrams obtained on the injection of 64M5Fab (Fig. 6A) and 64M5scFv (Fig. 6B) for each oligonucleotide coupled on the surfaces are shown. No binding was detected for sensor chips either derivatized with streptavidin alone or coupled with the undamaged oligonucleotide (data not shown).

To measure the binding kinetics of each antibody as to the T[6-4]T-oligonucleotides, sensorgrams were analyzed using a BIA evaluation 2.1 program. Such real time analyses of the antibody-antigen interaction allowed fast and reproducible measurements of both the association and dissociation rate constants. The calculated equilibrium dissociation and association rate constants of 64M5Fab and 64M5scFv are shown in Table I. The association rate constants for each antibody binding to several oligonucleotides containing T[6-4]T are virtually identical, suggesting that both antibodies can recognize only the d2mer-bio unit of the (6-4) photoproduct, d(T[6-4]T)-bio. The association rate constants for 64M5scFv as to T[6-4]T are only one order of magnitude smaller than those for 64M5Fab. However, the dissociation rate constants of the 64M5scFv and 64M5Fab fragment as to the d2mer-bio containing T[6-4]T were larger than that of the d8mer-bio containing T[6-4]T by three orders of magnitude. Although both antibodies recognize the d2mer unit of T[6-4]T as the epitope, the binding affinity as to the antigen analogs was affected by the oligonucleotide length.

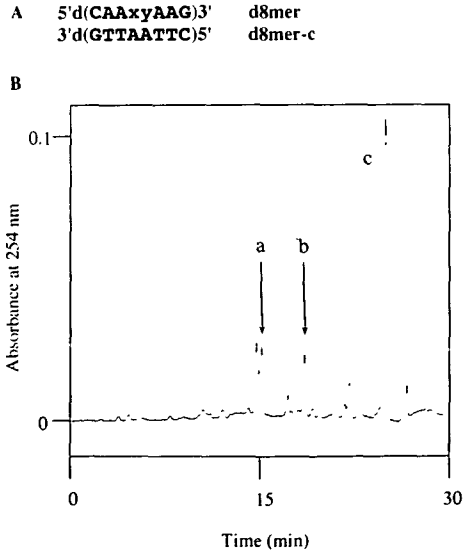

Fig. 2. **A: Oligonucleotide sequences used in the ELISA.** xy refers to either a *cis-syn* cyclobutane thymine dimer, the (6-4) photoproduct, the Dewar isomer, or the undamaged TpT. d8mer-c is the complementary strand of the d8mer. **B: HPLC chromatogram of irradiated d(CAATTAAG).** a, b, and c indicate the peaks of d(CAAT[c,s]TAAG), d(CAAT[6-4]TAAG), and d(CAATTAAG), respectively. A μ-Bondasphere C-18 column was used, with a linear gradient of CH_3CN in TEAA from 0 to 8% for 20 min at the flow rate of 1.0 ml/min.

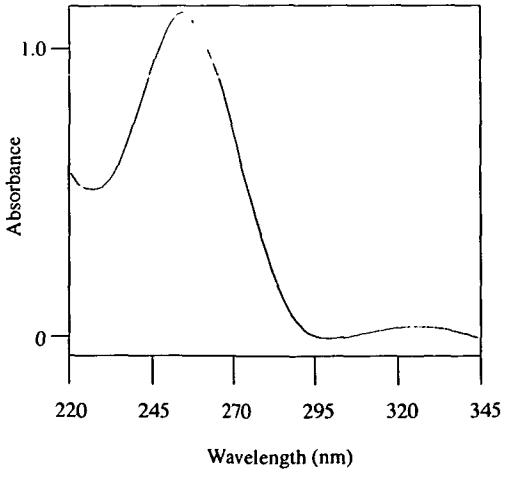

Fig. 3. **UV absorption spectrum of d(CAA'T[6-4]TAAG).**

DISCUSSION

The work presented here involved the use of synthetic DNA antigen analogs containing photoproducts, and confirmed the selectivity and affinity of the monoclonal antibody (64M5) established by Mori *et al.* (*12*), and those of its single-chain Fv derivative (64M5scFv) constructed by Morioka *et al.* (submitted for publication). There are two basic strategies for preparing oligonucleotides containing DNA photoproducts. One approach involves the use of TpT derivative building blocks (*17-19*). The other approach is direct-irradiation. Smith and Taylor reported the preparation of a hexadeoxyribonucleotide containing T[6-4]T at a single site by direct irradiation with UV-light (*20*). The yield of the photoproduct was reported to depend on the base sequence (*21*). Although the use of a building block is more advantageous, the direct irradiation approach is more rapid for small-scale preparation, and we prepared T[6-4]T-containing oligonucleotides by conventional UV irradiation of the parent molecules.

We have shown that these antibodies exhibit high specificity to T[6-4]T in DNA, but not to the Dewar isomer or the cyclobutane thymine dimer (Fig. 5). There have been a few reports of DNA recognition by ssDNA-specific or dsDNA-specific antibodies, and the modes of recognition of

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5'd(   xy   )-Biotin1 3'      d2mer-bio
5'd(  AxyA  )-Biotin2 3'      d4mer-bio
5'd( AAxyAA )-Biotin2 3'      d6mer-bio
5'd(CAAxyAAG)-Biotin2 3'      d8mer-bio
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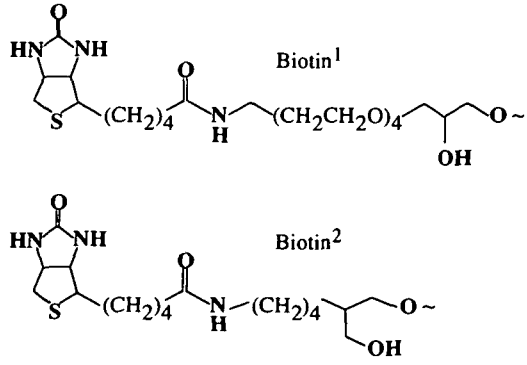

Fig. 4. **Oligonucleotide sequences used to examine the kinetics of the antibodies.** xy refers to either a *cis-syn* cyclobutane thymine dimer, the (6-4) photoproduct, the Dewar isomer, or the undamaged TpT.

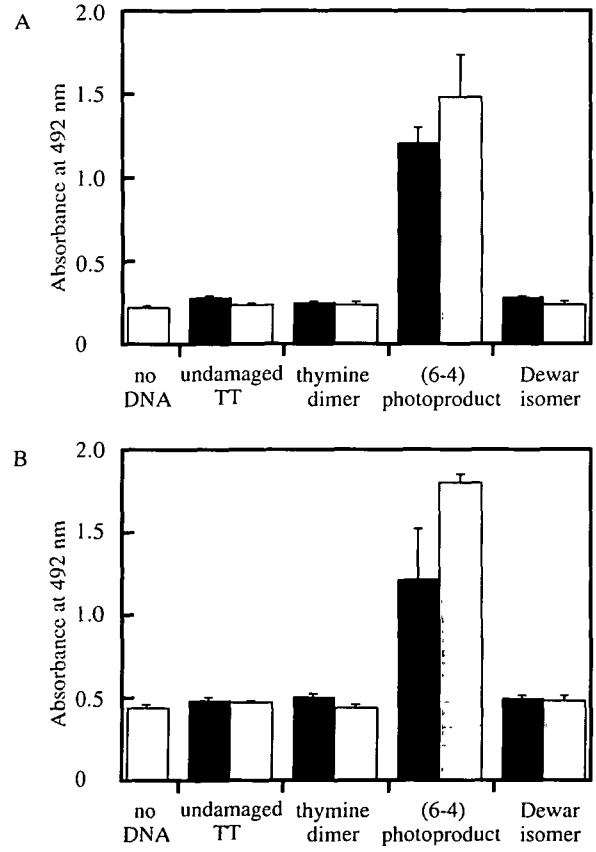

Fig. 5. **ELISA of the binding affinities of 64M5 (A) and 64M5scFv (B) to the UV-induced photoproducts.** The black bars indicate binding of the single-stranded octadeoxyribonucleotides containing either the undamaged TT (CAATTAAG) or a photoproduct, and the gray bars indicate that of the double-stranded octadeoxyribonucleotides, respectively.

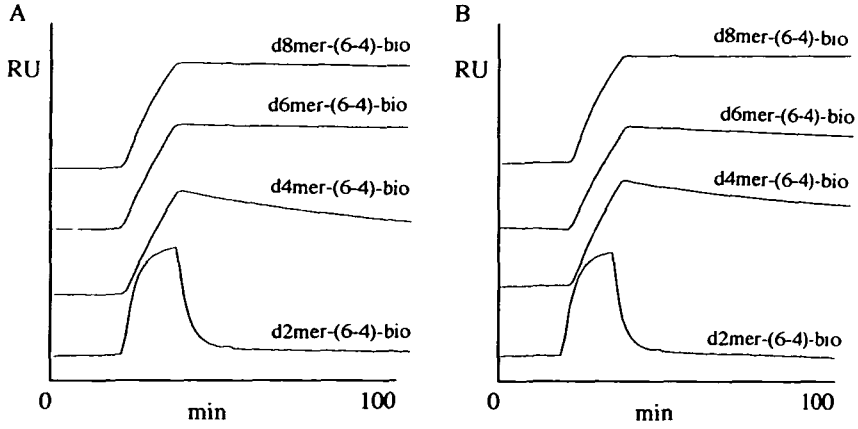

Fig. 6. **SPR sensorgrams of 64M5Fab (A) and 64M5scFv (B) binding to the immobilized biotinylated oligonucleotides containing the (6-4) photoproduct.** The coupling densities were: d2mer-(6-4)-bio, 36.4 RU; d4mer-(6-4)-bio, 50.7 RU; d6mer-(6-4)-bio, 89.9 RU; and d8mer-(6-4)-bio, 71.5 RU. These sensorgrams were obtained after injections of 15 nM of either 64M5Fab or 64M5scFv at the flow rate of 100 μl/min. Equivalent volumes of these antibodies were injected over a non-oligonucleotide surface to obtain blank sensorgrams for subtraction of the bulk refractive index background.

TABLE I. **Kinetic constants for the binding of 64M5Fab and 64M5scFv to oligonucleotides containing the (6-4) photoproduct of TpT.** 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 BIA evaluation 2.1 software (Pharmacia).

Oligonucleotide	64M5Fab		64M5scFv	
	k_{ass} ($M^{-1}\cdot s^{-1}$)	k_{diss} (s^{-1})	k_{ass} ($M^{-1}\cdot s^{-1}$)	k_{diss} (s^{-1})
d2mer-(6-4)	$1.9\pm0.1\times10^{5}$	$4.2\pm0.1\times10^{-2}$	$2.1\pm0.1\times10^{4}$	$2.3\pm0.1\times10^{-2}$
d4mer-(6-4)	$2.7\pm0.2\times10^{5}$	$8.6\pm0.6\times10^{-4}$	$2.2\pm0.3\times10^{4}$	$5.1\pm0.8\times10^{-4}$
d6mer-(6-4)	$2.9\pm0.5\times10^{5}$	$9.1\pm0.8\times10^{-5}$	$3.1\pm0.3\times10^{4}$	$3.8\pm1.1\times10^{-5}$
d8mer-(6-4)	$5.6\pm0.2\times10^{5}$	$8.0\pm0.4\times10^{-5}$	$5.3\pm1.0\times10^{4}$	$2.4\pm0.6\times10^{-5}$

these antibodies seem to be different. The three-dimensional structures of several anti-ssDNA antibody Fab fragments (*22, 23*) have been determined. In the case of ssDNA-specific antibodies, for example, in the Fab (BV04-01)-trinucleotide [$d(pT)_3$] complex, the thymine bases of the DNA penetrate into a deep cleft formed by the combining site of BV04-01 and are stacked with the side chains of aromatic amino acids (*23*). The N-3 and O-4 atoms of the central thymine nucleotide in the complex hydrogen bond with a serine residue of the Fab fragment. In dsDNA, these atoms of the thymines are involved in Watson-Crick hydrogen bonds with the adenine bases in the opposite DNA strand. As a result, it seems difficult for BV04-01 to interact with the thymine bases in dsDNA. In the case of dsDNA-specific antibodies, such as Jel 72 (*24*) and Jel 241 (*25*), the combining regions reside on a relatively flat surface. In the case of 64M5 antibody molecules, ELISA data indicated that both 64M5 and 64M5scFv bound to ssDNA and dsDNA containing T[6-4]T. NMR studies showed that the structure of T[6-4]T in the dsDNA has a distinct base orientation, due to the covalent linkage between the two pyrimidine rings, and the normal Watson-Crick hydrogen bonding is absent between the 3′-side T [6-4]T and its opposite base (*21*). Thus a DNA duplex may be destabilized by local denaturation in the region adjacent to the T[6-4]T, and these antibody molecules may recognize the 3′-side T[6-4]T in a single-stranded region even in duplex DNA.

Surface plasmon resonance-based methodology, such as BIAcore biosensor technology, has been effectively used for the detailed study of protein-DNA interactions (*26, 27*). To characterize the antibody molecules that recognize T[6-4]T, we determined both the association and the dissociation rate constants with several d2-d8mer-bio oligonucleotides containing T[6-4]T (Fig. 4), with real time monitoring using a BIAcore biosensor (Fig. 6). The use of intact antibody 64M5 was not possible in the measurement of the kinetic constants, since the BIAcore dissociation rate constants were off-scale. It was difficult to regenerate sensor chips under the conditions used, probably due to the high stability of the complex involving the bifunctional protein. Therefore, we attempted to analyze the binding constants using the 64M5Fab fragment, which quickly bound to T[6-4]T ($k_{ass}=1.9\text{-}5.6\times10^{5}\ M^{-1}\cdot s^{-1}$) regardless of the length of the oligonucleotide (Table I). However, the dissociation rate constants decreased with the size of the oligonucleotides, *e.g.*, that for the d8mer ($k_{diss}=8.0\times10^{-5}\ s^{-1}$) is smaller than that for the shortest oligonucleotide (d2mer, $k_{diss}=4.2\times10^{-2}\ s^{-1}$).

On the other hand, the association rate constants for 64M5scFv as to these oligonucleotides were one order of magnitude smaller than those for 64M5Fab, while the dissociation rate constants for 64M5scFv were almost the same as those for 64M5Fab with each oligonucleotide length (Table I). As reported by Roggenbuck *et al.* (*28*), BIAcore analysis showed that the association rate of scFv03 was one order of magnitude smaller than that of the Fab-03 fragment, although their dissociation rates were similar. The reason for these differences was proposed to be a conformational change of the paratopes, derived from the steric hindrance due to the linker peptide or from the lack of constant domains. Since 64M5scFv exhibited the same binding specificity as the corresponding Fab and Mab forms, we believe that the conformation of the 64M5scFv-DNA complex is identical to the others. This is supported by the identical off rates for scFv and Fab. Thus, we attribute the difference in association rate constants either to differences between the unliganded forms of scFv and Fab or to the existence of inactive forms of scFv.

In conclusion, these results indicated that (i) the 64M5 molecule specifically recognizes a (6-4) photoproduct in DNA and binds to a certain span of an oligonucleotide containing the lesion; (ii) the regions flanking the (6-4) photoproduct are likely to be involved in stabilization of the binding to the antibody; and (iii) the structure of the paratope in 64M5scFv seems to be partially denatured, but the binding to the oligonucleotides may induce a conformational change in the 64M5scFv molecule. The proposed antigen-binding mechanism for 64M5 can be tested by site-specific mutagenesis experiments and three-dimensional structure analysis. The gene for 64M5scFv has been cloned for further mutagenesis to investigate the detailed interactions of the present antibodies with photo-damaged DNA. These studies will provide useful information on protein-DNA interactions as well as for the diagnosis and treatment of disorders caused by DNA lesions.

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REFERENCES

1. Freidberg, E.C., Walker, G.C., and Siede, W. (1995) *DNA Repair and Mutagenesis*, ASM Press, Washington, DC
2. Gentil, A., Lepage, F., Margot, A., Lawrence, C.W., Borden, A., and Sarasin, A. (1996) Mutagenicity of a unique thymine-thymine dimer or thymine-thymine pyrimidine pyrimidone (6-4) photoproduct in mammalian cells. *Nucleic Acids Res.* **24**, 1837-1840
3. Cleaver, J.E., Cortes, F., Karentz, D., Lutze, L.H., Morgan, W.F., Player, A.N., Vuksanovic, L., and Mitchell, D. L. (1988) The relative biological importance of cyclobutane and (6-4) pyrimidine-pyrimidone dimer photoproducts in human cells. *Photochem. Photobiol.* **48**, 41-49

4. Szymkowski, D.E., Lawrence, C.W., and Lawrence, R.D. (1993) Repair by human cell extracts of single (6-4) and cyclobutane thymine-thymine photoproducts in DNA. *Proc. Natl. Acad. Sci. USA* **90**, 9823-9827
5. Todo, T., Takemori, H., Ryo, H., Ihara, M., Matsunaga, T., Nikaido, O., Sato, K., and Nomura, T. (1993) A new photoreactivating enzyme that specifically repairs UV light-induced (6-4) photoproducts. *Nature* **361**, 371-374
6. Reardon, J.T., Nichols, A.F., Keeney, S., Smith, C.A., Taylor, J.S., Linn, S., and Sancar, A. (1993) Comparative analysis of binding of human damaged DNA-binding protein (XPE) and *E. coli* damaged recognition protein (UvrA) to the major UV photoproducts. *J. Biol. Chem.* **268**, 21301-21308
7. Kai, M., Takahashi, T., Todo, T., and Sakaguchi, K. (1995) Novel DNA binding proteins highly specific to UV-damaged DNA sequences from embryos of *Drosophilia melanogaster*. *Nucleic Acids Res.* **23**, 2600-2607
8. Rosenstein, B.S., and Mitchell, D.L. (1987) Action spectra for the induction of pyrimidine (6-4) pyrimidone photoproducts and cyclobutane pyrimidine dimers in normal human skin fibroblasts. *Photochem. Photobiol.* **45**, 775-780
9. Mitchell, D.L. and Rosenstein, B.S. (1987) The use of specific radioimmuno assay to determine action spectra for the photolysis of (6-4) photoproducts. *Photochem. Photobiol.* **45**, 781-786
10. Roza, L., van der Wulp, K.J.M., MacFarlane, S.J., Lohman, P.H. M., and Baan, R.A. (1988) Detection of cyclobutane thymine dimers in DNA of human cells with monoclonal antibodies raised against a thymine dimer-containing tetranucleotide. *Photochem. Photobiol.* **48**, 627-633
11. Mizuno, T., Matsunaga, T., Ihara, M., and Nikaido, O. (1991) Establishment of a monoclonal antibody recognizing cyclobutane-type thymine dimer in DNA. *Mutat. Res.* **254**, 175-184
12. Mori, T., Nakane, M., Hattori, T., Matsunaga, M., Ihara, M., and Nikaido, O. (1991) Simultaneous establishment of monoclonal antibodies specific for either cyclobutane pyrimidine dimer or (6-4) photoproduct from the same mouse immunized with ultraviolet irradiated DNA. *Photochem. Photobiol.* **54**, 225-232
13. Matsunaga, T., Hatakeyama, Y., Ohta, M., Mori, T., and Nikaido, O. (1993) Establishment and characterization of a monoclonal antibody recognizing Dewar isomer of (6-4) photoproducts. *Photochem. Photobiol.* **57**, 934-940
14. Nakabeppu, Y. and Sekiguchi, M. (1981) Physical association of pyrimidine dimer DNA glycosylase and apurinic/apyrimidinic DNA endonuclease essential for repair of ultraviolet-damaged DNA. *Proc. Natl. Acad. Sci. USA* **78**, 2742-2746
15. Inaoka, T., Ishida, M., and Ohtsuka, E. (1989) Affinity of single- or double-stranded oligodeoxyribonucleotides containing a thymine photodimer for T4 endonuclease V. *J. Biol. Chem.* **264**, 2609-2614
16. Franklin, W.A., Lo, K.M., and Haseltine, W.A. (1982) Alkaline lability of fluorescent photoproducts produced in ultraviolet light-irradiated DNA. *J. Biol. Chem.* **257**, 13535-13543
17. Taylor, J.S., Brockie, I.R., and O'Day, C.L. (1987) A building block for the sequence-specific introduction of *cis-syn* thymine dimers into oligonucleotides. Solid-phase synthesis of TpT-[c,s]pTpT. *J. Am. Chem. Soc.* **109**, 6735-6742
18. Taylor, J.S. and Brockie, I.R. (1988) Synthesis of a trans-syn thymine dimer building block. Solid phase synthesis of CGTAT-[t,s]TATGC. *Nucleic Acids Res.* **16**, 5123-5136
19. Iwai, S., Shimizu, M., Kamiya, H., and Ohtsuka, E. (1996) Synthesis of a phosphoramidite coupling unit of the pyrimidine (6-4) pyrimidone photoproduct and its incorporation into oligodeoxynucleotides. *J. Am. Chem. Soc.* **118**, 7642-7643
20. Smith, C.A. and Taylor, J.S. (1993) Preparation and characterization of a set of deoxyoligonucleotide 49-mers containing site-specific *cis-syn*, trans-syn-I, (6-4), and Dewar photoproducts of tymidylyl(3′-5′)-thymidine. *J. Biol. Chem.* **268**, 11143-11151
21. Kim, J.-K. and Choi, B.-S. (1995) The solution structure of DNA duplex-decamer containing the (6-4) photoproduct of thymidylyl(3′-5′)thymidine by NMR and relaxation matrix refinement. *Eur. J. Biochem.* **228**, 849-854
22. Cygler, M., Boodhoo, A., Lee, J.S., and Anderson, W.F. (1987) Crystallization and structure determination of an autoimmune anti-poly (dT) immunoglobulin Fab fragment at 3.0 Å resolution. *J. Biol. Chem.* **262**, 643-650
23. Herron, J.N., He, X.M., Ballard, D.W., Blier, P.R., Pace, P.E., Bothwell, A.L.M., Voss, E.W., and Edmundson, A.B. (1991) An autoantibody to single-stranded DNA: Comparison of the three-dimensional structures of the unliganded Fab and a deoxynucleotide-Fab complex. *Proteins Struct. Funct. Genet.* **11**, 159-175
24. Mol, C.D., Muir, A.K.S., Lee, J.S., and Anderson, W.F. (1994) Structure of an immunoglobulin Fab fragment specific for poly(dG)-poly(dC). *J. Biol. Chem.* **269**, 3605-3614
25. Braun, R.P. and Lee, J.S. (1987) Equilibrium binding parameters of an autoimmune monoclonal antibody specific for double-stranded DNA. *J. Immunol.* **139**, 175-179
26. Fisher, R.J., Fivash, M., Casas-Finet, J., Erickson, J.W., Kondoh, A., Bladen, S.V., Fisher, C., Watson, D.K., and Papas, T. (1994) Real-time DNA binding measurements of the ETS1 recombinant oncoproteins reveal significant kinetic differences between the p42 and p51 isoforms. *Protein Sci.* **3**, 257-266
27. Barbas, S.M., Ditzel, H.J., Salonen, E.M., Yang, W.-P., Silverman, G.J., and Burton, D.R. (1995) Human autoantibody recognition of DNA. *Proc. Natl. Acad. Sci. USA* **92**, 2529-2533
28. Roggenbuck, D., Konig, H., Niemann, B., Schoenherr, G., Jahn, S., and Porstmann, T. (1994) Real-time biospecific interaction analysis of a natural human polyreactive monoclonal IgM antibody and its Fab and scFv fragments with several antigens. *Scand. J. Immunol.* **40**, 64-70