# Recombinant Phabs Reactive with 7,8-Dihydro-8-oxoguanine, a Major Oxidative DNA Lesion†

Ivan A. Bespalov, Andrei A. Purmal, Mary P. Glackin, Susan S. Wallace,* and Robert J. Melamede

*Department of Microbiology and Molecular Genetics, The Markey Center for Molecular Genetics, The University of Vermont, Stafford Hall, Burlington, Vermont 05405*

*Received July 26, 1995; Revised Manuscript Received November 21, 1995*⊗

ABSTRACT: Antibody Fabs that bind to DNA damages provide useful models for understanding DNA damage-specific protein interactions. BSA and RSA conjugates of the nucleoside and nucleotide derivatives of the oxidative DNA lesions, 7,8-dihydro-8-oxoguanine (8-oxoG) and 7,8-dihydro-8-oxoadenine (8-oxoA), were used to immunize mice. RNA from the responders was isolated and used to repertoire clone and phage display Fabs that bind to these haptens. Direct binding and competitive enzyme-linked immunosorbent assay (ELISA) demonstrated that phage Fabs (Phabs) specific for 8-oxopurine−BSA conjugates and 8-oxoguanine were produced although the Phabs did not react with 8-oxopurines in DNA. Amino acid sequence comparisons among clones having different binding properties suggested that a relatively small portion of the binding surfaces defined by the complementarity determining regions (CDR) accounted for hapten binding specificity, whereas other regions appeared to stabilize hapten binding by interacting with protein or DNA epitopes. Chain shuffling between 8-oxopurine−BSA binding Fabs and a DNA binding Fab showed that the heavy chain of the DNA binder conferred DNA binding capacity to the light chain of only one of the 8-oxopurine−BSA binders. Homology modeling of the 8-oxoG-specific clone g37 showed significant similarities to two previously isolated monoclonal antibodies specific for single-stranded nucleic acids. In the 8-oxoG Fab, which did not bind to DNA, the presumptive DNA binding canyon was blocked by heavy chain residues in the CDR2 region and appeared to lack part of the canyon wall due to the different placement of the light chain framework region.

Reactive oxygen species, such as those generated by ionizing radiation, oxidative metabolism, and inflammatory reactions, produce a variety of DNA base damages (Dizdaroglu, 1991; Halliwell & Gutteridge, 1985). When unrepaired, many of these are mutagenic and thus play an important role in a number of diseases including cancer. Purine bases can be readily oxidized at the 8 position to produce 7,8-dihydro-8-oxoadenine (8-oxoA)[1] and 7,8-dihydro-8-oxoguanine (8-oxoG). 8-OxoG, one of the most abundant lesions, is considered to be the hallmark of oxidative damage (Shigenaga *et al.,* 1989; Loft *et al.,* 1993).

Both 8-oxoG and 8-oxoA are well studied. Neither block DNA synthesis (Shibutani *et al.,* 1991; Guschlbauer *et al.,* 1991), and 8-oxoA does not appear to be a critical premutagenic lesion; however, 8-oxoG is (Wood *et al.,* 1990, 1992). NMR and X-ray crystallographic studies of oligonucleotides containing 8-oxoG have demonstrated that 8-oxoG in the *anti* form base pairs with C (Oda *et al.,* 1991; Lipscomb *et al.,* 1995), while in *syn,* it forms Hoogsteen pairs with A (Kouchakdjian *et al.,* 1991; McAuley-Hecht *et al.,* 1994), providing a structural basis for the observed G → T transversions induced by this lesion (Wood *et al.,* 1990). 8-OxoG can produce mutations by two mechanisms. When formed directly in DNA, 8-oxoG can mispair with adenine during replication and thus lead to G to T transversions (Shibutani *et al.,* 1991). Additionally, 8-oxodeoxyguanosine triphosphate (8-oxo-dGTP) can be produced from dGTP present in precursor pools and thus can be incorporated into DNA during replication (Maki & Sekiguchi, 1992; Purmal *et al.,* 1994), pairing with both A and C. Misincorporated 8-oxo-dGTP can therefore lead to T to G transversions (Cheng *et al.,* 1992). Cells have evolved defense mechanisms that counter both of these mutagenic routes. In *Escherichia coli,* formamidopyrimidine (FAPY) DNA glycosylase, the product of the *mutM* gene, removes 8-oxoG from DNA (Tchou *et al.,* 1991). A similar antimutator effect is attributable to the MutY protein which recognizes and removes A opposite 8-oxoG (Michaels *et al.,* 1992). Finally, the product of *mutT* hydrolyzes 8-oxo-dGTP to 8-oxodeoxyguanosine monophosphate (8-oxo-dGMP) to remove the oxidized base from the precursor pool (Maki & Sekiguchi, 1992).

In theory, enzyme-linked immunosorbent assays (ELISA) are ideal for measuring oxidative DNA damages because of their simplicity and sensitivity, as opposed to techniques such as HPLC/EC or GC/MS which require expensive equipment limiting their use. Conventional hybridoma technology (Park

† This work was supported by U.S. Department of Energy Grant DE-FG02-88ER60742, the Vermont Cancer Center, and the Lake Champlain Cancer Research Organization.

* To whom correspondence should be addressed.

⊗ Abstract published in *Advance ACS Abstracts,* February 1, 1996.

[1] Abbreviations: AFU, arbitrary fluorescence units; BSA, bovine serum albumin; CDR, complementarity determining region; EC, electrochemical; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; Fab, fragment antigen binding; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline; SB, super broth; Mops, 3-(*N*-morpholino)propanesulfonic acid; SCR, structurally conserved region; Tris, tris(hydroxymethyl)aminomethane; TPBS, PBS plus 0.025% Tween 20; PCR, polymerase chain reaction; Phab, phage-bound Fab; TAE, Tris−acetate; SLE, systemic lupus erythematosus; RSA, rabbit serum albumin; TEAB, triethylammonium bicarbonate; 8-oxoA, 7,8-dihydro-8-oxoadenine; 8-oxoG, 7,8-dihydro-8-oxoguanine; dUMP, deoxyuridine 5′-monophosphate.

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*et al.*, 1992) as well as polyclonal sera (Degan *et al.*, 1991) have been used to produce antibodies that detect 8-oxoguanine in urine, presumably shed as a result of cellular DNA repair. We have produced polyclonal antibodies specific for 8-oxoG and 8-oxoA in DNA (Ide *et al.*, submitted). Anti-DNA antibodies are pathologically produced in certain autoimmune diseases such as lupus erythematosus and rheumatoid arthritis and have been isolated from these patients (Stollar, 1981; Radic & Weigert, 1994) and from autoimmune mice (Theofilopoulos & Dixon, 1985). In fact, there is accumulating evidence suggesting that some of these anti-DNA autoantibodies may be specific for 8-oxoguanine (Blount *et al.*, 1989, 1990).

Autoantibodies to undamaged DNA have been well characterized. Rheumatoid arthritis autoantibodies are primarily reactive with single-stranded DNA while lupus autoantibodies can react with either single-stranded, duplex DNA, or both (Voss & Casperson, 1988). Over 250 sequences of anti-DNA antibodies are available from autoimmune mice, and the crystal structures of a number of anti-DNA antibodies have been resolved (Radic & Weigert, 1994). The anti-DNA antibodies that have been best characterized to date react with single-stranded DNA. Mutational analysis of anti-DNA antibodies has shown that changes to the basic amino acid, arginine, followed by asparagine and lysine, are particularly important for DNA binding (Radic & Weigert, 1994). Positively charged amino acids have the potential to form ionic bonds with the negatively charged phosphodiester backbone of DNA; arginine, the most versatile amino acid for DNA binding, can form up to five hydrogen bonds. Sequence analysis has suggested that there is a preferential use in the anti-DNA antibodies of L chains having long CDR1 loops with tips containing the basic residue, arginine, or asparagine (Radic & Weigert, 1994). The length of H-CDR3 is also greater for anti-DNA antibodies than for conventional antibodies (Wu *et al.*, 1993; Radic & Weigert, 1994). Site-specific mutagenesis has shown that reversion of the single arginine, R96H, in one anti-DNA antibody, completely eliminates DNA binding (Radic *et al.*, 1993).

Analysis of the crystal structures of anti-nucleic acid monoclonal antibody Fabs shows that those that bind single-stranded nucleic acids contain a pocket between the heavy and light chain variable regions which bind the nucleic acid (Herron *et al.*, 1991; Pokkuluri *et al.*, 1994) while duplex (Mol *et al.*, 1994b) and triplex (Mol *et al.*, 1994a) DNA binding Fabs have a relatively flat surface binding site. Similar conclusions were reached using models of anti-DNA Fv domains determined from sequence data (Barry *et al.*, 1994). For the single-stranded DNA binding Fab, Hed 10, electrostatic interactions predominate (Cygler *et al.*, 1987) while for BV04-01, which binds single-stranded DNA, and Jel 103, which binds single-stranded RNA, specific base-stacking and hydrogen-bonding interactions occur with amino acid residues in LCDR1 and LCDR2 (Herron *et al.*, 1991; Pokkuluri *et al.*, 1994). The phosphate interactions differ in these two Fabs with L chain interactions dominating in Jel 103 and heavy chain interactions dominating in BV04-01. Thus both sequence and structural data, combined with site-directed mutagenesis, have begun to pinpoint the important DNA binding amino acid residues in anti-DNA antibodies.

Recently, phage display repertoire cloning has emerged as a powerful technique for producing monoclonal Fabs (Sastry *et al.*, 1989; Huse *et al.*, 1989; Barbas & Lerner, 1991). These Fabs, because they are produced in *E. coli*, can provide inexpensive immunological reagents in a form that is readily amenable to basic molecular engineering techniques and structural analysis. As part of our efforts to understand the interactions of biologically important proteins including DNA repair enzymes and DNA polymerases with DNA damages, we have isolated and characterized monoclonal Fabs reactive with 8-oxoG and 8-oxoA using repertoire cloning and phage display.

## MATERIALS AND METHODS

*Bacterial Strains, Media, and Electroporation*. *E. coli* XL1-Blue recA$^-$ *rec*A1, *lac*$^-$, *end*A1, *gyr*A96, *thi*, *hsd*R17, supE44, *rel*A1, {F′ proAB, *lac*I$^q$, *lac*ZΔM15, Tn10} (Stratagene) was used for cloning and Fab production. Helper phage, VCSM13, was purchased from Stratagene. XL1-B was grown in super broth containing tetracycline (10 $\mu$g/mL). Super broth medium (SB; 30 g of tryptone, 20 g of yeast extract, 10 g of NaCl/L, pH adjusted to 7) was supplemented with 10 $\mu$g/mL tetracycline for XL1-B growth. For plasmid selection, SB was supplemented with 50 $\mu$g/mL carbenicillin and, for phage production, 50 $\mu$g/mL carbenicillin and 70 $\mu$g/mL kanamycin. Electrocompetent cells were prepared from 1 L of XLB prepared according to the Bio-Rad protocol. Electroporation of *E. coli* (Dower *et al.*, 1988) was performed using 0.2 cm cells with the Bio-Rad gene pulser apparatus (25 uF, 2.5 kV, 200 ohms).

*Buffers and Restriction Enzymes*. PBS (0.14 M NaCl, 1 mM $KH_2PO_4$, 20 mM $Na_2HPO_4$, and 3 mM KCl, pH 7.4), TPBS (PBS plus 0.5% Tween), binding buffer (0.1 M sodium bicarbonate, pH 8.6), and elution buffer (0.1 M HCl, adjusted to pH 2.2 with glycine, containing 1 mg/mL BSA) were used. Restriction enzymes were purchased from Boehringer Mannheim and the manufacturer's 10× buffers were used. The following conditions were used for plasmid digestions: 15 $\mu$g of pComb3 plasmid was digested with *Xho*I (10 units/$\mu$g) and *Spe*I (3 units/$\mu$g) for insertion of Fab heavy chains (4 h); 15 $\mu$g of pComb3 plasmid was digested with *Xba*I (10 units/$\mu$g) and *Sac*I (5 units/$\mu$g) for insertion of Fab light chains (4 h). PCR products were digested with the appropriate restriction enzymes as described above. The enzymes were then heat inactivated at 70 °C for 15 min, and the products were centrifugally concentrated and washed two to three times with 1 mL of $H_2O$ (Centricon 100) to remove the digested ends of the PCR products. The digested PCR products were ethanol precipitated prior to overnight ligation at room temperature.

*Agarose Gel Electrophoresis*. Agarose gels were run with TAE buffer, generally at 60−80 V. For plasmid preparations, 0.5% gels (Bio-Rad) were run. For PCR products, 2% gels (SeaChem 3:1) were run. DNA was recovered from agarose gels by electroelution using a Stratagene electroeluter. Cut agarose gel pieces were eluted at 105 V for 30 min−1 h in TAE buffer. DNA was then recovered from the membrane trap by repeated pipetting using the trapped volume of TAE followed by repeated pipettings using $H_2O$. The DNA solution was then centrifugally concentrated (Centricon 100) twice with the second resuspension in 2 mL of $H_2O$. The final volume remaining after the second centrifugal concentration was approximately 25 $\mu$L.

*Nucleic Acid Quantitation.* For double-stranded DNA quantitation, 2 μL of the solution to be quantified was added to 2 mL of ethidium bromide (0.5 μg/mL, pH 11.5). The DNA concentration was determined by comparing the AFU (arbitrary fluorecence units) using an Optical Technologies fluorometer with that of 2 μL of a standard solution of calf thymus DNA (previously determined by spectrophotometry). RNA was quantitated using a Milton Roy spectrophotometer (40 μg/OD 260).

*Synthesis of Modified Ribonucleosides and Deoxyribonucleoside Monophosphates and Preparation of Antigens.* To prepare antigens, nucleoside derivatives of 8-oxoA and 8-oxoG were synthesized according to the methods of Ikehara *et al.* (1968) and Ikehara and Maruyama (1975) and nucleoside derivatives of 8-oxoG by the method of Kasai and Nishimura (1984), respectively. For the synthesis of 8-oxoadenosine, 8-bromoadenosine, which is commercially available (Sigma), was refluxed in acetic anhydride in the presence of sodium acetate. The resulting *N*-acetyl-8-oxoadenosine was incubated in concentrated ammonia at room temperature to remove the acetyl group. After removing acetamide by chloroform extraction, 7-hydro-8-oxoadenosine was recrystallized from water. 7-Hydro-8-oxoguanosine was prepared by essentially the same procedure as 8-oxoA, except that 8-bromoguanosine was synthesized by bromination of guanosine. Hapten−protein conjugates of 8-oxoadenosine and 8-oxoguanosine were prepared by the periodate method of Erlanger and Beiser (1964) using BSA. The deoxyribonucleoside monophosphates of these modified bases were covalently attached to BSA and RSA through the 5′-phosphate group using the water-soluble carbodiimide conjugation procedure of Halloran and Parker (1966). Coupling efficiencies were determined spectrophotometrically; there were about five to six hapten molecules/protein carrier.

To prepare X-irradiated DNA, calf thymus DNA was X-irradiated with a Phillips X-ray generator set at 50 kVp and 2 mA and connected to a Machlett X-ray tube with a beryllium window. The dose rate, determined by Fricke dosimetry, was 100 Gy/min. Two milliliter samples were irradiated under air, with stirring, in PBS.

*Immunization Procedure.* Mice (male BALB-c) were immunized with a mixture that contained 20 μg of each conjugate in RIBI adjuvant. The mice were inoculated subcutaneously with two 100 μL injections (100 μL each armpit) and were boosted with the same mixture twice, at 3 week intervals. The mice were euthanized 3 days after the final boost. The spleens were removed and immediately placed into Trizol reagent (MRC).

*RNA Extraction and cDNA Synthesis.* RNA was extracted from cells by the method of Chomczynski and Sacchi (1987) using the Trizol reagent according to the manufacturer's instructions. Briefly, spleens were homogenized in 5 mL of Trizol reagent. The disrupted cell mix was then centrifuged to remove debris, and the supernatant was extracted with 0.2 mL of chloroform/mL of supernatant. The aqueous phase was saved and then centrifugally concentrated (Centricon 100, Amicon) twice. The final solution was in RNase-free water (Promega). First-strand cDNA was reverse transcribed using Superscript (BRL). Generally, 5 μg of total RNA and 1 μg of oligo(dT) (15-mer) primer were heated to 70 °C for 10 min. The mixture was then quick cooled on wet ice, and 10× buffer and 500 units of enzyme were added. The reaction was then incubated at 40 °C for 1−1.5 h and terminated at 70 °C for 15 min. RNase H (Boehringer Mannheim) was added (3 units), and the reaction was incubated at 37 °C for 30 min. The reaction was terminated at 70 °C for 10 min. The mix was then centrifugally concentrated, and the buffer was exchanged with water as described above.

*Mouse Primers.* To prepare the mouse library, the following primer sequences, provided by D. Burton, were used: Fab heavy chain 5′ primers, Hc1-8-agg tcc a(g/a)c t(g/t)c tcg agt c(t/a)g, IgHc9-agg tii aic tic tcg agt c(a/t)g g; Fab heavy chain 3′ class specific primers, IgG1-agg ctt act agt aca atc cct ggg cac aat, IgG2a-ggt ctg act agt ggg cac tct ggg ctc, IgG3-ggg ggt act agt ctt ggg tat tct agg ctc; Fab light chain 5′ primers, Lc1-cca gtt ccg agc tcg ttg tga ctc agg aat ct, Lc2-cca gtt ccg agc tcg tgt tga cgc agc cgc cc, Lc3-cca gtt ccg agc tcg tgc tca ccc agt ctc ca, Lc4-cca gtt ccg agc tcc aga tga ccc agt ctc ca, Lc5-cca gat gtg agc tcg tga tga ccc aga ctc ca, Lc6-cca gat gtg agc tcg tca tga ccc agt ctc ca, Lc7-cca gtt ccg agc tcg tga tga cac agt ctc ca; light chain 3′ primer, kappa-gcg ccg tct aga att aac act cat tcc tgt tga a. The following common extension primers were used for preparative PCR: VH1ext gag aga gag aga gag aga agg tcc arc tkc tcg a, VH9ext gag aga gag aga gag aga agg tii aic tic tcg a, IG1ext gag aga gag aga gag aga agg ctt act agt, IG2ext gag aga gag aga gag aga gtt ctg act agt, IG3ext gag aga gag aga gag aga ggg ggt act agt, VLext gag aga gag aga gag aga cca cwt cyt agc tcg, and CLext gag aga gag aga gag aga gcg ccg tct aga.

*PCR Conditions.* Mouse cDNA was PCR amplified with an Idaho Technologies 1605 air thermocycler. PCR from the cDNA was carried out in 10 μL reactions using the following conditions: an initial denaturation cycle of 94 °C for 10 s followed by 32 rounds of 94 °C for 0 s, 64 °C for 0 s, and 72 °C for 45 s followed by a final extension step of 72 °C for 1 min. The reaction products were subjected to a second round of PCR, using extension primers (for better subsequent restriction cutting). The extension reactions were carried out in 50 μL reactions according to the following protocol: 94 °C for 10 s followed by 32 rounds of 94 °C for 10 s, 64 °C for 10 s, and 72 °C for 45 s followed by a final extension step of 72 °C for 1 min.

*Library Construction and Screening.* Vector pComb3 was prepared using the Wizard plasmid kits (Mini or Maxi) according to the manufacturer's instructions (Promega). Libraries for phage display of Fab repetoires were largely produced as described by Barbas and Lerner (1991). Cut and gel-purified pComb3 (1.5 μg) was ligated with 300 ng of cut, gel-purified light chain PCR products overnight at room temperature (1 unit/μg DNA, BRL). The light chain library (1.5 μg) was electroporated into XL1-blue and grown overnight. Plasmid DNA was recovered the following morning (MaxiPreps-Promega). The light chain library was then cut for heavy chain insertion and gel purified. The heavy chain PCR library was then ligated overnight at room temperature with the cut vector that contained the light chain library. Then 1.5 μg of the combinatorial library was electroporated and grown overnight in the presence of helper phage to produce the combinatorial library displayed as a gene III fusion of Fab proteins on the surface of the phage (Phabs).

The panning procedure used, described by Barbas and Lerner (1991), is a modification of that first described by

Parmely and Smith (1988). Microtiter plate (Coster 3690) wells (two per panning) were coated with 50 $\mu$L of a 5 $\mu$g/mL antigen in binding buffer overnight at 4 °C. Nonspecific binding was blocked with 3% BSA in PBS for 1 h and then washed 5× with PBS/Tween. Phage (50 $\mu$L of $\sim 1 \times 10^{12}$/mL) were added to each well for binding enrichment (2 h at 37 °C). The panning mixtures were supplemented with corresponding normal deoxynucleosides (100 $\mu$M) and deoxynucleotides (500 $\mu$M). The phage solution was removed, and the wells were washed five times with TPBS. Elution buffer (50 $\mu$L) was added, and incubation at room temperature was continued for 15 min. The solution was then pipetted up and down 20 times, removed, and neutralized with 3 $\mu$L of 2 M Tris base. The eluted phage were then used to infect 2 mL of freshly grown XL1-blue (OD 600 = 1) for 15 min at room temperature. SB (10 mL), 10 $\mu$g/mL tetracycline, and 20 $\mu$g/mL carbenicillin were added. The phage were titered, and the culture was incubated for 1 h at 37 °C with shaking. SB (100 mL) containing 10 $\mu$g/mL tetracycline and 50 $\mu$g/mL carbenicillin was added and the culture incubated for an additional 1 h at 37 °C with shaking. Helper phage (VCSM13) were added to the culture ($10^{12}$ pfu) which was incubated for an additional 2 h at 37 °C with shaking. Carbenicillin was added to a final concentration of 50 $\mu$g/mL and the culture incubated overnight at 37 °C with shaking. After overnight growth of 100 mL cultures, the phage were collected, precipitated with 4% PEG 8000 and 0.5 M NaCl (final concentration), resuspended in 5 mL of PBS plus 3% BSA, and used for another round of panning. Generally, three to five rounds of panning were performed before individual clones were isolated and tested for specific Phab production by ELISA.

*Soluble Fab Production.* Soluble Fabs were produced by eliminating the heavy chain−gene III fusion by *Nhe*I/*Spe*I digestion and self-ligation of the vector. Fab producing cells were grown, induced, and harvested as described (Barbas & Lerner, 1991). Soluble Fabs were released from the periplasm by three cycles of freeze-thawing. Alternatively, Fab binding was examined by assaying aliquots of the growth medium.

*Enzyme-Linked Immunosorbent Assay (ELISA).* ELISA assays were performed using Costar high binder plates, in general according to Hubbard *et al.* (1989). For primary antigen recognition either soluble Fabs or phage-bound Fabs (Phabs) were used. When soluble Fabs were used to detect antigens, the secondary antibody was specific for the primary Fab. Mouse Fabs were detected using goat anti-mouse IgG (Fab specific) antibodies conjugated to horseradish peroxidase (Sigma). When Phabs were assayed, the secondary enzyme-linked antibody was specific for the phage coat protein (Pharmacia, Recombinant Phage Antibody Detection Module). The competitive ELISA was performed as described by Friguet *et al.* (1985).

*HPLC Conditions.* Fifteen microliters of DNA was digested by nuclease P1 (Pharmacia), treated with shrimp alkaline phosphatase (USB), and applied on an Adsorbosphere C18 (5 $\mu$m, 4.6 × 250 mm) column (Alltech). The nucleosides were separated by isocratic elution in 45 mM sodium acetate and 10 mM sodium citrate, pH 5.1, containing 10% methanol, and detected using an electrochemical detector (Bioanalytical System) at an input potential of 0.85 V. Authentic 8-oxo-dA and 8-oxo-dG (Wagner *et al*., 1992) were used as standards for peak identification and quantification.

*DNA Sequencing and Analysis.* DNA sequence analysis was performed on an ABI automated DNA sequencer. Dideoxy sequencing reactions were performed according to the manufacturer's instructions using their fluorescent-labeled DNA sequencing kit. The reaction products were purified using Centrisep columns according to the manufacturer's instructions (Princeton Separations). The DNA sequencing primers used (sequences provided by Dennis Burton) were SeqT3 ata aac cct cac taa ag and Kef gaa ttc taa act agc tag tcg.

*Chain Shuffling*. Heavy and light chains of clones were individually shuffled by cutting vectors with the appropriate restriction enzyme pairs as described above for producing the library. However, the heavy chain of g62 had an *Xba*I site. Since *Xba*I is used to insert light chains, multiple cloning steps were required to make the g62H/d1290L hybrid. The light chain of d1290 was first moved into g37 creating a g37H/d1290L and then the heavy chain from g62 was moved into this construct to produce g62H/d1290L.

*Homology Modeling.* The crystallographic coordinates for Jel 103 (entry 1MRC; Pokkuluri *et al.*, 1994), FAB R19.9 (entry 1FAI; Lascombe *et al*., 1992), and FAB 36-71 (entry 6FAB; Strong *et al*., 1991), all of which have very high sequence homology to clone g37, were obtained from the Brookhaven Protein Data Bank (Bernstein *et al.*, 1977; Abola *et al*., 1987). The computer programs Homology and Insight II (Biosym Technologies, San Diego, CA) were used to model the three-dimensional structure of g37 and the molecular mechanics package Discover (Biosym) with the cvff force field was used for energy minimization and molecular dynamics calculations. The sequences were aligned using the numbering system of Kabat and Wu (1991), and structurally conserved regions (SCR) of the template proteins were identified (see Table 1) using Homology.

After the SCRs were determined, the coordinates of these regions were assigned to g37 from the crystallographic coordinates of 1MRC. Coordinates for the loops connecting these regions were obtained from the coordinates of the three proteins used as templates. The resulting structure was subjected to short rounds of energy minimizations of the individual loop sections, using the steepest descent method and a nonbonded cutoff of 8 Å, to a maximum gradient of 5 kcal $mol^{-1}$ $Å^{-1}$. The loops were then optimized with five cycles of combined molecular dynamics and minimization. Each cycle of dynamics was performed using the leap-frog algorithm, equilibrated for 1.0 ps. Subsequent dynamics runs were continued for 1 ps at 300 K, using a 1 fs time step. Each round of dynamics was followed by energy minimization as above. The entire structure was subjected to a final round of minimization to remove remaining structural conflicts.

## RESULTS

*Characterization of Purine-Specific Phabs*. Figure 1 demonstrates the binding specificities of phage bound Fabs (Phabs) produced by four clones that were selected for by panning against 8-oxoA−BSA and 8-oxo-dAMP−RSA (a62 and a65) or against 8-oxoG−BSA and 8-oxo-dGMP−RSA (g48 and g37). Phabs from these clones demonstrated a spectrum of antigen binding specificities that may be divided

Table 1: Sequence Alignment and Structurally Conserved Regions[a]

```
     L1                                                                  L50
G37: ELVMTQSPSSLSASLGERVSLTCRASQEI-----SGYLSWLQQKPDGTIKRLIYA A
MRC: DVVMTQTPLSLPVSLGDQASISCASSQSLVHSNGNTYLHWYLQKPGQSPKLLIYK V
FAB: DIQMTQIPSSLSASLGDRVSISCRASQDI-----NNFLNWYQQKPDGTIKLLIYF T
FAI: DIQMTQTTSSLSASLGDRVTISCRASQDI-----SNYLNWYQQKPDGTVKLLIYY T

                                                          L100
G37: STLDSGVPKRFSGSRSGSDYSLTISSLESEDFADYYCLQYASYPPTFGG GTKLEIK
MRC: SNRFSGVPDRFSGSGSGTDFTLKISAVEAEDLGVYFCSQSTHVPRTFGG GTKLEIK
FAB: SRSQSGVPSRFSGSGSGTDYSLTISNLEQEDIATYFCQQGNALPRTFGG GTKLEIK
FAI: SRLHSGVPSRFSGSGSGTDYSLTISNLEHEDIATYFCQQGSTLPRTFGG GTKLEIK

                                                    L150
G37: RADAAPTVSIFPPSSEQLTSGGASVVCFLNNFYPKDINVKWKI DGKERQNGVLNSW
MRC: RADAAPTVSIFPPSSEQLTSGGASVVCFLNNFYPKDIAVKWKI DGKERQNGVLNSW
FAB: RADAAPTVSIFPPSSEQLTSGGASVVCFLNNFYPKDINVKWKI DGSERQNGVLNSW
FAI: RADAAPTVSIFPPSSEQLTSGGASVVCFLNNFYPKDINVKWKI DGSERQNGVLNSW

                                                L200
G37: TDQDSKDSTYSMSSTLTLTKDEYERHNSYTCEATHKT STSPIVKSFNRNEC
MRC: TDQNSKDSTYSMSSTLTLTKDEYERHNSYTCEATHKT STSPIVKSFNR
FAB: TDQDSKDSTYSMSSTLTLTKDEYERHNSYTCEATHKT STSPIVKSFNRNEC
FAI: TDQDSKDSTYSMSSTLTLTKDEYERHNSYTCEATHKT STSPIVKSFNRNEC

     H1                                                             H50
G37: QVKLLESGPELVRPGASVKMSCKASGYTFTNYWMHWVKQRPGQGLEWIGE I--NPS
MRC: QVQLQQSGAELVKPGASVKLSCKASGYTFTSYWMQWVKQRPGQGLEWIGE I--DPS
FAB: EVQLQQSGVELVRAGSSVKMSCKASGYTFTSNGINWVKQRPGQGLEWIGY N--NPG
FAI: QVQLQQSGAELVRAGSSVKMSCKASGYTFTSYGVNWVKQRPGQGLEWIGY I--NPG

                                                          H100
G37: NGRTNYNEKFKSKATLTVDKSSSTAYMQLSSLTSEDSAVYYCA---------RNWDG
MRC: ASYTNYNQKFKGKATLTVDTSSSTAYMQLSSLTSEDSAVYYCA--------NLRGYF
FAB: NGYIAYNEKFKGKTTLTVDKSSSTAYMQLRSLTSEDSAVYFCA---RSEYYGGSYKF
FAI: KGYLSYNEKFKGKTTLTVDRSSSTAYMQLRSLTSEDAAVYFCARSFYGGSDLAVYYF

                                                    H150
G37: VYWGQGTLVTVSAAKTTPPSVYPLAPGCGDTTGSSVTLGCLVKGYFPE SVTVTWNS
MRC: DYWGQGTTLTVSSAKTTPPSVYPLAP---|TTGSSVTLGCLVKGYFPE SVTVTWNS
FAB: DYWGQGTTLTVSSAKTTPPSVYPLAPGSAAQTNSMVTLGCLVKGYFPE PVTVTWNS
FAI: DSWGQGTTLTVSSAKTTPPSVYPLAPGCGDTTGSSVTLGCLVKGYFPE SVTVTWNS

                                          H200
G37: GSLSSSVHTFPALLQSGLYTMSSSVTVPSSTWP SQTVTCSVAHPASSTTVDKKLEP
MRC: GSLSSSVHTFPALLQSGLYTMSSSVTVPSSTWP SQTVTCSVAHPASSTTVDKKLEP
FAB: GSLSSGVHTFPAVLQSDLYTLSSSVTVPSSPRP SETVTCNVAHPASSTKVDKKIVPRD
FAI: GSLSSSVHTFPALLQSALYTMSSSVTVPSSTWP SQTVTCSVAHPASSTTVDKKL
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[a] Amino acid sequences of clone g37 and Jel 103 (titled MRC), FAB 36−71 (titled FAB), and FAB R19.9 (titled FAI) which were used as templates for homology modeling. The areas of these three molecules defined as structurally conserved across all three structures and used as a template for the three-dimensional model of g37 are shaded.

into two catagories. Phabs from clones a62, a65, and g48 bound to 8-oxopurine, purine, and BSA epitopes, although the 8-oxo moiety and guanine were preferred over adenine. Phabs from clone g37 exhibited a clear preference for binding to 8-oxoG epitopes. The preference of Phabs from clone g37 was unique compared to the other clones in that the binding specificity for the 8-oxoG epitope was seen when the hapten was presented in the context of either BSA or RSA. Phabs from clones a62, a65, and g48 recognized the 8-oxoG residue only when presented by BSA and thus must contain paratopes specific for BSA. Phabs from clone a62 also recognized urea epitopes and had significant cross-reactivity with BSA.

We also examined the ability of these purine-specific Phabs to detect 8-oxopurines in DNA. None of the clones bound to X-irradiated calf thymus DNA (Figure 1). Both 8-oxoA and 8-oxoG were present in the X-irradiated DNA used (data not shown), as determined by electrochemical detection of HPLC-separated deoxyribonucleosides (Floyd *et al.*, 1986). Additionally, 8-oxoA and 8-oxoG can be immunochemically detected in this same X-irradiated DNA sample by using polyclonal sera (Ide *et al.*, submitted) in an ELISA. Fabs were also isolated from clone d1290 that bound to untreated DNA but not to any modified base, whether in DNA or conjugated to a protein (Bespalov *et al.*, submitted).

The binding characteristics of clones g37 and a65 were also determined by competition experiments. 8-Oxoguanosine conjugated to RSA and 8-oxodeoxyguanosine monophosphate conjugated to BSA (8-oxo-dGMP−BSA) strongly inhibited g37 binding to 8-oxoguanosine−BSA bound to plates (inhibition constant, $10^{-9}$ M) as shown in Figure 2. Importantly, dGMP−RSA did not inhibit the binding of g37 Phabs. Similarly, hapten inhibition by 8-oxoG-containing

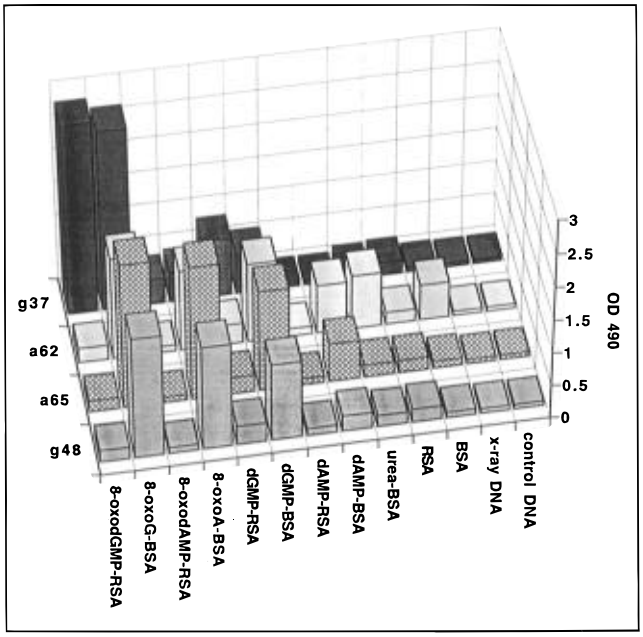



FIGURE 1: Binding specificities of mouse Phabs from clones a62, a65, g37, and g48 as demonstrated by direct-binding ELISA. The antigens bound to the plate (0.1 $\mu$g/well) are indicated in the figure; the Phabs used (50 $\mu$L of PEG resuspension) are indicated in the figure, and the reactions were performed as described in Materials and Methods.

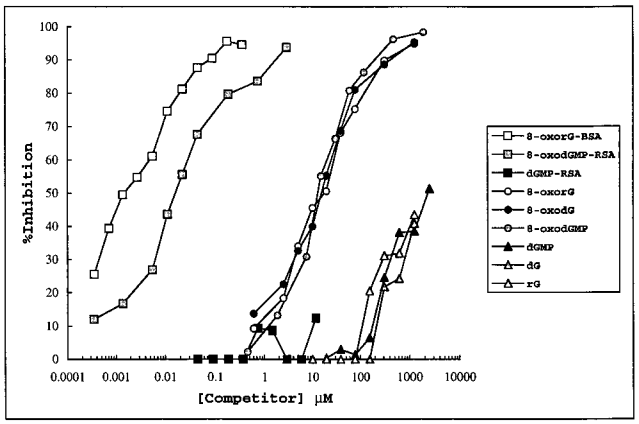



FIGURE 2: Specificity of Phab g37 using competitive ELISA. 8-Oxoguanosine−RSA (250 ng/well) was immobilized on 96-well microtiter plates, and competition assays were performed as described in Materials and Methods. Competitor symbols are indicated in the figure.

nucleosides and nucleotides was observed, while normal G nucleosides and nucleotides were less efficient inhibitors by 2 orders of magnitude. Since the conjugates were almost 4 orders of magnitude more effective at inhibiting binding of g37 Phabs than the 8-oxopurines alone, common epitopes in BSA and RSA must stabilize Phab binding. Our working model is that the Phabs described in this paper have a binding site that specifically interacts with the hapten and a stabilizing site(s) that interacts with protein epitopes. Thus, hapten−protein conjugates are much better competitors of Phab binding than are the damaged purines alone.

Figure 3 shows that clone a65 also appears to have specificity for the 8-oxopurines; however, the affinity of a65 for 8-oxoguanosine−BSA is lower than that of clone g37. Both 8-oxoguanosine and 8-oxoadenosine conjugated to BSA inhibited clone a65, but 8-oxo-dGMP−BSA was a poor inhibitor. 8-Oxopurine nucleoside and nucleotides and

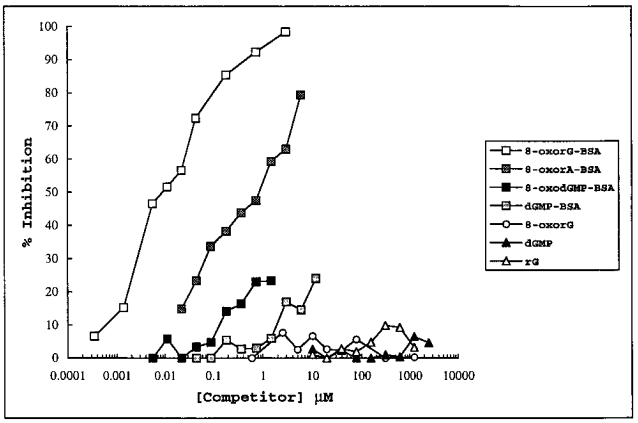



FIGURE 3: Specificity of Phab a65 using competitive ELISA. 8-Oxoguanosine−BSA (250 ng/well) was immobilized on 96-well microtiter plates, and competition assays were performed as described in Materials and Methods. Competitor symbols are indicated in the figure.

Table 2: Heavy Chain Amino Acid Sequences[a]

| | CDR1 | | | |
|---|---|---|---|---|
| | 10 | 20 | 30 | 40 |
| a62 | QVKLLESGAE | LVRPGVSVKI | SCKGSGCTFT | --DYNIH--W VKQSHAKSLE |
| a65 | QVKLLESGAE | LVRPGVSVKI | SCKGSGYTFT | --DYNMH--W VKQSHAKSLE |
| g37 | QVKLLESGPE | LVRPGASVKM | SCKASGYTFT | --NYWMH--W VKQRPGQGLE |
| g48 | QVKLLESGAE | LVRPGVSVKI | SCKGSGYTFT | --DYNMH--W VKQSHAKSLE |
| d1290 | QVKLLESGAE | LARPGASVKM | SCKASGYTFT | --SYTMH--W VKQRPGQGLE |
| Guo-1 | QVQLQQSGAE | LTKPGASVKM | SCKASGYTFT | --NYWMN--W VKQRPGQGLE |
| BV04-01 | EVQPVETGGG | LVQPKGSLKL | SCAASGFSFN | --TNAMN--W VRQAPGKGLE |
| Jel103 | QVQLQQSGAE | LVKPGASVKL | SCKASGYTFT | --SYWMQ--W VKQRPGQGLE |

| | CDR2 | | | | |
|---|---|---|---|---|---|
| | 49 | 52a bc | 60 | 70 | 80 82abc |
| a62 | WIG---VIST | --YYGDASYN | QKFKG---KA | TMTVDKSSST | AYMELARLTS |
| a65 | WIG---VIHT | --YYGDATYN | QKFKG---KA | TMTVDKSSST | AYLELGRLTS |
| g37 | WIG---EINP | --SNGRTNYN | EKFKS---KA | TLTVDKSSST | AYMQLSSLTS |
| g48 | WIG---VIST | --YYGDATYN | QKFKG---KA | TMTVDKSSST | AYLELARLAS |
| d1290 | WIG---YINP | --SSGYTNYN | QKFKD---KA | TLTADKSSST | AYMQLSSLTS |
| Guo-1 | YIG---AINP | --YNAYTEYN | QRFKD---KA | ILTADKSSST | AYMQLNNLTS |
| BV04-01 | WVA---RIRS | KSNNYATYYA | DSVKD---RF | TISRDDSQNM | LYLQMNNLKT |
| Jel103 | WIG---EIDP | --SDSYTNYN | QKFKG---KA | TLTVDTSSST | AYMQLSSLTS |

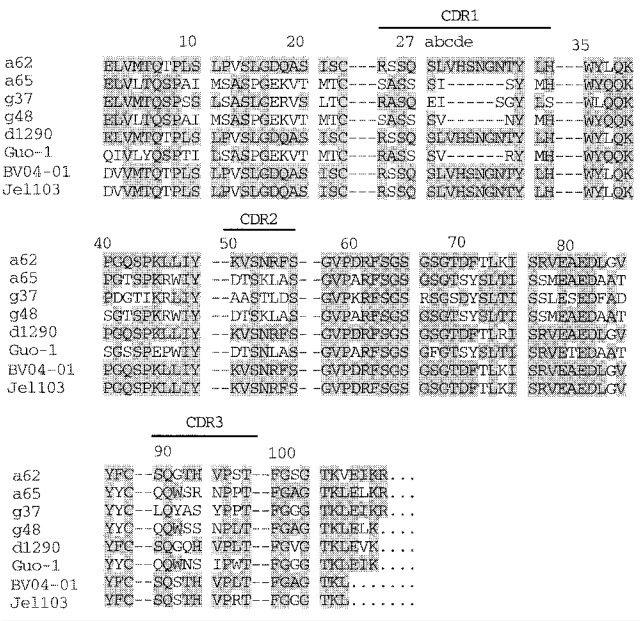
| | CDR3 | | | |
|---|---|---|---|---|
| | 90 | 95 100ab cd | | 110 |
| a62 | EDSAIYYCAR | --NGGRGWHF | --DV----WGAGTTVTVS | S.. |
| a65 | EDSAIYYCAR | --NYLYAM-- | --DY----WGQGTSVTVS | S.. |
| g37 | EDSAVYYCAR | --NWDG---- | --VY----WGQGTLVTVS | S.. |
| g48 | EDSAIYYCAR | --NYLYAM-- | --DY----WGQGTSVTVS | S.. |
| d1290 | EDSAVYYCAR | --RYF----- | --DV----WGAGTTVTVS | S.. |
| Guo-1 | EDSAVYYCSR | --GIYNGYYV | YFDY----WGQGTTLTVS | S. |
| BV04-01 | EDTAMYYCVR | --DQTGTAWF | --AY----WGQGTLVTVS | A. |
| Jel103 | EDSAVYYCAN | --LRGYF--- | --DY----WGQGTTLTVS | S. |

[a] Amino acid sequences of mouse heavy chain Fv regions of clones a62, a65, g37, and g48 that have affinity for 8-oxoA and 8-oxoG and of clone d1290 that binds to undamaged DNA. The sequences of Guo-1 (Cottet *et al.*, 1994) and BV04-01 (Herron *et al.*, 1991), DNA binding monoclonal antibodies, and Jel 103 (Pokkuluri *et al.*, 1994), an RNA binding monoclonal antibody, are provided for comparison.

normal nucleosides and nucleotide haptens did not inhibit a65 Phab binding. These results are in contrast to Phab g37, which was inhibited from binding 8-oxoG−BSA by 8-oxo-dGMP-RSA and 8-oxopurine haptens. Since a65 Phabs did not bind RSA conjugates in the direct assay, they were not tested as competitors. Clones a62 and g48 behaved similarly to a65 in the competitive assay, and these data are not shown.

*Amino Acid Sequences of Fabs.* Table 2 shows the alignment of the heavy chain and Table 3 the alignment of the light chain amino acid sequences of damage-specific Fabs isolated from clones g37, a62, a65, and g48, as well as the sequences from clone d1290 which binds untreated DNA (Bespalov *et al.*, submitted). Also included are the heavy and light chain sequences, acquired from GenBank (Altschul *et al.*, 1990), of Guo-1 (Cottet *et al.*, 1994), which has been

Table 3: Light Chain Amino Acid Sequences[a]




[a] Amino acid sequences of mouse light chain Fv regions of the same clones described in Table 2.

shown to bind single-stranded DNA and dCMP−BSA, Jel 103 (Pokkuluri *et al.*, 1994), which binds to single-stranded RNA and poly(rI), and BV04-01 (Herron *et al.*, 1991), which binds to single-stranded DNA and poly(dT). The latter two Fabs have highly resolved crystallographic structures in the Protein Data Bank (Bernstein *et al.*, 1977; Abola *et al.*, 1987).

Phabs g48, a65, and a62 are similar to each other, binding with similar efficiencies to purines conjugated to BSA, but not RSA, and none of them bound to DNA. All three have almost identical heavy chain CDR1, CDR2, and framework sequences, but their CDR3 regions differ. Thus, the same germ line heavy chain gene segment (J558 family) appears to have been involved in VDJ recombination. Phabs g48 and a65 have almost identical heavy and light chain sequences; thus they must be encoded by the same rearranged genes which have accumulated somatic mutations and/or PCR generated errors. Evidently, the amino acid differences between Phabs g48 and a65 at positions H-52 (H-CDR2, Ser/ His), L-27b (L-CDR1, Ile/Val), L-31 (L-CDR1, Ser/ Asn), L-93 (L-CDR3, Arg/Ser), and L-96 (L-CDR3 Pro/Leu) do not significantly alter either the specificity or the affinity of these Phabs.

The amino acid sequence of clone g37 compared with DNA binding antibodies revealed significant similarities and potentially important differences in the primary sequence of both the heavy and light chains. In a sequence comparison among anti-double-stranded and anti-single-stranded DNA antibodies, Radic and Weigert (1994) deduced that particular amino acid residues found in heavy chains are involved in DNA binding. Support for this comparison was confirmed by mutational analysis (Radic *et al.*, 1993) using an anti-double-stranded antibody which also reacted with single-stranded DNA. Radic *et al.* showed that the introduction of arginine into position H31 of CDR1, positions H56 or H64 of CDR2, or position H76 of framework region 3 increased antibody binding to DNA. In contrast, the loss of arginine from positions H53 or H96 resulted in decreased DNA binding. We have also compared our Fab sequences to anti-DNA antibodies isolated by others to determine if any key positions might be identified. Clone g37 has many heavy chain similarities to DNA binders such as Guo-1; although neither clone has arginine at position H31, they both have polar asparagine which can also form hydrogen bonds. Position H53 of clone g37 is serine, as it is in clone d1290, in Jel 103 (Table 2), and in other DNA binders (Radic & Weigert, 1994), except for Guo-1 which has tyrosine. Arginine is found in clone g37 at position H56 but not in the DNA binders, d1290 or Guo-1, or the RNA binder, Jel 103, all of which have tyrosine. Position H64 is a positively charged lysine residue and is found in all the clones shown in Table 3. Similarly, the framework region of all the clones has a serine at residue H76. In contrast, of the clones shown in Table 3, only Jel 103 has arginine at H96. The size of the heavy chain CDR1 and CDR2 regions do not differ significantly among the clones listed in Table 3. The CDR3 regions are similar in size among the purine binders isolated here, and they are significantly smaller than the CDR3 regions of the DNA binders Guo-1 and BV04-01 but not d1290 and Jel 103. Although it has been suggested by others (Wu *et al.*, 1993; Radic & Weiger, 1994) that DNA binders appear to have longer than average HCDR3 regions, Table 2 shows several exceptions to this generalization. Accordingly, the size of the CDR3 region may not be responsible for the lack of DNA binding that characterizes these clones.

There are many significant similarities among the light chain sequences shown in Table 3. The light chain of clone a62 is almost identical to clones d1290, Jel 103, and BV04-01. The light chain sequences of a65, g48, and Guo-1 are also very similar. Clone g37 has the most unique light chain and also exhibits the greatest degree of specificity for 8-oxoG. The light chain CDR2 and CDR3 regions of all the antibodies listed in Table 3 are identical in size, but the light chain CDR1 regions of a65, g37, g48, and Guo-1 are significantly smaller than the rest. The light chain CDR1 regions are on average longer than those of non DNA binding antibodies (Radic & Weigert, 1994). The longer length of d1290 L-CDR1 is in keeping with this observation.

The binding characteristics of Phabs a62, a65, g37, and g48 show that they have appropriate binding sites for modified nucleotides (sides). The primary sequence similarity of these clones to Fabs known to bind DNA suggests that the 8-oxopurine-specific Fabs might also have protein domains that could be involved in polynucleotide binding. However, these domains may not be present in the correct combinations to allow specific binding to DNA containing a particular base damage.

*Chain Shuffling.* The presence of amino acids characteristic of DNA binders, or their conservative substitutions at critical positions, suggests that amino acids at other positions may otherwise impair possible DNA binding. Amino acids that interfere with DNA binding may be localized on the heavy or the light chain. As stated earlier, our data are compatable with a model in which the BSA/RSA binding site(s) may be distinct from the 8-oxoG binding site, and the characteristics of the protein binding site(s) may conflict with DNA binding. In an effort to correlate binding phenotypes with the genotypes of a particular heavy or light chain, heavy and light chains from various clones were shuffled (Kang *et al.*, 1991). The heavy chain from the DNA binding clone d1290 paired with the light chain from clone a62 that bound 8-oxoA−BSA and 8-oxoG−BSA. The new

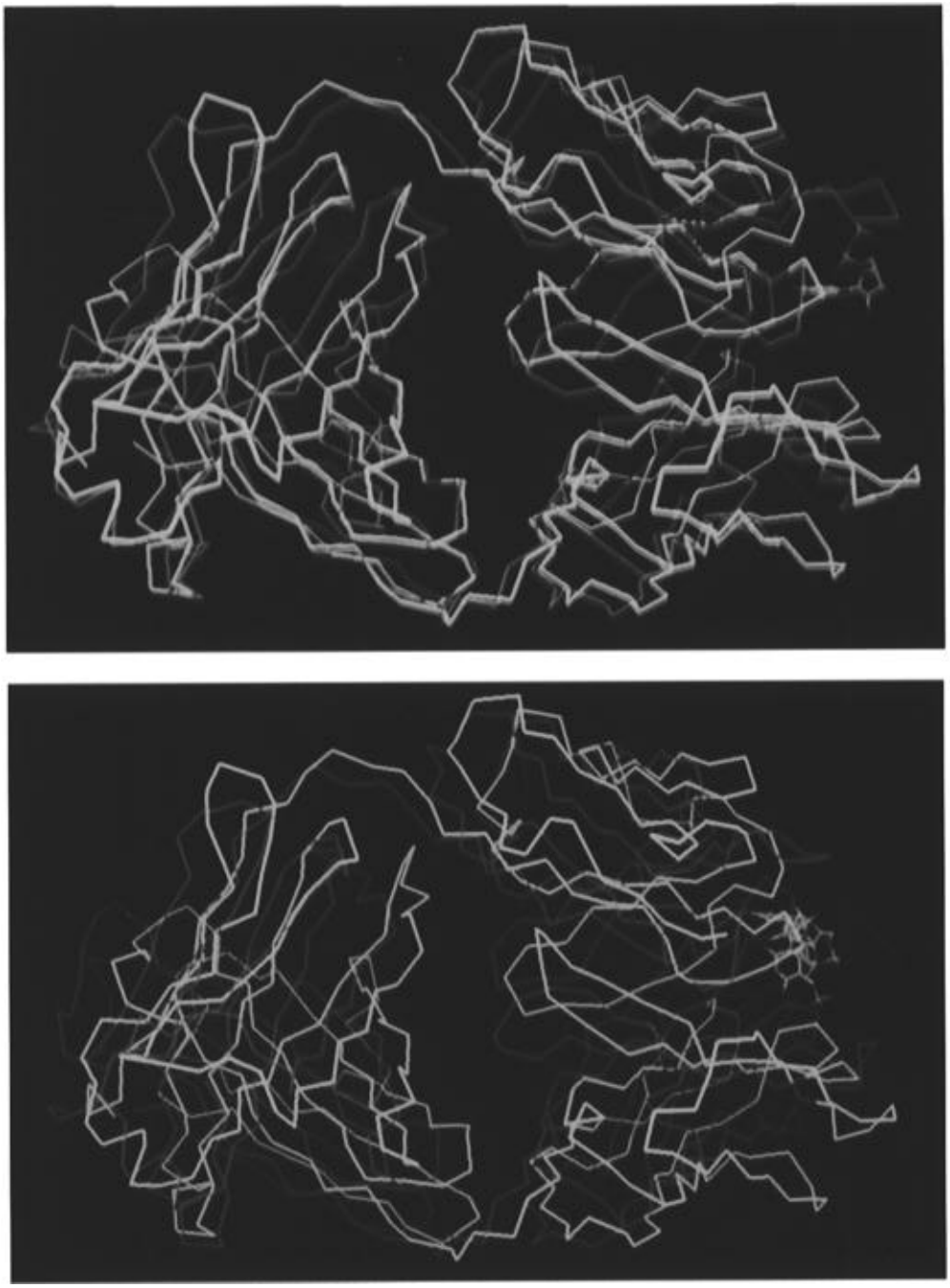


FIGURE 4: (a, top) Modeled α-carbon trace of g37, in yellow, superimposed on the α-carbon trace of Jel 103, in blue, with GDP bound to Jel 103. The α-carbon positions of tyrosine 31 in both proteins and amino acid 91, a tyrosine in g37, and a serine in Jel 103 are shown in red. (b, bottom) Superimposed α-carbon traces of g37, in yellow, and BV04-01, in red, including a trinucleotide of deoxythymidylic acid, $d(pT)_3$.

hybrid pair d1290H/g62L bound to DNA but not to 8-oxoG−BSA or other conjugates (data not shown). Hybrids d1290H/g37L and d1290H/a65L bound neither DNA nor base damage conjugates (data not shown). Either specificity for both these ligands was lost or the hybrid molecules did not successfully form. The reciprocal hybrid, a62H/d1290L, did not bind to DNA or 8-oxoG−BSA or other conjugates (data not shown). Similar results were obtained with the reciprocal hybrids g37H/d1290L and a65H/d1290L (data not shown).

*Homology Modeling of Clone g37.* In order to gain insight into the antigen binding sites of clone g37, which is specific for 8-oxoG, we employed homology modeling and computational analysis to deduce its structure. This approach has been successfully used by Barry *et al.* (1994) for six DNA binding antibodies. Chothia *et al.* (1989) have defined cannonical structures, based on primary amino acid sequence, for all the complementarity determining regions except for HCDR3. However, only HCDR1 of clone g37 has a sequence which agrees with these cannonical structures. Accordingly, there was little predetermined structural information about these normally well-characterized CDR regions necessitating the development of an homology model.

Figures 4 and 5 show a three dimensional model of g37 produced by homology modeling. The overall structure is similar to most antibodies as anticipated. However, the placement of several loops and the size and placement of LCDR1 are markedly different from those found in DNA binding Fabs. Figure 4a shows the superposition of the

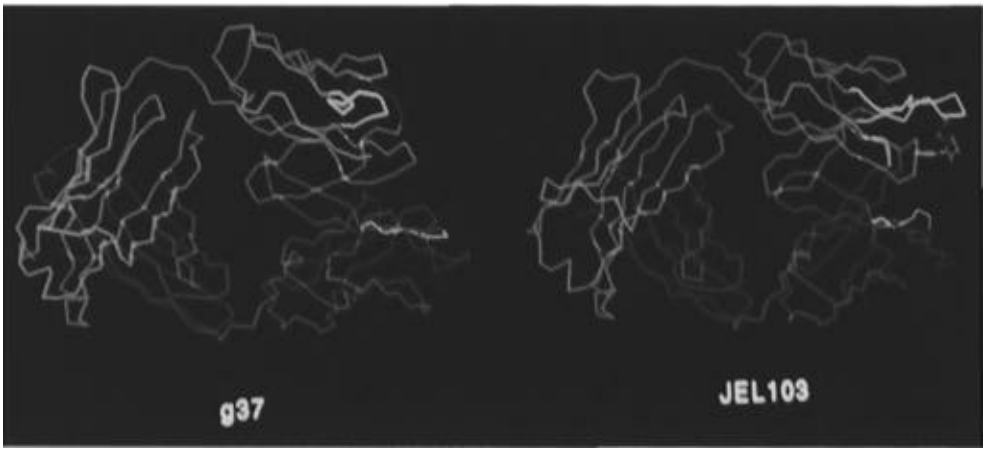



FIGURE 5: α-Carbon traces for g37 and Jel 103 shown in the same orientation with each light chain in green and the heavy chains in blue. The CDR regions of each chain are highlighted with CDR1 of each chain in yellow, CDR2 in red, and CDR3 in purple.

α-carbon traces of Jel 103 Fab on the homology-modeled structure of g37. Jel 103, the RNA binder which specifically binds to single-stranded poly(rI) (Pokkuluri *et al.*, 1994), has overall sequence similarity to g37 and was one of three structures used to model g37 (Table 1). The others, FAB R19.9 (Lascombe *et al.*, 1992) and FAB 36-71 (Strong *et al.*, 1991), which also share sequence homologies to g37 (Table 1), are specific for *p*-azobenzenearsonate and *p*-azophenylarsonate, respectively. The backbone of structures Jel 103 and g37 significantly overlaps except in the antigen binding region of Jel 103, an area identified by soaking mononucleotides into the crystal. Guanosine 5′-diphosphate, which binds to Jel 103, is shown in Figure 4a to delineate this binding site. Overlap of the two structures is expected since Jel 103 was one of the structures used to model g37. Figure 4b shows the superposition of the α-carbon backbones of BV04-01 and g37. BV04-01, which binds to single-stranded DNA (Herron *et al.*, 1991), was cocrystallized with a trinucleotide of deoxythymidylic acid (also shown in Figure 4b) and was not used to homology model g37. BV04-01 has the same light chain variable sequences as Jel 103 except for position 96L and differs from d1290 at positions 91L and 92L. However, there are significant differences in the light chain variable regions between g37 and BV04-01 and the other nucleic acid binding Fabs, Jel 103 and d1290. It is apparent from Figure 5 that the differences in sequence between clone g37 and Jel 103 result in different placement of some complementarity determining regions. Not only is LCDR1 in g37 much shorter but it does not extend over the binding area. Also, LCDR2 is moved closer to the binding area, HCDR2 is closer to the light chain, and LCDR3 and HCDR1 are closer to each other in g37.

The interactions between Jel 103 with guanosine mononucleotide and BV04-01 with the center thymine base of the trinucleotide are similar (Pokkuluri *et al.*, 1994); in both cases the base stacks with Tyr32L and forms nucleotide-specific hydrogen bonds with Ser 91L. In BV04-01, the base also stacks with tryptophan at 100aH (Herron *et al.*, 1991). In Jel 103, Arg 96H hydrogen bonds to the sugar. Clone g37 also has tyrosine at 32L but has tyrosine not serine at 91L. Tyr 32L in Jel 103 and g37 is not in identical positions, and the α-carbon position of each is depicted in red in Figure 4a. The backbones of Ser 91L in Jel 103 and Tyr 91L in g37 overlap in the superimposed structures, but the different amino acids result in different environments at these positions. There is no amino acid at position 100aH in g37. It should be pointed out that g37 can distinguish between G and 8-oxoG, binding preferentially to 8-oxoG. The contacts in Jel 103 for the α-phosphate group also include Tyr 32L which is present in g37. Asn 30L, Lys 50L, and Lys 169L in Jel 103 contact both phosphates (Pokkuluri *et al.*, 1994). Clone g37 has polar serine at 30L and lysine at 169L but instead of lysine at 50L has hydrophobic alanine and only Ala 50L is near the region on g37 that superimposes with the binding site of Jel 103. The phosphate group of the middle thymine interacts with Arg 52H of BV04-01 (Herron *et al.*, 1991) which is replaced by asparagine in g37.

Because of the similarities in sequence and structure between g37 and the crystallized Jel 103 and BV04-01, it is a reasonable first approximation to suggest that the 8-oxoG binding site in g37 is similarly positioned to the G and the T binding sites in Jel 103 and BV04-01, respectively, especially since all three structures contain a similarly positioned canyon, the putative binding site for single-stranded polynucleotides (Pokkuluri *et al.*, 1994; Barry *et al.*, 1994). The width of the canyon in Jel 103 appears to be just under 6 Å at L100 → H44 (5.7 Å) and at L95 → H60 (5.65 Å) enough to accommodate a single-stranded polynucleotide. Although the width of the interior of the g37 canyon at L100 → H44 is only slightly larger (7.1 Å), the mouth of the g37 canyon is much larger (L95 → H60, 15.3 Å) perhaps too large to form stabilizing electrostatic interactions with a polynucleotide. This canyon also appears to be partially blocked by heavy chain residues in the CDR2 heavy chain region. Electrostatic interactions which might stabilize g37 interactions with BSA could inhibit its binding to DNA.

## DISCUSSION

We have used phage display/repertoire cloning technology to produce monoclonal Fabs that bind to different oxidative DNA base damages. In this paper, we have focused on 8-oxoguanine due to its importance as a biological marker for oxidative DNA damages (Shigenaga *et al.*, 1989; Loft *et al.*, 1993; Lunec *et al.*, 1994). Four clones were characterized; three exhibited specificity for 8-oxopurine, purine, and BSA epitopes while one clone, g37, was highly specific for 8-oxoG.

Sequence analysis of these clones showed significant similarities and differences to DNA binders in the data base

and to a DNA binder, d1290, isolated by us. The most striking similarity was found in the light chain of a62, a purine−BSA binder, which was almost identical to the light chains of the nucleic acid binders, d1290, Jel 103, and BV04-01. The use of almost identical V$\kappa$1 sequences in these four monoclonal antibodies must reflect strong selective pressure for these sequences since their random occurrence would be a very low probability event. Both a62 and d1290 were isolated by repertoire cloning whereas BV04-01 (Ballard *et al.*, 1984) and Jel 103 (Pokkuluri *et al.*, 1994) were isolated by conventional monoclonal technology; therefore, selection seems a more likely explanation for the use of these sequences rather than a PCR or cloning artifact. Since a62 was selected by panning against an 8-oxoG BSA conjugate, d1290 and BV04-01 were selected as DNA binders, and Jel 103 was selected as an RNA binder, the common denominator selected for might be the ability to bind bases. In agreement with this suggestion, the crystal structures of Jel 103 and BV04-01 indicate that their light chains are involved with specific and similar binding to pyrimidine and/or purine bases. The promiscuous pairing of these light chains with dissimilar heavy chains further suggests unique selective pressures on these light chains.

Previous sequence analysis of DNA binding monoclonal antibodies (Marion *et al.*, 1992) has indicated that a number of germline sequences from both VH and VL genes code for anti-DNA antibodies. Yet, preferential use of VH and VL genes and VH−VL pairs has been reported. The preferential use of $V_H$588−V$\kappa$1 pairs seen in the Fabs reported in this paper has been observed in the past (Kofler *et al.*, 1988; Smith and Voss, 1990). The use of particular VH−VL pairs has been taken to indicate that there is receptor-mediated clonal selection of B cells to make anti-DNA antibodies (Marion *et al.*, 1992). Since the Fabs that we generated were elicited with hapten−conjugates, and clones specific for just the carrier would have been procedurally eliminated, the hapten moieties elicited an immune response from the same families reported for spontaneously produced anti-DNA antibodies.

The structural characteristics of these Phabs were further characterized by chain shuffling. The heavy and light chains of the DNA binder, d1290, were shuffled with the heavy and light chains of the 8-oxopurine binders, g37, a62, and a65. Although a62 did not bind to DNA, d1290H/a62L did. Since Fabs produced from a62 light chains combined with d1290 heavy chains bound to undamaged DNA, the DNA binding capacity of d1290 may reside primarily in the heavy chain. Structural differences in the light chains of g37 and a65 compared with light chains of d1290 or a62 may interfere with the DNA binding capacity of d1290 heavy chains. A comparison of the light chain sequences from a62 and d1290 shows that these light chains are very similar in primary amino acid sequence and thus potentially in tertiary structure. Apparently, the conformation of the a62 light chain is so similar to that of d1290 that, by combining the light chain of a62 with the DNA binding heavy chain of d1290, the resulting hybrid binds to DNA. A similar observation was made by Calcutt *et al.* (1993), who chain shuffled between two DNA binders and with heavy chains from DNA binders and light chains from their library and found that only a few light chains function in DNA binding. The similarity of light chain sequences from clone d1290 and a62 precludes, but does not exclude, the assignment of DNA binding to the heavy chain. The reciprocal hybrid, a62H/d1290L, did not bind the 8-oxoG−BSA conjugate. Since the light chain of d1290 differs from the light chain of a62 by only three amino acids (Table 4), one or more of these must be involved in binding to the hapten conjugate or in preventing the heavy and light chains from pairing. It is possible that two of these residues, Thr L92 and Ser L96, in the LCDR3 might be in the putative nucleotide binding site vicinity. Since binding to conjugates and not haptens alone was measured in these experiments, in theory the hybrid might bind unconjugated haptens; that is, it might retain specificity for 8-oxopurines.

Table 4: Amino Acids That Distinguish the Light Chains of Clones a62 and d1290

| clone | residue L74 | residue L92 | residue L96 |
|---|---|---|---|
| a62 | Lys | Thr | Ser |
| d1290 | Arg | Gln | Leu |

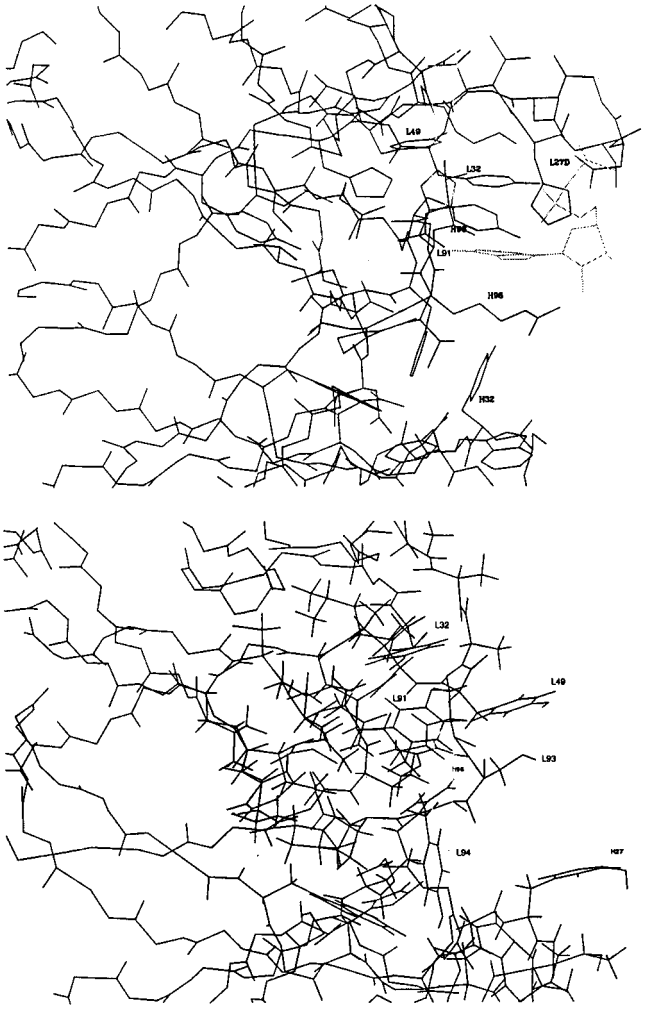



FIGURE 6: (a, top) Backbone diagram of Jel 103 with GDP bound taken from the PDB crystal structure (Entry 1MRE; Pokkuluri *et al.*, 1994). GDP is shown stippled. The side chains of amino acids within 10 Å of GDP are shown and labeled with chain and sequence number. (b, bottom) Backbone diagram of the model of clone g37. Side chains of amino acids within 10 Å of the putative nucleotide binding area are shown and labeled.

Clone g37, the most specific clone, was further characterized by homology modeling and comparison to two nucleic acid binders, Jel 103 and BV04-01. For all three, the base binding specificity appears to reside primarily in the light chain with residues in the CDR1 and CDR3 variable regions. In the crystal structure of Jel 103 (Entry 1MRE; Pokkuluri

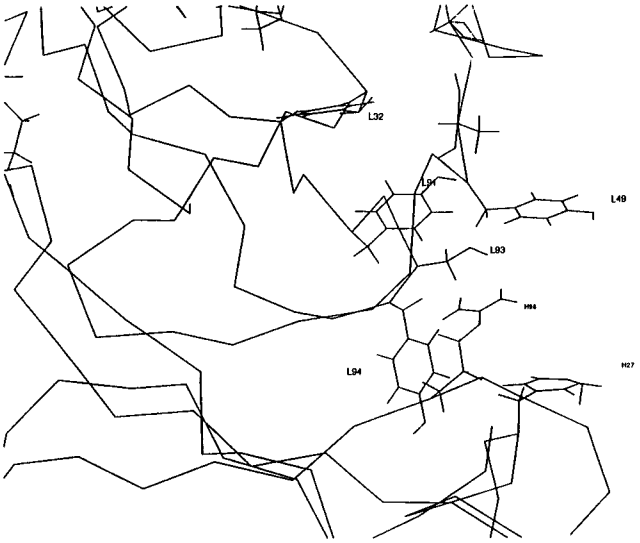



FIGURE 7: α-Carbon trace of the putative nucleotide binding area showing the side chains of nearby residues and of Tyr L32 and L91 which are further from this site.

*et al.*, 1994) GDP is bound through a number of interactions in an area that might be described as a "cleft" (Figure 6a). Ser L91 hydrogen bonds to the base, docking the ligand. The CDR1 region of the light chain extends out over the base binding area, stabilizing Tyr L32 on which the base stacks. Beneath the ligand, Arg H96 extends out under the base and forms a hydrogen bond to the sugar ring.

Figure 6b shows the area of the g37 model which superimposes on the binding sites of Jel 103. This molecule has a flatter surface in the putative binding area. The markedly shorter CDR1 region of the light chain does not extend out from the surface. Tyr L32 and Tyr L91 of g37 are at a distance from the binding area; the presence of two tyrosine residues in close proximity reorients both residues, and in fact, Tyr L91 is buried in the protein. However, a number of residues are available to interact with a nucleotide (Figure 7). Tyr L49 and H27 are potentially available for stacking although not immobilized by hydrogen bonds. The ring of Tyr L94 is perpendicular to H27 which may stabilize it. Ser L93 and Arg H98 are also in the vicinity of the putative binding region and may provide stabilizing hydrogen bonds, and these residues may provide 8-oxoG binding specificity.

A DNA binding pocket, a so-called canyon (Pokkuluri *et al*., 1994), is present in the crystallized structures of Jel 103 and BV04-01 between the tips of the heavy and light chain variable regions. In g37, which does not bind to DNA, the mouth of the canyon is much larger than in Jel 103 and BV04-01 and appears to be partially blocked by residues in the CDR2 region of the heavy chain.

Taken together, these data show that specificity for a DNA base (hapten binding site) can be determined primarily by the light chain and can be separated from a general polynucleotide binding site shared by heavy and light chains but with a predominant heavy chain influence. This conclusion is suggested not only by the homology modeling but by chain shuffling. DNA binding could only be conferred on a conjugate binding light chain (a62) which did not bind DNA, by partnering it with the heavy chain of a DNA binder (d1290). The light chain of the conjugate binder (a62) had a light chain sequence nearly identical to that of the DNA binder (d1290) so that it was able to complex with the d1290 heavy chain forming the proper canyon for DNA binding.

Antibodies are currently available that recognize 8-oxoguanine in blood and urine (Degan *et al*., 1991; Park *et al.*, 1992), and a polyclonal antibody recognizes this lesion in DNA (Ide *et al*., submitted). For many experimental designs, it is desirable to measure oxidative DNA damages when present in DNA. Our ultimate goal is to engineer Fabs to address this need. While we have produced monoclonal Phabs that can very specifically bind to 8-oxoguanosine and 8-oxoguanosine monophosphate preferentially over the normal base counterparts, they do not bind 8-oxoG in the context of DNA. However, this is the first reported immunization of mice with specific modified nucleotide conjugates to perform repertoire cloning for antibody isolation. As such, we view these studies as the starting point for establishing alternative methods of immunization and to provide raw materials for directed engineering.

## ACKNOWLEDGMENT

We are grateful to Dennis Burton and the members of his laboratory for all their help. The computational analysis and computer graphics utilized the UVM Computer Modeling Facility funded by the U.S. Department of Energy.

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BI9517244