ABSTRACT

Four synthetic peptides from the sequence of human O'5-methylguanine-DNA methyltransferase (MGMT), corresponding to three different hydrophilic regions and one corresponding to the sequence containing the alkyl acceptor residue cysteine 145, were used to immunize rabbits. The antibody against Peptide III (residues 171-184) was highly specific, and MGMT protein could be detected on Western blots of soluble protein extracts containing as little as 1 fmol of active MGMT. Antibodies against all of the peptides were able to immunoprecipitate denatured MGMT, while only the antibody against Peptide III was able to react with active enzyme. The antibody against Peptide III did not cross-react with methyltransferase from mice. The use of synthetic peptides has led to the production of a highly sensitive, specific antibody that recognizes native and denatured human MGMT. This antibody should prove useful in studies involving the detection, purification, and characterization of this enzyme.

INTRODUCTION

MGMT (EC 2.1.1.63) is an important DNA repair enzyme found in all normal tissues studied. As first described in Escherichia coli (1, 2), the protein catalyzes the removal of alkyl groups from the O'5 position of alkylguanine residues and transfers the alkyl group to a cysteine residue within the MGMT polypeptide chain. This reaction is irreversible and inactivates the transferase, restoring the DNA to its normal structure. By virtue of its ability to repair alkylated guanine residues, it is able to reduce the toxic and mutagenic effects of many classical tumor initiators. It is also able to increase the resistance of tumors to the cytotoxic effects of chemotherapeutic alkylating agents such as 1,3-bis(2-chloroethyl)nitrosourea that produces lesions involving O'5-guanine (for a review see Refs. 3 and 4). Since a significant fraction of tumor cell lines, termed Mer' or Mex' (5, 6), are deficient in MGMT activity and are highly sensitive to these alkylating agents, there is great interest in determining MGMT levels in tissue sections of tumor biopsies as a means of predicting drug response. Specific antibodies against the MGMT molecule would provide a practical means of assessing MGMT concentrations in tissues in a rapid manner. These antibodies would also be valuable tools for research into the mechanisms of carcinogenesis by and tumor resistance to alkylating agents and for further biochemical studies of the protein itself. Specific antibodies against human MGMT have been reported; however, these only recognize the denatured enzyme (7). In this study, synthetic peptides were used to produce polyclonal antibodies that could detect as little as 1 fmol of MGMT on Western blots and react with the native enzyme.

MATERIALS AND METHODS

Synthesis of Peptides. The peptides used in this study (MDK-CERKRTTLDS; YFHPOAEIEEFFPVP; HEGHRLGKPGLGGS; PVPIIPCHVRVCSS; Peptides 1-IV, respectively) were synthesized by Applied Biosystems (Foster City, CA). Purity of the peptides was confirmed by high-pressure liquid chromatography, amino acid analysis, and mass spectroscopy (Applied Biosystems). A carboxy-terminal cysteine was added to each peptide to facilitate coupling to keyhole limpet hemocyanin. Internal cysteines were blocked with acetonitrile-ethyl to prevent disulfide bridge formation.

Immunization Protocol. For each peptide, three New Zealand White rabbits (Robinson Ranch, Winston Salem, NC) received s.c. injections in four sites of 250 μg of peptide conjugated to keyhole limpet hemocyanin in 2 ml of a 1:1 emulsification of 115 mM phosphate buffer (pH 7.4) and complete Freund's adjuvant. Rabbits were bled on Day 35 post-immunization and boosted with 250 μg of peptide in 2 ml of incomplete Freund's adjuvant. They were then bled on Days 7 and 35 post-secondary immunization. Animals were rested for 81 days and boosted with 250 μg of peptide in 2 ml of incomplete Freund's adjuvant containing 2.5 mg of dried Salmonella minnesota. Animals were bled 30 days post-tertiary immunization.

Enzyme-linked Immunosorbent Assay Procedure. Nine-six-well enzyme-linked immunosorbent assay plates (Dynatech Laboratories, Chantilly, VA) were coated with 500 ng of peptide in 50 μl of 0.1 M sodium carbonate buffer (pH 9.6) overnight at 4°C. Plates were rinsed 3 times with rinse buffer (115 mM phosphate buffer containing 0.05% Brij 35 detergent and 0.02% gelatin). Non-specific binding to plates was blocked with blocking buffer (10% goat serum and 0.02% gelatin in 115 mM phosphate buffer) for 30 min. The buffer was removed; 50 μl of antibody diluted in blocking buffer were added to triplicate wells for each dilution; and the plates were incubated for 1 h. The plates were then rinsed 8 times with rinse buffer: 50 μl of biotinylated goat anti-rabbit IgG (BRL, Gaithersburg, MD), at a concentration of 1 μg/ml in blocking buffer, were added to each well; and the plates were incubated for 1 h.

The plates were again rinsed 8 times with rinse buffer; 50 μl of a 1:1000 dilution of avidin alkaline phosphatase in blocking buffer were added to each well; the plates were incubated for 1 h and then rinsed 8 times with rinse buffer and twice with 20 mM Tris buffer (pH 7.6). One hundred μl of phosphatase substrate were added to each well, and the plates were incubated until color had developed, followed by the addition of 50 μl of 10 mM 1-cysteine/well. Phosphatase substrate was 4 mg of p-nitrophenyl phosphate/ml of 10% (w/v) diethanolamine buffer (pH 9.6). Absorbance was read at 405 nm using an automated plate reader (Titerdek; Flow Laboratories). Purification of Antibodies. Antipeptide antibodies were purified from rabbit sera by affinity chromatography on resins prepared by coupling
free peptides to cyanogen bromide-activated Sepharose as previously described (8). IgG antibodies were purified from preimmune rabbit and immune sera by protein A-Sepharose chromatography for direct comparison. The yield of purified IgG from immunized rabbits was approximately 0.2-1.0 mg/ml following affinity purification. All purified antibody preparations were examined by size exclusion high-pressure liquid chromatography essentially as described (8) and found to consist of a single major protein peak eluting at a position corresponding to IgG. Affinity-purified material was used for all experiments described except where noted.

Recombinant MGMT. Human recombinant MGMT was purified from E. coli containing the plasmid pKT100 as described (9). The cloning of mouse MGMT cDNA and purification of mouse recombinant MGMT will be published elsewhere.4

Extract Preparation. A cell pellet containing approximately 10⁸ cells was suspended in 2 volumes of 50 mM Tris-HCl (pH 7.5), 0.1 mM NaCl, 2 mM EDTA, 1 mM dithiothreitol, and 0.02% sodium azide, with 0.2 mM phenylmethylsulfonyl fluoride and aprotinin (20 trypsin inhibitor units/liter; Sigma Chemical Co., St. Louis, MO). The cells were disrupted by intermittent sonication for a total of 90 s and centrifuged for 45 min in a Beckman-type 50-Ti rotor at 40,000 rpm. Supernatants that were to be assayed for MGMT were stored in liquid nitrogen until used.

MGMT Assay. MGMT activity was determined by measuring the appearance of radiolabeled protein after incubating cell extracts with ³H-methylated DNA as described previously (10). Briefly, [methyl-³H]-nitrosourea-treated calf thymus DNA containing 2 pmol O⁶-[methyl-³H]guanine was incubated with increasing amounts of cell extract (0.1-0.5 mg protein) for 30 min at 37°C in 200 μl of TEDG buffer. The reaction was stopped by adding 0.5 ml of 5% trichloroacetic acid, and the DNA was hydrolyzed by heating to 80°C for 30 min. After cooling on ice for 5 min, 100 μg of bovine serum albumin were added as a carrier, and the protein precipitate was collected on glass fiber filters (Whatman GF-F). The filters were washed and placed in scintillation vials. The precipitates were solubilized with NCS tissue solubilizer (Amersham, Arlington Heights, IL) before adding nonaqueous scintillation fluid for quantitating the radioactivity due to methyl-³H-labeled DNA. MGMT activity was calculated from the slope of the linear part of the assay.

SDS-PAGE and Electroblotting. Cell extracts were resolved by SDS-PAGE in a 0.75-mm-thick 10% slab gels according to the method of Laemmli (11) in a Bio-Rad (Richmond, CA) minigel apparatus at 200 V for 45 min. Proteins were electroblotted onto polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA) for 2 h at 140 mA in a Bio-Rad Mini Trans-Blot apparatus according to the method of Towbin, Staehelin, and Gordon (12).

Immunoprobing of Blots. The blotted membranes were blocked with 5% bovine serum albumin (Amersham) in 20 mM Tris-HCl (pH 8.2) with 0.9% NaCl and processed further either as a sheet or cut into strips. The sheet or strips were probed for 2 h with the specific antibody in a buffer containing 20 mM Tris-HCl (pH 8.2), 0.1% bovine serum albumin, 0.9% NaCl, 1% normal goat serum (Gibco, Grand Island, NY), and 5% of a concentrated gelatin solution supplied by Amersham. Membranes were then washed three times in the same buffer without serum or gelatin; antibody binding was visualized by using Amersham’s gold-labeled secondary antibody and silver enhancement kit according to the manufacturer’s instructions. Densitometric analysis of film negatives was performed on a CAMAG TLC Scanner II (Muttenz, Switzerland) using CATS 3 software. Regression analysis was performed for each antibody-antigen curve yielding linear plots with correlation coefficients of 0.983 and 0.986 for anti-Peptide III and 4.A1, respectively, when band intensity in mV was plotted versus fmol of MGMT loaded.

Immunoprecipitation of [methyl-³H]MGMT. For each assay, 0.8 pmol MGMT purified 20-fold from extracts by ammonium sulfate precipi-

Results
Selection of Peptides. Three 14-amino acid peptides were chosen for this study based on a hydrophilicity analysis (13) of the amino acid sequence of human MGMT predicted from the cDNA (14). These peptides contained amino acids 1-14, 69-82, and 171-184 (Peptides I-III, respectively) of the predicted MGMT sequence (Fig. 1). A fourth sequence, centered around the alkyl acceptor residue cysteine 145 (9) and containing amino acids 138-152 (Peptide IV), was also prepared. Together these sequences constitute more than 25% of the MGMT molecule and include sequences from the amino and carboxy terminal regions, one internal hydrophilic region, and the evolutionarily conserved alkyl acceptor site. No other sequences with identity to the four peptides were revealed by searching of the NBRF data base (Release 21; National Biomedical Research Foundation, Washington, D.C.) using FASTA (Wisconsin Genetics Package, Version 6.1) (15).

Immune Response against Synthetic Peptides. All of the peptides produced a strong immune response as evidenced by the

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the antibody against Peptide III appeared to be highly specific for the MGMT protein.

Sensitivity of Anti-Peptide III Antibody. To examine the sensitivity of this antibody, different dilutions of an MGMT-containing extract from CEM cells were analyzed by Western blotting. MGMT could be reproducibly detected on Western blots using affinity-purified antibody against Peptide III when dilutions containing as little as 1 fmol of active MGMT were analyzed (Fig. 3a). A linear relationship between fmol of MGMT loaded and band intensity revealed by densitometry of Western blots exists for each antibody preparation:

\[ F(X) = -5.39 + 0.34X \]

for anti-Peptide III antibody, and

\[ F(X) = -12.05 + 2.93X \]

for MAb 4.1 (Fig. 3b). Comparison of the intensity:mol ratios over the linear portion of these curves shows that, at the antibody concentrations assayed, detection of MGMT by anti-Peptide III serum is 18 (18.01 ± 3.01)-fold more sensitive than with MAb 4.1.

Immunoprecipitation of Inactive MGMT. Each of the four antibody preparations was tested for its ability to precipitate methylated MGMT. When incubated with \(^3\)H-labeled methyl-
ated MGMT, antibodies against all four of the peptides were able to immunoprecipitate the MGMT protein in a dose-dependent manner (Table 1). Antibodies against Peptides II and III were most effective in this assay, precipitating 20-fold more MGMT than the control preimmune IgG when 20 μg of antibody were added. At the higher concentration tested (40 μg), these two antibodies precipitated essentially all available MGMT. Antibodies against Peptides II and III also precipitated approximately 2-fold more MGMT than antibodies against Peptides I and IV at identical concentrations.

**Immunoprecipitation of Active MGMT.** When tested for their ability to react with native MGMT in solution, only the antibody raised against Peptide III was able to precipitate a significant amount of the MGMT activity present (Fig. 4). The immunoprecipitation of native MGMT by the antibody to Peptide III was dose dependent, although rather high concentrations of antibody were required (approximately a 400:1 molar ratio). Antibodies raised against the other three portions of the MGMT molecule were completely unreactive against the active protein in this assay.

Based on these results, an affinity column was prepared by coupling 9 mg of anti-Peptide III antibody to an Actigel-ald (Sterogene, Arcadia, CA) resin. Active MGMT was applied to the column, eluted with a high pH buffer [0.1 M 3-(cyclohexylamino)-1-propanesulfonic acid, pH 11], and rapidly neutralized. In two experiments, 10–15% of the applied activity was recovered in the elution fractions. Only an additional 10–25% of the MGMT activity was recovered in the unbound fraction, suggesting that most of the MGMT was bound by the column, but the activity was destroyed in the elution buffer. The recovery of active MGMT from an affinity column demonstrates again the ability of anti-Peptide III antibody to bind native enzyme.

**Species Specificity.** Because of the conservation of certain features of the alkyltransferase between species, it was of interest to determine the cross-reactivity of these antibodies with MGMT from other sources. Western blot analysis showed that the antibody against Peptide III did not cross-react with purified recombinant mouse MGMT. In contrast, a MAb raised against human MGMT, 4.A1, did react with the mouse protein. Fig. 5 shows that MAb 4.A1 could detect as little as 0.01 μg of the mouse transferase, whereas the antibody against Peptide III was completely unreactive with 10-fold higher concentrations.

The antibody against Peptide IV, which spans the conserved active site, was able to immunoprecipitate the purified, methylated mouse transferase but did not precipitate the ada protein from *E. coli* (data not shown).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>MGMT precipitated (dpm)</th>
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<tbody>
<tr>
<td>Anti-Peptide 1</td>
<td>6,286 ± 125</td>
</tr>
<tr>
<td>Anti-Peptide 2</td>
<td>23,572 ± 466</td>
</tr>
<tr>
<td>Anti-Peptide 3</td>
<td>25,168 ± 2,171</td>
</tr>
<tr>
<td>Anti-Peptide 4</td>
<td>9,972 ± 373</td>
</tr>
<tr>
<td>Preimmune IgG</td>
<td>948 ± 26</td>
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<tr>
<td>Bovine serum albumin</td>
<td>981 ± 51</td>
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*Results are reported as the average dpm recovered in the protein G-Sepharose pellet ± SD of replicate samples. For this experiment, approximately 31,000 total dpm were available.*

**DISCUSSION**

MGMT is of interest in current cancer research, both because it affords protection against the carcinogenic effects of many compounds and because of its role in the resistance of tumors to certain chemotherapeutic alkylating drugs. The protein is present in small amounts in most mammalian cells and has been difficult to obtain in quantities sufficient for detailed analysis. The human MGMT cDNA has recently been cloned by several groups (14, 17, 18). The goal of the present investigation was to produce highly specific, sensitive immunoreagents to allow further study of this important protein.

The use of synthetic peptides as immunogens in this study differs from our earlier approach (7), where we used the intact human transferase eluted from acrylamide gels as the immunogen. This latter study resulted in several high-affinity MAbs to the MGMT molecule; however, these antibodies were only able to recognize the denatured protein, presumably because immunization was with material cut or eluted from SDS gels. By using peptides conjugated to keyhole limpet hemocyanin as the immunogen, it was possible to inject sufficient amounts of a defined antigen to immunize and, by designing the peptides appropriately, perhaps increase the chance of generating antibodies that recognized the native molecule. The use of synthetic peptides to generate antibodies to the human MGMT has been reported, but a detailed characterization of these has not been published (3).

Of the four synthetic peptides used to generate antibodies against the human MGMT molecule, three were chosen based on a hydrophilicity profile of the MGMT sequence. This method predicts that regions of a protein that are hydrophilic may reside on an exposed surface of the molecule and thereby be accessible to antibody binding. The success of the current study, in which antibodies raised against one of the peptides have been shown to recognize the native protein, lends support to the validity of this approach. The fourth peptide was centered around the alkyl acceptor residue, cysteine 145. This peptide contains the sequence PCHR5, which is conserved between the human and mouse MGMTs, and the similar *E. coli* transferases, ada and ogt. This sequence was included in the present study with the expectation that antibodies raised against a highly conserved and unique sequence would show a high degree of specificity for human MGMT and perhaps for alkyltransferases of other species as well. In addition, it was hoped that antibodies against the alkyl acceptor region would inactivate MGMT. The inability of anti-Peptide I and anti-Peptide IV
Fig. 5. Species specificity of anti-Peptide III antibody compared to MAb 4A1 by Western analysis. The indicated amount of purified mouse recombinant MGMT was blotted and probed with protein A-purified anti-Peptide III antibody (A) or MAb 4A1 (B). Control lanes showing the reaction of the antibodies with purified recombinant human MGMT were also included on the blots.

<table>
<thead>
<tr>
<th>ng protein</th>
<th>Human rMGMT</th>
<th>Mouse rMGMT</th>
<th>Human rMGMT</th>
<th>Mouse rMGMT</th>
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antibodies to react with MGMT on Western blots and of anti-Peptide I, anti-Peptide II, and anti-Peptide IV antibodies to react with native protein substantiates the difficulty of predicting the reactivity of antipeptide antibodies with native proteins (19, 20).

The ability of anti-Peptide II and anti-Peptide III antibodies to react with MGMT on Western blots (Fig. 2) indicates that the epitopes recognized by these antibodies are exposed on the denatured enzyme. This is perhaps not surprising because the short peptides used as immunogens may more closely resemble the peptide chain of the enzyme. The inability of anti-Peptide I and anti-Peptide IV antibodies to recognize MGMT on Western blots is therefore somewhat unexpected. Peptide IV does contain two cysteine residues that were blocked during synthesis, and this may have altered the antigenicity of this peptide. Nevertheless, all of the antipeptide antibodies were able to precipitate inactive MGMT from solution (Table 1). The fact that antibodies to Peptides I, II, and IV were able to precipitate the inactive enzyme but did not react with active enzyme may indicate that the enzyme undergoes a conformational change following its reaction with alkylated DNA. The bacterial enzyme also undergoes a conformational change after alkylation that allows it to act as a transcriptional enhancer (21). Because the inactive enzyme becomes very difficult to maintain in solution, our experiments used low concentrations of sodium deoxycholate which may have partially denatured the transferase, exposing antibody binding sites normally unavailable on the soluble molecule. Further studies are necessary to define the antibody-transferase interactions more clearly and the existence and function of any conformational changes in the enzyme following its inactivation.

The antibody to Peptide III was unique in that it was able to recognize MGMT under all conditions tested. These included the specific detection of MGMT denatured by SDS-PAGE, the precipitation of the inactive protein and the active protein in solution, and the binding and recovery of the active enzyme from an affinity column. This indicates that the epitope(s) present on Peptide III is (are) most likely on the external surface of MGMT and are stable to the conditions tested. Because anti-Peptide III antibody recognizes both the active and inactive forms of the transferase, it can be used to complement measurements of transferase activity to quantitate the total amount of MGMT protein present in solution. The antibody to Peptide III will also be useful for the detection of MGMT by immunoblot analysis. The sensitivity of this polyclonal antibody is at least 10-fold greater than that which we have previously reported using the MAb 4A1. The lack of cross-reactivity with mouse MGMT will be useful in distinguishing human from mouse MGMT in studies where both proteins are present.

Finally, antibodies to MGMT could be of significant clinical utility in the treatment of cancer patients. The level of MGMT has been shown in model systems to relate directly to the resistance of a tumor to alkylating agents (i.e., Refs. 22–24). The ability to detect MGMT levels in biopsy samples rapidly and quantitatively, e.g., by radioimmunoassay, enzyme-linked immunosorbent assay, or the immunoblot method used herein, would give physicians another tool in designing treatment strategies for particular patients. The production and characterization of specific polyclonal antibodies against synthetic peptides described in this paper may lead to the development of a clinically useful reagent.

ACKNOWLEDGMENTS

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\[
\text{KDa} \\
| 43 | \\
| 31 | \\
| 21.5 | \\
| 14.2 | \\
| A. anti-peptide III | B. 4A1 |


Production and Characterization of Antipeptide Antibodies against Human \( O^6 \)-Methylguanine-DNA Methyltransferase

Lawrence E. Ostrowski, Charles N. Pegram, Mathew A. von Wronski, et al.


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