Development of a Monoclonal Antibody-based Immunoassay for Cyclic DNA Adducts Resulting from Exposure to Crotonaldehyde

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ABSTRACT

In order to develop an immunoassay for DNA modifications resulting from exposure to crotonaldehyde, monoclonal antibodies specific for the 8R,6R- and 8S,6S-stereoisomers of 3-(2-deoxy-D-erythro-pentofuranosyl)-5,6,7,8-tetrahydro-8-hydroxy-6-methylpyrimido[1,2-a]purine-10(3H)one were produced. These cyclic N2-propanodeoxyguanosines are formed in DNA exposed to crotonaldehyde in vitro. Three of the four antibodies were most specific for one stereoisomer while the fourth was most specific for the other stereoisomer. Fifty % inhibition of binding in an enzyme-linked immunoabsorbent assay could be achieved with 0.2 picomol of either stereoisomer. A high-pressure liquid chromatography–enzyme-linked immunoabsorbent assay using two of these antibodies and capable of detecting 0.5 μmol of 1N2-propanodeoxyguanosine per mol of DNA was developed. The method was validated by comparison to results obtained with fluorescence assay.

INTRODUCTION

Recently the modified nucleosides 8R,6R- and 8S,6S-3-(2-deoxy-D-erythro-pentofuranosyl)-5,6,7,8-tetrahydro-8-hydroxy-6-methylpyrimido[1,2-a]purine-10(3H)one (Fig. 1, structures 1a and 1b) were identified in DNA which had been exposed in vitro to crotonaldehyde (CH2(CH==CHCHO; Refs. 1 and 2). The same modified deoxyguanosines may also form as a result of N hydroxylation of NPYR. Both crotonaldehyde and NPYR are tumorigenic in rats (3) with NPYR being the more potent of the two. Crotonaldehyde is also mutagenic toward Salmonella typhimurium (4, 5) and is commonly found in the human environment. NPYR is found in cooked bacon, mainstream and sidestream tobacco smoke, and as a trace contaminant of various foodstuffs (6). In order to study the role of structures 1a and 1b in the mechanism of action of crotonaldehyde and NPYR a sensitive assay is needed to measure the formation of structures 1a and 1b in vivo. In recent years immunoassays for a variety of carcinogen-DNA adducts have been developed, many involving the use of monoclonal antibodies (7–10). These assays are highly sensitive, being able to detect as little as 0.5 μmol adduct per mol deoxyguanosine. Carcinogen-DNA adducts have also been detected in humans with the aid of immunoassays (11, 12). In the present study monoclonal antibodies specific for structures 1a and 1b were developed and their binding specificities were characterized. Using these antibodies, an assay for structures 1a and 1b in DNA has been developed and validated by comparison with an assay based on HPLC-fluorescence.

MATERIALS AND METHODS

Chemicals. Structures 1–3 were synthesized as previously described by reaction of deoxyguanosine with crotonaldehyde, acrolein, or glyoxal, respectively (1, 2, 13). Calf thymus DNA (Sigma Chemical Co., St. Louis, MO) was modified in vitro with crotonaldehyde as follows. Twenty mg of DNA were dissolved in 4 ml of 0.1 m phosphate buffer (pH 7.0). Forty-five mg of crotonaldehyde (Aldrich Chemical Co., Milwaukee, WI) were added and the mixture was incubated in a 37°C shaking water bath for 48 h. The DNA was then precipitated with cold ethanol, redissolved, and reprecipitated three times. The modified DNA was stored at −20°C.

Coupling of Adduct to Protein. The ribose forms of structures 1a and 1b were synthesized by the reaction of crotonaldehyde with guanosine, under conditions similar to those described (1). The structures of the guanosine adducts were confirmed by their UV spectra, which were identical to those of structures 1a and 1b, and by their proton nuclear magnetic resonance spectra which showed characteristic chemical shifts for the ribosyl protons and the 1N2-propanodeoxyguanosine protons previously reported. The diastereomers were coupled to KLH using the periodate oxidation method as described by Meredith and Erlanger (14). The conjugate was dialyzed extensively against PBS and stored at −20°C.

Immunization. Female BALB/c x C57BL/6 F mice were immunized with 100 μg of structure 1a,b-KLH conjugate in 0.1 ml of saline emulsified with an equal volume of Freund’s complete adjuvant, given in a split dose, i.p. and s.c. A second injection was given at 2 weeks in incomplete Freund’s adjuvant. Four weeks after the second injection individual mice were boosted with 100 μg of conjugate in saline given i.p. on days 1, 2, 3, and 4. On day 5 the mouse was sacrificed and the spleen was removed for fusion.

Fusion Protocol. The fusion partner FO (15) was maintained in RPMI 1640 supplemented with 1 mm sodium pyruvate, 25 μg/ml gentamicin, and 15% fetal bovine serum. The FO cells were maintained in log-phase growth for 3 days prior to fusion. The FO cells were centrifuged and resuspended in HBSS. A single cell suspension was made by forcing the spleen through a fine mesh stainless steel screen followed by centrifugation. RBC were lysed by resuspending the cell pellet in 5 ml of cold Tris-ammonium chloride (pH 8.0) and incubating for 5 min on ice. The cells were washed, resuspended in 10 ml of HBSS, and counted. The spleen cells were mixed with the FO cells at a ratio of 1:1 in a 17 x 100-mm polypropylene test tube (Falcon Labware, Oxnard, CA; 2006) and centrifuged. The liquid was removed from the pellet and 1 ml of the fusing solution [50% HBSS, 45% polyethylene glycol (Eastman Kodak Co., Rochester, NY; M, 1450) and 5% dimethyl sulfoxide] was added. The cell pellet was broken into small clumps by gentle agitation and incubated for 5 min at 37°C. The fused cells were centrifuged and the supernatant was discarded. The cells were resuspended in 5 ml of complete RPMI 1640 and centrifuged, and the cell pellet was resuspended in 50 ml of complete RPMI 1640 supplemented with hypoxanthine (0.1 mm), aminopterin (0.4 μM), thymidine (16 μM), insulin (5 μg/ml), transferrin (5 μg/ml), and sodium selenite (5 ng/ml). The cells were cultured in 96-well tissue culture plates at 150 μl/well and incubated at 37°C in 5% CO2 in air. On day 6 the cultures were fed with 50 μl of complete RPMI 1640 supplemented with hypoxanthine and thymidine. By day 14 the colonies were sufficiently large to be screened for antibody production.

ELISA. Wells of a 96-well polystyrene ELISA plate were coated with 10 ng of structure 1a,b-KLH conjugate, diluted in PBS, for 1 h at room temperature. The wells were washed twice with PBS containing 0.1% Tween 20 and twice with distilled H2O. From this point all reagents were diluted in PBS containing 0.1% bovine serum albumin. Wells were filled with 50 μl of PBS-BSA followed by 50 μl of culture supernatant and incubated at room temperature for 1 h. For the competitive ELISA, adduct standards or test samples were mixed with the monoclonal antibody and added to the well. The wells were washed

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2 To whom requests for reprints should be addressed.

The abbreviations used are: NPYR, N-nitrosopyrrolidine; KLH, keyhole limpet hemocyanin; HBSS, Hank’s balanced salt solution; PBS, phosphate-buffered saline; BSA, bovine serum albumin; HPLC, high-pressure liquid chromatography; ELISA, enzyme-linked immunosorbent assay.
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Fig. 1. Cyclic deoxyguanosine adducts formed by reaction with: structures 1a and 1b, crotonaldehyde; structures 2a and 2b, acrolein; structure 3, glyoxal.

Table 1 Monoclonal antibody specificity

<table>
<thead>
<tr>
<th>Compound</th>
<th>CA1</th>
<th>CA2</th>
<th>CA3</th>
<th>CA4</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a*</td>
<td>12.8*</td>
<td>17</td>
<td>0.2</td>
<td>10</td>
</tr>
<tr>
<td>1b</td>
<td>0.2</td>
<td>0.4</td>
<td>10</td>
<td>0.9</td>
</tr>
<tr>
<td>1, base*</td>
<td>8.6</td>
<td>69</td>
<td>143</td>
<td>30</td>
</tr>
<tr>
<td>2a</td>
<td>90</td>
<td>9</td>
<td>5</td>
<td>112</td>
</tr>
<tr>
<td>2b</td>
<td>0.8</td>
<td>0.2</td>
<td>0.9</td>
<td>7.9</td>
</tr>
<tr>
<td>3</td>
<td>113</td>
<td>156</td>
<td>22</td>
<td>41.470</td>
</tr>
<tr>
<td>Deoxyguanosine</td>
<td>2.564</td>
<td>698</td>
<td>14</td>
<td>5,370</td>
</tr>
<tr>
<td>Deoxyadenosine</td>
<td>&gt;80,000</td>
<td>&gt;80,000</td>
<td>&gt;80,000</td>
<td>&gt;80,000</td>
</tr>
<tr>
<td>Deoxycytosine</td>
<td>&gt;110,000</td>
<td>&gt;110,000</td>
<td>&gt;110,000</td>
<td>&gt;110,000</td>
</tr>
<tr>
<td>Thymidine</td>
<td>&gt;100,000</td>
<td>&gt;100,000</td>
<td>&gt;100,000</td>
<td>&gt;100,000</td>
</tr>
<tr>
<td>O'-Methyldesoxyguanosine</td>
<td>56,790</td>
<td>51,270</td>
<td>235</td>
<td>&gt;100,000</td>
</tr>
<tr>
<td>7-Methylguanosine</td>
<td>&gt;50,000</td>
<td>&gt;50,000</td>
<td>&gt;50,000</td>
<td>&gt;50,000</td>
</tr>
</tbody>
</table>

* The absolute configurations of the diastereomers 1a and 1b are not known. The configurations shown in Fig. 1 are arbitrary.
* Values are pmol of compound needed for 50% inhibition of binding in the competitive ELISA.
* A single isomer is obtained by acid hydrolysis of structures 1a and 1b.

Cloning of Hybridomas. Hybrid cultures which were positive in the ELISA screening test were cloned by limiting dilution in hanging drop cultures as described by Bell et al. (16). Drops with viable colonies were selected microscopically and transferred to a 96-well culture plate and expanded. The class and subclass of the antibodies were determined by using a subisotyping kit from HyClone Laboratories (Logan, UT).

DNA Adduct Immunoassay. The assay of DNA samples for the presence of adduct was performed by enzyme hydrolyzing 1-2 mg of DNA with deoxyribonuclease I, phosphodiesterase, and alkaline phosphatase as described by Muller and Rajewsky (17). The hydrolyzed DNA was analyzed on a C18-Bondapak reverse phase analytical HPLC column (Millipore, Milford, MA) with a linear gradient from 100% 100 mM phosphate buffer, pH 5.6, to 100% of 10% methanol in H2O in 1 h at 1.5 ml/min. Fractions of 3 ml were collected. The fractions were lyophilized and reconstituted in 500 µl of PBS containing 0.1% BSA and tested for structures 1a and 1b using the competitive ELISA described above.
RESULTS AND DISCUSSION

A single mouse was boosted with the structure 1a,b-KLH conjugate for 4 consecutive days and the fusion was performed on the fifth day. Fourteen days after fusion, 113 of 288 wells showed growth of hybrid colonies. Thirteen of these wells, when tested in the ELISA, showed reactivity with structure 1a,b-KLH conjugate, in the presence of free KLH. For 4 of these 13 wells this binding could be inhibited with a mixture of structures 1a and 1b. These cell lines were cloned and named CA1, CA2, CA3, and CA4. The immunoglobulin isotypes of these antibodies was clearly strongest for structures 1a and 1b. These cell lines were cloned and named CA1, CA2, CA3, and CA4. The immunoglobulin isotypes of these antibodies were found to be: CA1, IgG2a; CA2, IgG2b; CA3 and CA4, and IgG1. Their binding specificities were determined by competitive ELISA (Table 1; Fig. 2). The reactivity of the 4 antibodies was clearly strongest for structures 1a and 1b. Cross-reactivity was also seen with the free base form of structure 1a. These results suggest that CA1 is most specific for the hydroxyl group at position 8 of the modified deoxyguanosine, since the 3 most reactive molecules, structures 1b, 2b, and 1a all have the hydroxyl at position 8. Adduct 2a with the hydroxyl at position 6 is less reactive than the above three molecules. CA3 appears to have the same specificity except that it prefers the hydroxyl group in the opposite stereochemical configuration. The specificity of antibody CA2 is unique in that it is most specific for structure 2b which is formed from acrolein.

For the development of the immunoassay for structures 1a and 1b in DNA two antibodies were chosen; CA1 because of its high apparent affinity for structure 1b and CA3 because it had the highest affinity of the four antibodies for structure 1a. Because of the cross-reactivity of these antibodies for deoxyguanosine it was necessary to purify the DNA hydrolysate by HPLC prior to ELISA. Fig. 3 shows a chromatogram obtained by HPLC analysis of calf thymus DNA, which had been modified in vitro with crotonaldehyde and enzyme hydrolyzed. The compounds 1a and 1b elute in fractions 29 and 32 under these conditions. Fractions 26 through 35 were lyophilized, reconstituted, and tested in the ELISA. The results of these assays (Fig. 3) show that antibody CA3 was reactive only with material from fraction 29 which contains structure 1a. The low level of reactivity with fraction 32 which contains structure 1b is consistent with the cross-reactivity of CA3 with structure 1b (see Table 1). Analogous results were obtained with antibody CA1. The strongest reactivity occurred with fraction 32, with a lower level of reactivity with fraction 29. This is consistent with the specificity pattern of CA1 which has the highest affinity for structure 1b and lower affinity for structure 1a. To verify the accuracy of the assay for structures 1a and 1b, calf thymus DNA which had been modified in vitro with crotonaldehyde was analyzed for structures 1a and 1b by HPLC-fluorescence. A series of dilutions of modified DNA in unmodified DNA were made and the resulting samples were tested by HPLC-ELISA. The results of this analysis are shown in Table 2. The two methods are in good agreement. There is a linear relationship between the calculated level of DNA modification and the results of the ELISA with correlation coefficients of 0.987 and 0.997 for structures 1a and 1b, respectively. The theoretical sensitivity of this assay, based on the inhibition data, is 0.5 μmol structures 1a or 1b per mol deoxyguanosine. While it appears that structures 1a and 1b are formed in nearly equal

<table>
<thead>
<tr>
<th>Fraction Number</th>
<th>Fluorescence Immunoassaya</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.05</td>
</tr>
<tr>
<td>10</td>
<td>0.05</td>
</tr>
<tr>
<td>15</td>
<td>0.05</td>
</tr>
<tr>
<td>20</td>
<td>0.05</td>
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<tr>
<td>25</td>
<td>0.05</td>
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<tr>
<td>30</td>
<td>0.05</td>
</tr>
<tr>
<td>35</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Fluorescence Immunoassay. To analyze the in vitro modified calf thymus DNA, 2.8 mg of DNA were enzyme hydrolyzed and analyzed by HPLC as described above. The fractions containing structures 1a and 1b were concentrated to 1 ml and analyzed by HPLC with a Partisil SCX column, using isocratic elution with 1 mM ammonium phosphate buffer, pH 2.0, at 1 ml/min. The effluent was monitored with a Perkin-Elmer fluoro spectrophotometer. Concentration was determined by comparison of the fluorescence intensity to that of a synthetic standard.

Table 2. Comparison of fluorescence and immunoassay data for assay of structures 1a and 1b in DNA

<table>
<thead>
<tr>
<th>Structure</th>
<th>Fluorescence</th>
<th>ImmunoAssay</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>1b</td>
<td>0.05</td>
<td>0.05</td>
</tr>
</tbody>
</table>

* Mean of duplicate determinations.

Fig. 3. HPLC analysis of 2 mg of calf thymus DNA containing 168 μmol of structures 1a and 1b per mol of deoxyguanosine. The sample was enzyme hydrolyzed and analyzed on reverse phase HPLC as described in "Materials and Methods." Fractions 26 thru 35 were tested in the ELISA with antibodies CA1 (o) and CA3 (b). dAdo, deoxyadenosine; dCyt, deoxycytidine; dGuo, deoxyguanosine; dThy, deoxythymidine.
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amounts in vitro, the ability to assay for each isomer separately will allow us to determine if either is formed or removed preferentially in vivo. Studies designed to investigate the extent of formation structure 1a and 1b in rats exposed to crotonaldehyde and NPYR are in progress. In addition, the use of antibody CA2 to assay structure 2b in animals exposed to acrolein or its precursors is being investigated.

REFERENCES

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