Immunological Detection of Two Types of Cyclobutane Pyrimidine Dimers in DNA

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ABSTRACT

A hyperimmune rabbit antiserum to ultraviolet-irradiated DNA was observed to contain two activities directed against irradiated DNA. Radioimmunoassay studies demonstrated that both antigenic determinants were pyrimidine dimers as evidenced by the reduction in antibody binding to enzymatically photoreactivated irradiated DNA. Enzymatic photoreactivation specifically monomerizes pyrimidine dimers in DNA. Under conditions in which antibody binding was measured with native DNA, binding was observed with DNA that contained both thymine-thymine and cytosine-containing dimers but not against native DNA that contained only thymine-thymine dimers. Under these assay conditions, competitive binding to irradiated DNA increased as a linear function of fluence of ultraviolet radiation. Under a second set of conditions in which binding was measured with single-stranded DNA, antibodies were bound to DNA which contained only thymine-thymine dimers. The fluorescence response for the increase in binding to thymine-thymine dimers was nonlinear and increased as a function of fluence squared. Binding to thymine dimers appeared to require bivalent attachment to two dimers that occurred in close proximity.

INTRODUCTION

Previous measurements of UVR-induced damage in DNA have relied upon chromatographic (1) and enzymatic (17) methods to detect lesions in radiolabeled DNA. To obtain adequate labeling of DNA requires large quantities of labeled precursors. Immunological detection of DNA lesions is an alternate method which circumvents the need for large quantities of radioactive material and has the potential of being more sensitive (20) than are current chromatographic or enzymatic techniques. A 10-fold increase in sensitivity is necessary so that assays of DNA damage can be carried out at sublethal fluences.

Levine et al. (9) in 1966 reported on antibodies that were specific for UV-irradiated DNA. Subsequently, antibodies in rabbits that recognize antigenic determinants in UV- (14–16, 20) or γ-irradiated (10) DNA and a mouse monoclonal antibody to UV-irradiated DNA (23) have been described. The antigenic determinants in all of these UVR studies appear to be pyrimidine dimers.

This report demonstrates that not only can antibodies be used to measure the presence of pyrimidine dimers in DNA but they can also be used to distinguish between 2 classes of dimers, the T-T dimers and the C-Py dimers.

MATERIALS AND METHODS

Source of Antiserum. The antiserum used in these studies was a generous gift of Dr. Eng Tan and was obtained from Rabbit 9091 which had received multiple injections of heavily irradiated (at 254 nm) native DNA complexed with methylated bovine serum albumin. The details of the immunization procedures were published by Natali and Tan (16). They reported that the antiserum exhibits activity against UV-irradiated, but not unirradiated, DNA.

Radioactive Labeled DNA. Tritiated DNA was obtained by growing Escherichia coli K-12 (W3110) in medium which contained [1H]thymidine at a concentration of 2 μg/ml and a final specific activity of 4.5 Ci/mmol. The medium and culturing procedures have been described in detail elsewhere (12). Labeled cells were collected by centrifugation and lysed, and the DNA was isolated by sedimentation in CsCl gradients as described previously (11). Fractions from the gradient which contained DNA were pooled and dialyzed exhaustively against PBS. The specific activity of the DNA was 10^7 dpm/μg.

Sources of UVR. UVR at 254 nm was obtained from a General Electric 15-watt Germicidal lamp. The fluence rate was 7.5 watts/sq m as determined with a Jagger meter (6). Monochromatic 313-nm radiation was obtained by described previously (13). The fluence rate was 50 watts/sq m as measured with a calibrated YSI Kettering Model 65 radiometer (Yellow Springs Instrument Co., Yellow Springs, Ohio).

A broad-spectrum source of UVR was obtained from a Westinghouse FS-40 T12 fluorescent sunlamp, which emits wavelengths between 280 and 400 nm with a peak emission at 313 nm (5). The fluence rate was 2 watts/sq m, as measured with the Kettering Model 65 radiometer.

Irradiation of Antigens. Tritiated E. coli DNA was exposed to 254 nm radiation to induce T-T, T-C, and C-C dimers (4, 22) and designated TA(254). Additional labeled antigen was prepared by exposing the DNA to acetophenone and monochromatic 313-nm radiation. Acetophenone sensitization is highly specific for the dimerization of thymine (8). This labeled antigen, which contains only T-T dimers, was designated TA(AcO). CAs were obtained by exposing unlabeled calf thymus DNA (calbiochem) in phosphate buffer (0.05 M, pH 7.2) to graded fluences from the FS-40 sunlamps or to acetophenone and monochromatic 313-nm radiation. In those experiments when single-stranded DNA was required, the DNA was heated at 100° for 10 min followed by rapid cooling in an ice bath. All DNAs were sheared by sonication prior to use in immune assays.

RIA. The formation of antigen-antibody complexes with irradiated, tritiated antigens, TA(254) and TA(AcO), was measured using formalin-fixed Staphylococcus aureus (Enzo Bio Chem, Inc., New York, N. Y.) as a solid-phase absorbent (7). Binding assays were carried out in a total volume of 0.27 ml with each reagent added in the following sequence and volumes: (a) 50 μl of a 1:1 mixture of Nonidet P-40 detergent (5% w/v in PBS) and BSA, (50 mg/ml in PBS); (b) 10 μl of TA(254) or TA(AcO); in PBS, ~1500 cpm; (c) 0.2 ml of unlabeled calf thymus DNA in PBS (irradiated or unirradiated) at the desired concen-

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2 The abbreviations used are: UV, ultraviolet radiation; T-T, thymine-thymine dimer; C-Py, cytosine-containing pyrimidine dimer; PBS, phosphate-buffered saline (g/liter: Na2HPO4·7H2O, 2.68; NaH2PO4·H2O, 1.38; NaCl, 8.8; pH 7.4); RIA, radioimmunoassay; CA, competing antigen; BSA, bovine serum albumin; PPRE, photoreactivating enzyme.

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was 6.2 for TA(254) and 7.0 for TA(Ac<i>). At this percentage of consistently greater with single-stranded DNA (Chart 1ß). No exposed to acetophenone plus 313 nm radiation, TA(Ac<i>) was the native state. Conversely, binding of antibodies to DNA percentage of the total antigen was bound when the DNA was in obtained with TA(254), either single stranded or native, follow tritiated DNA exposed to 2000 J/sq m at 254 nm [TA(254)] or to separate pyrimidine dimers from thymine in hydrolysates of and TA(Ac<i>), as outlined above under "RIA." RESULTS Pyrimidine Dimer Assays by Paper Chromatography. Aliquots of TA(254) or TA(Ac<i>) were precipitated by the addition of trichloroacetic acid to a final concentration of 10%. The precipitate was collected by centrifugation and washed twice with 95% ethanol, once with diethyl ether, and then dried. Hydrolysis of the DNA and subsequent paper chromatography were as described by Cook and Worthy (2).

Immunoadsorption of Whole Antiserum. A slurry of DNA-cellulose (native DNA) (P-L Biochemicals, Inc., Milwaukee, Wis.) was washed 3 times with PBS and then resuspended in PBS at a final concentration of DNA of 10 μg/ml. One-half (2 ml) of the slurry was exposed to 6000 J/sq m at 254 nm. Aliquots of irradiated DNA cellulose were mixed with various volumes of unirradiated DNA cellulose to maintain a final concentration of DNA of 10 μg/ml with the amount of irradiated DNA increasing from 0 to 10 μg/ml. To 0.5 ml of each mixture of irradiated-unirradiated DNA-cellulose were added 0.1 ml of Nonidet P-40 detergent (5% w/v in PBS), 0.1 ml of BSA (50 mg/ml in PBS), and 10 μl of immune serum. The reaction was incubated for 2 hr at 37° with constant agitation sufficient to maintain the DNA-cellulose in suspension. Following incubation, the DNA-cellulose was collected by centrifugation, and 10 μl of the supernatant were tested for binding to TA(254) and TA(Ac<i>), as outlined above under "RIA."
Antibodies to Pyrimidine Dimers in DNA

CD

I

80-

60-

40-

20-

80-

60-

40-

20-

0.01 0.05 0.1 0.5 1.0

DNA

5.0 10.0

Chart 2. Inhibition of the binding of labeled, irradiated DNA to antiserum by nonradioactive, denatured calf thymus DNA. The calf thymus DNA was exposed to 250 (O), 500 (A), 1000 (A), or 2000 J/sq m from the FS-40 sunlamp and then heat denatured before use as a CA. The CA was inhibiting the binding of antiserum to: A, 1 ng of denatured TA(Acφ); B, 1 ng of native TA(254). The antiserum dilution was 5 x 10^-3 in the TA(Acφ) RIA and 2.5 x 10^-4 when TA(254) was used.

system and as a square function of fluence when TA(Acφ) DNA was used.

In another series of experiments, unlabeled calf thymus DNA treated with acetophenone and 313-nm radiation was used as a CA to test its ability to inhibit binding of antibodies to TA(Acφ) and TA(254). The results of this experiment are presented in Table 1. As would be expected, the acetophenone plus 313-nm-treated DNA was effectively inhibited the formation of antigen:antibody complexes with TA(Acφ). The same CA, however, was ineffective in competing for antibody binding to TA(254) even at 1000-fold excess of CA. Apparently, the antigenic determinants of TA(Acφ) and TA(254) recognized by rabbit antiserum are different.

Photoreactivation Experiments. Because photoproducts other than pyrimidine dimers were induced in DNA exposed to UVR (18), it cannot be assumed that the dimers alone were involved in the competition assays described in the previous section. The ability to monomerize pyrimidine dimers specifically with yeast PRE and long-wavelength radiation (21) was used to determine whether pyrimidine dimers were involved in the competitive binding observed. Calf thymus DNA was exposed to 2000 J/sq m from the FS-40 sunlamp and subsequently treated with PRE and light for increasing time periods. Following the PRE treatment, the DNA was tested for its capacity to compete with the TA(254) and TA(Acφ) antigens for antibody binding. In both cases, PRE treatment had reduced the capacity of the irradiated DNA to function as a competing antigen (Chart 4). Thus, pyrimidine dimers were involved in the formation of antigen:antibody complexes with both the TA(254)

Table 1

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<thead>
<tr>
<th>CA (ng)</th>
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* Acetophenone (0.01 M) and 5 x 10^5 J/sq m at 313 nm. The DNA was heat denatured before use as a competing antigen.

* The TA(Acφ) was tritium-labeled E. coli DNA exposed to 0.01 M acetophenone and 1 x 10^5 J/sq m at 313 nm. TA(Acφ) was heat denatured before use in the binding assay. The antiserum dilution was 5 x 10^-3.

* The TA(254) was tritium-labeled E. coli DNA exposed to 2000 J/sq m at 254 nm. TA(254) was used as double-stranded DNA in the assay, and the antiserum dilution was 2.5 x 10^-4.

Chart 3. Plot of the concentration of CA as a function of fluence required to obtain 50% inhibition of antiserum binding to TA(254) (O) or TA(Acφ) (A). The 50% inhibition levels were taken from Chart 2.

Chart 4. Reduction in the competitive capacity of UV-irradiated calf thymus DNA as a function of treatment time with yeast PRE and black light. The tritium-labeled antigen in the competition assay was TA(254) (O) or TA(Acφ) (A). The concentrations of labeled antigens and antiserum were as in Chart 2.
R. D. Ley

and the TA(Ac0) antigens. The photoreactivation rates observed, however, differ by a factor of at least 6 and were indicative of some difference in the nature of the DNA lesion involved in antibody binding with the TA(254) and TA(Ac0) antigens.

**ImmunoaBSorption.** Immunoabsorption studies were performed to determine whether the differences in binding observed with the TA(254) and TA(Ac0) antigens were due solely to properties of the antigens or whether multiple antibodies were present in the immune serum. In these studies, native DNA bound to cellulose was exposed to 254-nm radiation. Under these conditions, the properties of the UVR-induced antigenic determinant are similar to those present in the TA(254) antigen. Treatment of the antiserum with increasing concentrations of the immunoabsorbent resulted in a decrease in the amount of binding activity directed against the TA(254) antigen (Chart 5). A similar decrease in binding activity as a function of concentration of immunoabsorbent was also observed with the TA(Ac0) antigen (Chart 5). These data indicate that a single antibody is responsible for the binding observed with the TA(254) and TA(Ac0) antigens.

**DISCUSSION**

It is apparent from the data presented here that the 9091 antiserum originally described by Natali and Tan (16) contains at least 2 activities against UV-irradiated DNA. The antiserum was obtained from a rabbit that received multiple injections of antiserum originally described by Natali and Tan (16) contains specifically monomerizes pyrimidine dimers in DNA. The predominant activity, as measured with native DNA, appears to be directed at C-T and/or C-C dimers but not T-T dimers. This was concluded from the following observations: a) measurable binding was obtained with double-stranded TA(254) antigen (contains all 3 types of dimers) at relatively high antiserum dilutions, but no binding was apparent under similar conditions when the TA(Ac0) antigen was used. The TA(Ac0) contains only the thymine homodimer.3; and b) calf thymus DNA treated with acetoephene and 313-nm radiation which induced high levels of thymine homodimers did not compete for binding of antibodies against the TA(254) antigen (Table 1). Calf thymus DNA exposed to the FS-40 sunlamp, which induced the 3 types of dimers, competed effectively with the TA(254). The amount of binding that occurred at C-Py dimers appeared to increase as a linear function of dose; i.e., a doubling of fluence halves the amount of DNA required to obtain 50% inhibition of binding to the TA(254) antigen (Charts 2 and 3). The second activity in the immune serum is also specific for pyrimidine dimers but appears to recognize only T-T dimers. Measurable binding was observed with the TA(Ac0) antigen and, unlike the activity against the C-Py dimers, de- naturation of the DNA enhanced binding to the TA(Ac0) at all antiserum dilutions tested (Chart 1B). The binding measured with TA(Ac0) was also different in that the capacity of the CA to inhibit binding to TA(Ac0) increased as a nonlinear function of fluence. The slope of the curve obtained for fluence versus efficiency of competition was calculated to be -1.9. Thus, the binding capacity of the CA appeared to increase as a function of fluence squared (F2). With a F2 response, a doubling of the UVR exposure to the competing DNA resulted in a 4-fold reduction in the concentration of DNA required to obtain a given degree of inhibition. A F2 response for an increase in binding sites in the TA(Ac0) assay system would result if measurable binding of the antibody required bivalent attachment of one antibody to 2 thymine dimers that occur in close proximity.

It had been suggested that the antigenic determinant in UV- irradiated DNA is the conformational distortion associated with the dimer in DNA rather than the dimer itself (23). The data presented here suggest that a level of recognition does reside in the dimer itself, as judged by the ability to distinguish between C-Py dimers and the thymine homodimer. Furthermore, the differential binding that we have observed appears to be the property of a single antibody as judged by our inability to separate the 2 binding activities with immunoaBSorption (Chart 5).

The specificity of these RIAs may be used to measure the different rates of induction of C-Py and T-T dimers and, perhaps more importantly, the kinetics of repair of these 2 classes of dimers. There is some evidence that for organisms exposed to sunlight C-Py may be of particular biological importance (4). At the wavelengths of interest in skin carcinogenesis, i.e., 290 to 320 nm, C-Py dimers are likely to occur more frequently in exposed DNA than are T-T dimers. In addition, a substantial fraction of mutations induced by UVR is due to the cytosine residue in C-T dimers (19).

In addition to a high degree of specificity, the RIAs described herein also have a reasonable degree of sensitivity. The lowest fluence used in these studies, 250 J/sq m from the FS-40 sunlamp, is equivalent in terms of the number of dimers induced to 4 J/sq m at 254 nm.3 This is similar to the sensitivity of currently used chromatographic and enzymatic methods to measure pyrimidine dimers and comparable to the sensitivity of the RIAs developed recently by Mitchell and Clarkson (15). RIAs have the additional advantage that dimers can be measured in unlabeled DNA. This is particularly attractive for the

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**Chart 5.** Percentage of binding activity remaining in the immune serum following immunoabsorption with varying concentrations of irradiated, native DNA bound to cellulose. The immunoabsorbent was exposed to 6000 J/sq m at 254 nm. Binding activity was measured with TA(254) (○) and TA(Ac0) (□).
measurement of UVR-induced dimers in nondividing cell populations. Studies are in progress to increase the sensitivities of the assays with other antisera and monoclonal antibodies.

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REFERENCES

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