ABSTRACT

Monoclonal antibodies specific for DNA damaged by 8-methoxypsoralen (8-MOP) plus ultraviolet A (UVA) light were used to study adduct formation in human keratinocytes and mouse and rat skin in vitro. This antibody does not cross-react with unmodified DNA or free 8-MOP. Sensitive competitive enzyme-linked immunosorbent assays with color or fluorescence endpoints were used to quantify adducts on DNA isolated from treated keratinocytes or skin samples. Localization of 8-MOP-DNA adducts was studied by indirect immunofluorescence with fluorescein-conjugated anti-mouse-IgG antibodies. When cultured keratinocytes were treated with 8-MOP and UVA, immunofluorescence was localized in the nucleus. There was no fluorescence in untreated control cells or treated cells incubated with nonspecific serum. Comparison of intensity of immunofluorescence staining with quantitation of adduct levels by enzyme-linked immunosorbent assay indicated that the limit of sensitivity of the immunofluorescence technique is 9.0 fmol adduct/µg DNA or 2.9 adducts/10^6 nucleotides.

INTRODUCTION

PUVA has been used clinically in the treatment of a number of skin diseases including psoriasis, a hyperproliferative disease of the epidermis. It has also been used extracorporeally as an experimental cytoreductive treatment for the leukemic phase of cutaneous T-cell lymphoma, and as a form of immunosuppression (2). While it is generally assumed that the efficacy of this treatment results from DNA damage, a recent study has suggested an alternative mechanism involving interaction of 8-MOP with specific membrane-associated binding sites (3). Psoralens photoreact with pyrimidine bases forming both monofunctional and bifunctional adducts (interstrand cross-links) (4–6). This damage interferes with DNA replication, inhibiting DNA synthesis (7). In vitro, studies of cultured human cells have shown that these photoadducts are mutagenic (8–10). In vivo, squamous and basal cell carcinoma of the skin have occurred in animals treated with 8-MOP and UVA (11). Several case reports and epidemiological studies have suggested PUVA treatment may also be carcinogenic in humans (12–15). Recently, we have developed a panel of monoclonal antibodies which specifically recognize 8-MOP-DNA photoadducts (16). These antibodies have been used in highly sensitive competitive ELISA to quantify 8-MOP adducts on DNA from cells treated in culture (16). More recently we have begun to measure DNA adduct levels in the lymphocytes of patients undergoing extracorporeal photopheresis for cutaneous T-cell lymphoma (17).

Another group (18–20) has developed polyclonal antibodies to 8-MOP-DNA and used them for immunofluorescence localization of adducts. However, the sensitivity of their antibody has not been determined in terms of amount of adduct detectable. Here we report on the development of highly sensitive competitive enzyme-linked immunosorbent assays for quantitation of 8-MOP-DNA adducts at the femtomole level. In addition, indirect immunofluorescence techniques have been developed for visualization of 8-MOP-DNA adducts in both cultured cells and animals treated in vivo with 8-MOP and UVA. The sensitivity of this assay was determined by quantitation of DNA adduct levels by competitive ELISA.

MATERIALS AND METHODS

Chemicals. 8-MOP, calf thymus DNA, goat anti-mouse IgG-alkaline phosphatase conjugates, p-nitrophenyl phosphate, 4-methylumbelliferyl phosphate, DNase, RNase, proteinase K, EDTA, and hydrocortisone (0.4 µg/ml), penicillin (100 U/ml), streptomycin (0.1 mg/ml), and fungizone (2.5 µg/ml) supplemented with 10% heat-inactivated FCS.

Keratinocyte growth medium consisted of minimum essential medium, Earl's salts, trypsin (1:250), l-glutamine, antimycotic antibiotic solution, and nonessential amino acid mixture were obtained from Gibco, Grand Island, NY. FCS was obtained from Sterile Systems, Logan, UT.

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2 To whom requests for reprints should be addressed, at College of Physicians and Surgeons, Columbia University, Institute of Cancer Research, 650 West 168th Street, New York, NY 10032.

3 The abbreviations used are: PUVA, 8-methoxypsoralen plus ultraviolet A light (320–340 nm); 8-MOP, 8-methoxypsoralen; PBS, phosphate buffered saline; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum.
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Treatment of Animals. BALB/c female mice or Sprague-Dawley rats were shaved and residual hair removed by treatment with Nair (Carter-Wallace Inc, New York, NY). Two days later animals were injected intradermally (i.d.) with 100 ng 8-MOP/cm² (0.1% 8-MOP in 20% ethanol, 34% ethylene glycol) or i.p. with 30 ng 8-MOP/g body weight. After 1 h, animals were exposed to 12J/cm² UVA. Animals were sacrificed and dorsal skin was removed surgically. For DNA extraction studies, the excised skin was incubated in 0.5% trypsin at 4°C for 12-14 h. Epidermal sheets were peeled from the dermis and DNA was extracted from epidermis by standard phenol and RNaSe procedures.

Quantification of DNA-8-MOP Photoadducts by Competitive ELISA. 8-MOP-DNA adducts were quantitated by competitive ELISA with color or fluorescence endpoints using antibody 8G1 (16) by procedures described elsewhere (23). Briefly, for the color assay, Immunol 2, 96-well U-bottom microwell plates (Dynatech Laboratories, Inc., Alexandria, VA) were coated with 10 ng denatured 8-MOP-modified calf thymus DNA in PBS by drying at 37°C overnight. For the fluorescence assay, polystyrene U-bottom black plates (MicroFLUOR-B; Dynatech) were coated with 0.5 ng denatured 8-MOP-DNA/well. Coated plates can be stored at 4°C for several months. After automatically washing the plates with PBS-Tween (0.05%) (Multiwash; Flow Laboratories, McLean, VA), wells were incubated with 200 µl 1% FCS in PBS-Tween. Monoclonal antibody 8G1 was diluted 1:3 x 10⁸ for the color ELISA and 1:6 x 10⁸ for the fluorescence ELISA. For the standard curve, serial dilutions of in vitro-modified 8-MOP-DNA (50 µl) were mixed with the diluted antibody (50 µl) and added to the plate. The antigen-antibody mixture was incubated at 37°C for 90 min, then the plates were washed. Goat anti-mouse IgG-alkaline phosphatase conjugate was then added (100 µl, 1:250 for color ELISA and 1:500 for fluorescence ELISA) and incubation continued for another 90 min. The wells were then washed with PBS-Tween and with 0.01 M diethanolamine buffer, pH 8.6. For the color assay, the substrate was p-nitrophenyl phosphate (1 tablet, Sigma 104)/5 ml 1 M diethanolamine, pH 8.6. Absorbance at 405 nm was read on a Titertek Multiskan MC (Flow Laboratories). For the fluorescence assay, the substrate was 4-methylumbelliferone phosphate (50 µg/ml 0.1 M diethanolamine, pH 9.6), and fluorescence was read on a 96-well fluorescence reader (MicroFLUOR; Dynatech). All assays were done in duplicate on at least two different days.

Immunofluorescence Studies. Mouse or rat skin, snap frozen in OCT (Lab-Tek Products, Naperville, IL), was cut with a cryostat (American Optical, NY) in 4-µm sections, placed on slides and fixed with 70% ethanol at room temperature until used. Immunofluorescence staining of human keratinocytes and mouse or rat skin was carried out essentially as described by Huitfeldt et al. (24). To enhance antibody interaction with antigenic sites shielded within the chromatin structures, PBS-washed slides were first treated with RNase A (100 µg/ml) at 37°C for 1 h, then washed twice in PBS. Slides were then incubated with proteinase K (10 µg/ml) at room temperature for 10 min, and washed twice with PBS. Finally, 4 N HCl was added for 20 min at room temperature. Specific monoclonal antibody (9D8) (16) was diluted 1:10 to 1:100 in 20% FCS PBS-Tween and slides treated at 37°C for 30 min. After washing with PBS, goat anti-mouse IgG conjugated with fluorescein isothiocyanate, diluted 1:40 in 20% FCS PBS-Tween, was added for 30 min at 37°C. After washing, slides were mounted in p-phenylenediamine (1 g/liter) to prevent fading (24). Slides were examined with an Olympus IM microscope. A filter block with a 530 barrier filter and a BG12 excitation filter was used for fluorescein observation.

RESULTS

Human keratinocytes cultured on chambered slides or on dishes were treated under identical conditions with 8-MOP and irradiated with 12 J/cm² UVA. Immediately after irradiation, slides were fixed in ethanol for immunofluorescence staining and DNA isolated from the cells grown on dishes for quantitation by ELISA. Four previously characterized antibodies to 8-MOP-DNA (16) were tested for use in immunofluorescence staining and antibody 9D8 was determined to be the most sensitive. Conditions were also selected that were optimal for immunostaining. As described in “Materials and Methods,” treatment of the fixed slides with proteinase K and RNase followed by HCl treatment to denature the DNA and make it more accessible to antibody binding gave the best immunostaining. Specific nuclear staining could be detected after treatment with 10, 5, 2.5, 0.5, and 0.25 µg/ml 8-MOP and 10 J/cm² UVA. Typical results for treatment with 10 and 0.25 µg/ml 8-MOP are given in Fig. 1, a and b, respectively. With treatment at 10, 5, and 2.5 µg/ml nuclear staining was homogeneous while with 0.5 and 0.25 ng/ml staining was granular and weaker. No staining of the cytoplasm could be seen. Dimethyl sulfoxide-treated control cells had no specific staining (Fig. 1c). Additional control experiments were carried out with 8-MOP-treated cells. They included use of a nonspecific antibody instead of the anti 8-MOP-DNA-specific antibody, elimination of the specific antibody to test for nonspecific binding of the fluorescein isothiocyanate conjugate, DNase treatment before staining (Fig. 1d), and staining with anti-8-MOP-DNA antibody preabsorbed with 8-MOP-DNA (Fig. 1e). In all controls nuclear staining was eliminated. Therefore positive staining was due to specific binding of antibody to 8-MOP-DNA. Table 1 summarizes the data on treatment and positive immunostaining of keratinocytes. In addition, Table 1 gives quantitative data on adduct levels in several samples; these data are discussed below.

Before treatment of animals with 8-MOP, the optimum time of irradiation after i.p. administration had to be determined. A mouse was treated with [³H]8-MOP and blood samples removed at various time points. Measurement of [³H] indicated that peak levels of 8-MOP occurred 1 h after i.p. injection (data not shown). Therefore, mice or rats were injected i.p. with 30 µg 8-MOP/g body weight in corn oil 1 h before UVA irradiation. Total UVA dose was 12 J/cm² and immediately after irradiation skin was removed both for immunofluorescence staining and DNA isolation. Immunohistochemical staining of cryostat sections of mouse skin from i.p. treatment indicated that adducts were localized mainly in keratinocytes of the epidermal layer (Fig. 2, a and b). Similar results were obtained with mice treated i.d. with 100 µg 8-MOP/cm² (not shown). Rats were also treated i.p. with 30 µg 8-MOP/g body weight. Fig. 2c indicates that positive staining was localized in basal cells (arrow). A control animal receiving UVA without 8-MOP had no positive nuclear staining but did show nonspecific staining of the outer layer. The arrow indicates the position of the basal cells (Fig. 2d).

Quantitation of 8-MOP-DNA Adducts. Previous studies (16) indicated that antibody 8G1 was the most sensitive antibody for detection of 8-MOP-DNA adducts in a competitive ELISA with color detection (50% inhibition at 17 fmol). An even more sensitive assay (50% inhibition at 4 fmol) was developed with a fluorescent endpoint. Details of this assay are given in “Materials and Methods” and involve use of higher dilutions of the specific antibody to test for nonspecific binding of the fluorescein isothiocyanate conjugate, DNase treatment before staining (Fig. 2d), and staining with anti-8-MOP-DNA antibody preabsorbed with 8-MOP-DNA (Fig. 1e). In all controls nuclear staining was eliminated. Therefore positive staining was due to specific binding of antibody to 8-MOP-DNA. Table 1 summarizes the data on treatment and positive immunostaining of keratinocytes. In addition, Table 1 gives quantitative data on adduct levels in several samples; these data are discussed below.

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Fig. 1. Primary human keratinocytes treated with various concentrations of 8-MOP or untreated controls. After fixation, nuclear staining was enhanced as described in "Materials and Methods." Anti 8-MOP-DNA antibody 9D8 was applied at a dilution of 1:10–1:100 and final incubation was with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (1:40 dilution) as follows: A, 10 μg/ml 8-MOP treatment; 450 ×; B, 0.25 μg/ml 8-MOP treatment, 450 ×; C, dimethyl sulfoxide-treated control, 450 ×; D, 10 μg/ml 8-MOP treatment followed by treatment with DNase before immunostaining, 450 ×; E, 10 μg/ml 8-MOP treatment, anti-8-MOP-DNA antibody was absorbed with 8-MOP-DNA before staining. All samples received 12 J/cm² UVA irradiation.

were then measured by color or fluorescence ELISA. Animals were treated only with nonlabeled 8-MOP. Table 2 compares modification levels as determined by the three techniques. In general there is good agreement with the ELISA data and that determined by radioactivity. The lowest treatment level of keratinocytes that gave positive immunofluorescence staining was 0.25 μg/ml 8-MOP. Adduct levels for this sample (Table 1) allowed determination of the limit of sensitivity of immunostaining. As little as 9.0 fmol 8-MOP-DNA adduct/μg DNA corresponding to 2.9 adducts/10⁶ nucleotides could be detected.

DISCUSSION

The development of highly specific monoclonal antibodies to 8-MOP-modified DNA has allowed the detection and quantitation of these adducts in various biological samples. These antibodies do not cross-react with nonmodified DNA or free 8-MOP. They have been well-characterized in terms of cross-reactivity with specific 8-MOP-DNA adducts and other psoralen-modified DNAs (16). They react preferentially with the 4',5'-monoadduct but there is some reactivity with cross-linked

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adducts. DNA modified with angelicin and trimethylpsoralen also react with the antibody but at concentrations several orders of magnitude higher than with 8-MOP-DNA. Highly sensitive competitive ELISA with color or fluorescence detection of enzyme activity have been validated by simultaneous determination of 8-MOP levels with $[^3H]8$-MOP. While there is a higher variation in the values as determined by ELISA due to their inherent variability, the data correspond well with those determined from radioactivity.

These monoclonal antibodies are also useful for studies on tissue localization of DNA adducts. Immunofluorescence staining of treated keratinocytes was limited to the nucleus at all treatment levels examined. At high doses of 8-MOP intense nuclear staining was observed while diffuse fluorescence was seen at 500 and 250 ng/ml 8-MOP. At low doses of 8-MOP, not all cells gave equal staining. Treatment with proteinase K, RNase, and HCl before immunostaining increased the sensitivity of the assay. Current limits of sensitivity are around 9.0 fmol 8-MOP-DNA adduct/μg DNA which corresponds to about 2.9 adducts/10⁶ nucleotides. We are currently investigating the use of computer-assisted, video-enhanced microscopy to increase this sensitivity. It has been reported that as few as 300 adducts per cell (3 adducts/10⁷ nucleotides) can be seen with a highly sensitive antibody to $O^6$-methylguanosine (25).

PUVA has been used clinically for the treatment of vitiligo psoriasis for many years but more recently several new experimental treatments with this combination therapy have been developed (2). Thus, the number of people exposed to this treatment will increase. This population provides an ideal model system for the development and validation of molecular epidemiological approaches to monitoring humans for exposure to environmental carcinogens (26). This approach attempts to combine laboratory techniques for measuring human exposure with traditional epidemiology in the hope of obtaining more relevant data on risk assessment. PUVA patients receive defined, quantifiable exposures to 8-MOP and UVA. The techniques presented here should allow measurement of 8-MOP DNA adducts in lymphocytes of exposed individuals. In addition, skin biopsy samples can be examined by quantitative

### Table 1 Comparison of immunofluorescence staining of keratinocytes to 8-MOP-DNA adduct levels as measured by competitive ELISA

<table>
<thead>
<tr>
<th>8-MOP dose (μg/ml)</th>
<th>Relative immunofluorescence staining</th>
<th>8MOP-DNA adduct level (fmol adduct/μg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>++++</td>
<td>ND*</td>
</tr>
<tr>
<td>5.0</td>
<td>+++</td>
<td>ND*</td>
</tr>
<tr>
<td>2.5</td>
<td>+++</td>
<td>104 ± 31</td>
</tr>
<tr>
<td>0.5</td>
<td>++</td>
<td>39 ± 2</td>
</tr>
<tr>
<td>0.25</td>
<td>+</td>
<td>9 ± 6</td>
</tr>
<tr>
<td>0</td>
<td>−</td>
<td>ND, not determined</td>
</tr>
</tbody>
</table>

* All cells received 12 J/cm² UVA.

* As determined by competitive ELISA with color endpoint. Values, mean ± SD of triplicate assays on a single sample.

ND, not determined.
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<table>
<thead>
<tr>
<th>Table 2</th>
<th>Quantitation of 8-MOP-DNA adducts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>8-MOP treatment</td>
</tr>
<tr>
<td></td>
<td>[H]#</td>
</tr>
<tr>
<td>In vitro</td>
<td></td>
</tr>
<tr>
<td>Human keratinocyte</td>
<td>2.5 µg/ml</td>
</tr>
<tr>
<td>Human keratinocyte</td>
<td>0.5 µg/ml</td>
</tr>
<tr>
<td>In vivo</td>
<td></td>
</tr>
<tr>
<td>Mouse skin i.d.</td>
<td>100 µg/cm²</td>
</tr>
<tr>
<td>Mouse skin i.p.</td>
<td>30 µg/l</td>
</tr>
</tbody>
</table>

* Values, mean ± SD of triplicate assays on a single sample.
# Adduct levels were quantitated by ['H counting.

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Immunological Detection and Visualization of 8-Methoxypsoralen-DNA Photoadducts

Xiao Yan Yang, Vincent DeLeo and Regina M. Santella


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