# Immune Serum against Anti-DNA-8-Methoxypsoralen Photoadduct

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Specific immune serum against photo-damaged DNA with the photoadducts of 8-methoxypsoralen (8-MOP) was obtained. The antigenic determinant is a polynucleotide chain with a mono-photoadduct: a coumarin moiety of psoralen linked through a cyclobutane ring to the 5,6-bond of thymine. The specific rabbit antiserum was used in the immunofluorescence method, for the detection of photodamaged DNA in the kinetoplasts and nuclei of unicellular organisms *Crithidia luciliae*, in the nuclei of snap-frozen tissue of mammals, and in human blood lymphocytes. Detection by the immunofluorescence method was limited by psoralen availability *in situ*; the psoralen concentration should be in the range 0.05–0.2 μg/cm$^2$ on specimens submitted to a topical application or about 1 μg/ml in cell suspension. The long-wave ultraviolet light (UV-A) exposure dose applied to the nuclei should exceed 1 J/cm$^2$.

Immune serum against DNA-8-MOP-photoadduct antibodies was first obtained in 1976 [1] and its properties have been described [2]. This serum has been used in IF method for detection of DNA *in situ* changed after irradiation of specimens at 365 nm (UV-A) in the presence of 8-methoxypsoralen (8-MOP). A full account of experiments with specimens irradiated *in vitro* and with mice irradiated *in vivo* will be published elsewhere [2a]. This report concerns some details of the immunization procedure and characteristics of the immune serum. We describe here the limits of detection of photodamaged DNA *in situ* as a result of a combined action of psoralen and UV-A irradiation and the titers on the various antigenic substrates.

## Materials and Methods

### *Chemicals*

DNA from calf thymus was from several sources: Miles, Sigma, and Calbiochem; MBSA from Calbiochem, 8-MOP from the Food and Drug Administration, Washington D.C.; the other psoralen derivatives: psoralen, 5-methoxypsoralen (bergapten), 4,5′,8-trimethylpsoralen and angelicin were kindly donated by Prof. G. Rodighiero, University of Padua, Italy. We are indebted to Dr. P. Strickland, Frederic Cancer Research Facility, USA for one sample of Miles DNA (South Africa Labs.).

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### *Preparation of the antigenic DNA*

DNA was dissolved in 2 mM NaCl, pH 7, at a concentration 1.3 mg/ml, 30% (v/v) glycerol was added and the solution sonicated with an MSE 100 W oscillator for 2 × 20 s in an ice bath. DNA molecular weight was thus decreased 4-fold to 5 × 10$^5$ daltons, as checked by ultracentrifugation. The sonicated DNA was dialyzed once against 0.15 M NaCl, pH 7 and then 3 times against 2 mM NaCl, pH 7. To 5 ml of this DNA, diluted to 0.5 mg/ml, were added 10 μl of ethanolic 8-MOP at a ratio 40 μg 8-MOP/500 μg DNA and then irradiated with magnetic stirring in a 5-cm Petri dish covered with a quartz disc.

Irradiation was with an Osram HQV 125 high pressure mercury lamp, emitting principally at 365 nm. The fluence rate was 41.5 W/m$^2$ at a distance of 3 cm, measured with an International Light 442 Phototherapy Dosimeter. Protection against heat emitted by the lamp was attained by irradiation in the cold room at 6 °C and by the use of an electric fan. The extent of photoaddition of 8-MOP to DNA was checked by means of temperature profiles [3]. The irradiated polymer was dialyzed 4 times against 0.15 M NaCl, pH 7 and then com-

plexed with MBSA in a 1:1 ratio (by weight) and, after mixing with an equal volume of complete Freund's adjuvant, injected into rabbits.

*Immunization*

5 Male rabbits of white race, about 3 months old, were immunized according to Tan and Stoughton [4], alternately by intramuscular and intracutaneous injections. After 4 and 5 weeks, blood samples were taken and the sera were checked by immunofluorescence on CL cells UV-A irradiated in the presence of 8-MOP. The rabbits were sacrificed and their sera preserved at − 20 °C.

*Immunofluorescence test (IF)*

The principals of the immunofluorescence method have been previously described [2]. In these studies the following sera were also used: anti-DNA-pyrimidine-dimer and anti-horseradish peroxidase as immune sera to unrelated antigens; these two sera raised in rabbits served as negative controls. As positive controls of nuclear staining served either SLE human serum with a high titer (above 80) of anti-nuclear antibodies, tested on cryostat section, or SLE serum containing the anti-native-DNA antibodies, tested on CL cells.

*Preparations of specimens submitted to 8 MOP-UVA treatment*

*Crithidia* cells or lymphocytes, spread on slides as monolayers, dried and fixed in ethanol, and cryostat sections of snap-frozen tissues, were gradually soaked with an aqueous solution of 8-MOP (about 40 μg/ml), and air dried, leaving about 0.2−0.3 μg of solute per square centimeter, and then irradiated with an appropriate dose of UV-A. Control specimens were not treated or submitted either to 8-MOP saturation or to UV-A irradiation. The IF test was carried out directly after the 8-MOP-UVA treatment, which was made in as short a time as possible, in order to avoid overdrying specimens.

## Results

*Production of immune serum against DNA-8-MOP-photoadduct*

Two of the seven immunizations, made with the different antigenic DNA preparations were successful. In one trial, two of the five rabbits elicited high titer of antibodies as tested on CL cells. In the second positive trial, three of the five rabbits elicited antibodies. The results of both negative and positive immunizations indicate that the basic conditions for production of an antigenic DNA are as follows: (i) a short chain DNA, (ii) a high dose of UV-A irradiation, about 80 J/cm$^2$.

Both successful immunizations were obtained with Miles DNA, perhaps because this gave the shortest chain after sonication.

The DNA-8-MOP-photoadduct antigen, after an irradiation with 35 J/cm$^2$, gave invariably negative results in the immunization trials, even if heat denaturated following the 8 MOP-UVA treatment. After a dose of 35 J/cm$^2$ of UV-A the antigen showed an optimal psoralen photoaddition to DNA as inferred from the shape and Tm of temperature profiles, very similar to those obtained after 50 J/cm$^2$ or 80 J/cm$^2$ of UV-A dose.

Four weeks of immunization were sufficient to obtain an optimum level of antibodies. The next boosting did not cause an appreciable increase, and rabbits with a low titer did not show an increase in the antibody level in their sera. The immune sera were quite stable, at − 20 °C they could be stored for several years.

*Limits of detection of photodamaged DNA in situ*

The data presented in Table I show the optimal conditions, and the limits of detection (the titer) of photodamaged DNA on the various substrates. The best one was a cryostat section of monkey/rabbit esophagus. The fluorescence of tissue section nuclei disappeared when submitted to 8-MOP-UVA treatment with the dose below 0.05 μg/cm$^2$ of 8-MOP and 1 J/cm$^2$ of UV-A. *Crithidia luciliae* (CL) cells were equally a good substrate; here, the indication of the end point of serum reaction was the disappearance of the fluorescence of kinetoplasts of CL cells.

*Penetration of psoralens into cellular DNA*

Several psoralen derivatives have been tested on CL cells in order to establish the chemical nature of the antigenic determinant(s) of the DNA-8-MOP-photoadduct. The 8-MOP-UVA treated specimens, submitted to the IF test with anti-DNA-8-MOP-photoadduct serum, showed staining of the two

Table I. Detection of photodamaged DNA in specimens submitted to 8 MOP-UVA treatment.

| Substrate | 8 MOP-UVA 8-MOP dose | Treatment: UV-A exposure [J/cm$^2$] | IIF reaction with anti-DNA-8-MOP photoadduct, titer[a] |
|---|---|---|---|
| Crithidia luciliae cells fixed on slides | 0.20 μg/cm$^2$ | 7–45 | 320 |
| Crithidia as above | 0.05 μg/cm$^2$ | 3.5 | 320 |
| Crithidia as above | 0.05 μg/cm$^2$ | 1 | 160 |
| Lymphocytes fixed on slides | 0.20 μg/cm$^2$ | 14 | 16 |
| Crithidia luciliae cells in suspension[b] | 50 μg/ml | 21 | 160 |
| Lymphocytes in suspension[b] | 5–50 μg/ml | 21 | 80 |
| Cryostat sections (monkey and rabbit esophagus, monkey and guinea pig lip, mouse kidney) | 0.20 μg/cm$^2$ | 7 | 640 |

[a] The titer indicated was tested with the use of immune serum obtained from the rabbit with the highest titer of antibodies (No 91). The titer for sera from other rabbits was two to three times lower.

[b] CL cells and lymphocytes, $10^6$ cells/ml suspended in RPMI 1640 medium, preincubated with 8-MOP 30 min, and UV-A irradiated in Petri dishes.

Table II. The penetration of psoralen derivatives into cellular components of CL cells. Detection by IF test after 8 MOP-UVA exposure[a].

| Psoralen derivative | IF reaction with anti-DNA-8-MOP-photoadduct[b] | |
|---|---|---|
| | on nuclei | on kinetoplasts |
| 8-methoxypsoralen | 10, slightly visible | 320 |
| 5-methoxypsoralen | 20, slightly visible | 160 |
| psoralen | 40, slightly visible | 160 |
| 4,5′,8-trimethyl-psoralen | invisible | invisible |
| angelicin | 640 | 640 |

[a] The smears of CL cells were soaked with 0.2–0.3 μg/cm$^2$ of appropriate solute, then irradiated with 7 J/cm$^2$ of UV-A.

[b] The numbers indicate the titer of the immune serum obtained from the rabbit with the highest titer of antibodies. The other sera tested on the same substrates had lower titers, but not below 80 on the kinetoplasts.

cell components: nucleus and kinetoplasts. The level of fluorescence staining of both DNA sites was dependent mainly on the penetration property of psoralen derivative as shown in Table II.

The level of IF staining was related to the water solubility of the compounds: the highest solubility index produced the best fluorescence staining, while the 10-fold less soluble trimethylpsoralen did not react with nuclear material within the cell. These findings have been proved in *in vivo* experiments on mice (to be published). The intracutaneous injection of angelicin gave much better nuclear staining of the epidermal cells of mice than that submitted to the 8-MOP injection and UV-A.

## Discussion

Positive immunization with short-chain DNA irradiated with a high UV-A dose, and negative immunization trials with long-chain DNA, indicate that only the first is able to reveal antigenic determinants and induce the production of antibodies. The primary photoadducts of psoralen, formed in DNA, are completely shielded in the regular helical structure, since cross-links prevent DNA unfolding. The secondary photoproducts, which are presumably formed at high UV-A exposure, 30–80 J/cm$^2$, could be due to photolysis of the furan ring of psoralen mono-photoadduct [5]. They could form non-regular regions of DNA polymer, including primary photoadducts, and should prevent DNA folding. Thus, secondary photolysis products of psoralen-thymine photoadducts with an open furan ring would be merely destabilizing agents of

DNA-8-MOP-photoadduct, and they would induce the unmasking of antigenic determinants.

Our immune serum revealed IF nuclear staining after 8 MOP-UVA treatment with other linear psoralen derivatives. The methoxy groups at the 5 or 8 position of the coumarin moiety do not change the reaction with antibodies. A positive reaction was also obtained with the angular isomer of psoralen, *i.e.* angelicin, and this fact leads to the first conclusion that the antigenic determinant is not the whole psoralen molecule, but only its coumarin moiety. One can visualize the antigenic determinant as a coumarin moiety of psoralen with its pyrone ring attached to thymine by a cyclobutane ring. The hypothetical antigenic determinant is presented in Fig. 1. This determinant should be embedded in the polynucleotide chain in order to be recognized by an antibody, because the DNA-8-MOP-photoadduct, thoroughly digested by deoxyribonuclease, did not give a precipitin line in the immunodiffusion test [2].

Anti-DNA-8-MOP-photoadduct serum recognizes DNA-angelicin-photoadduct produced in the nuclei of CL and tisssue cells. It is known that angelicin does not form cross-links in normal DNA [3], and, from this, one may draw a second conclusion, *viz.* that our specific immune serum recognizes the *mono*-photoadduct of psoralen. Furthermore, it appears likely that the cross-link "seen" by the antibody is recognized merely as a mono-photoadduct.

The limit of detection of photodamaged DNA in the presence of psoralen, described in this report, looks promising, bearing in mind the application of the IF test *in vivo*, particularly in humans submitted to 8 MOP-UVA treatment (so called PUVA therapy). Formation of photoadducts between psoralen and pyrimidine bases is regarded as the most important effect of 8 MOP and longwave ultraviolet treatment [6]. Detection of photochanged DNA in PUVA-treated skin might be a useful test for assessing risk of skin cancer [7] and other UV-A induced damage in patients with psoriasis under chronic PUVA treatment.

However, it shows that the IF method is only applicable in tissue which accumulates a sufficient concentration of psoralen in the nuclei during the PUVA treatment.

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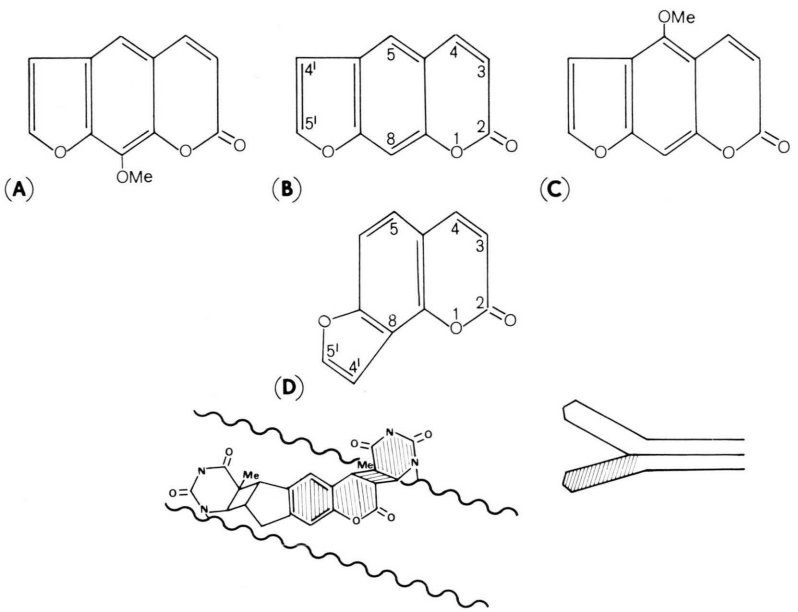


Fig. 1. Schematic representation of the antigenic determinant of anti-DNA-8-MOP-photoadduct. (A) The 8-methoxypsoralen which seerved as the component of antigenic DNA in the immunization procedure; (B, C, D): psoralen, 5-methoxypsoralen, angelicin, respectively, recognized by the immune serum anti-DNA-8-MOP-photoadduct after photoaddition of these molecules to DNA in the nuclear site. Bottom: complementary DNA strands with psoralen cross-link to two thymine molecule. The hatched rings show the components of the hypothetical antigenic site recognized by the specific antibody of our immune serum.

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