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Peroxidase Technique for the Detection of Photochemical Lesions in Intracellular Deoxyribonucleic Acid

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Summary

The widespread use of phototherapy for the prevention and treatment of neonatal hyperbilirubinemia has generated some concern as physiologic substances other than bilirubin may be photoactivated. Little information is available on the long term toxicity of these photodecomposition products. Recent observations of the *in vitro* DNA-modifying activity of phototherapy lights has encouraged us to develop laboratory procedures which can identify and quantitate these light-induced alterations. The purpose of the present study was to develop a technique capable of detecting photochemical changes in the genetic material of human cells in tissue culture. The results demonstrate that the antinucleoside peroxidase staining procedure is capable of detecting changes in the DNA of human cells exposed to physiologic (riboflavin) and nonphysiologic (methylene blue) photosensitizing agents in the presence of light with a fluence rate (450 nm) of 141 $\mu W \cdot cm^2$.

Speculation

In view of the known relationship between the ability of a number of environmental agents to modify DNA on the one hand and their potential to induce mutations and cancer on the other, the present study, which documents the DNA-modifying potential of phototherapy, raises questions regarding the safety of this therapeutic maneuver. The further development of techniques described here to identify and quantitate photochemical alterations in the genetic material of irradiated infants would enable us to intelligently define the risk-benefit ratio of phototherapy and to identify an *at risk* population for long term follow-up studies.

The effects of environmental agents on child health is a subject of considerable interest at the present time (1). Of special concern are the long term effects of such agents and more specifically their carcinogenic, mutagenic, and teratogenic po-

tential which is derived in part from the ability of these agents to modify intracellular DNA. Our laboratory has been systematically evaluating the DNA-modifying potential of an environmental agent commonly utilized in clinical pediatrics for the treatment and prevention of neonatal hyperbilirubinemia-high intensity illumination with visible light or phototherapy. Investigations thus far suggest that the DNA-modifying potential of phototherapy is dependent upon the generation of singlet oxygen and the subsequent oxidation of the guanine moieties of the DNA molecule (8, 9). Because of the frequent exposure of jaundiced infants to this therapeutic maneuver, we have been interested in developing laboratory procedures which can be used clinically to identify and quantitate alterations in the genetic material of irradiated infants. The purpose of the present communication is to describe a system in which the photooxidation of guanine in living human cells in tissue culture can be detected. The procedure described utilizes antibodies which are specific for free cytosine and have been used to detect free base residues in human cells exposed to physiologic (riboflavin) and nonphysiologic (methylene blue) photosensitizing agents.

MATERIALS AND METHODS

Riboflavin was purchased from Sigma Company and methylene blue from Allied Chemical Company.

PREPARATION OF ANTI-C ANTIBODY

The preparation of nucleoside conjugates (2) and the procedure for purification of rabbit anti-C antibody has been described previously (10). Peroxidase labeled sheep anti-rabbit antibody was prepared by the method of Lubit *et al.* (5).

PREPARATION OF CELLS

Human (KB) cells were grown in monolayers on chambered slides (Miles Laboratories) in Eagle's medium containing calf serum (10%) and glutamine. The cells were washed with phosphate-buffered saline containing 5% dextrose (D_5 PBS) and exposed to 25 or 50 μg riboflavin/ml or 10 μg methylene blue/ml in D_5 PBS. After incubation in the dark at 37° for 1 hr the cells were illuminated under a standard phototherapy unit (Dura Test Vita Lite). The unit was protected from direct sunlight and air-cooled to maintain the cultures at 23°. The sample distance from the light source was adjusted to maintain a fluence rate (450 nm) of 141 μW cm^{-2}. All photometric measurements were made with the IL600 A photometer coupled to the IL600 photodensitometer manufactured by International Light, Inc. After illumination the cells were fixed and stained as previously described (5).

RESULTS

Examination of the cells treated with anti-C antibody revealed peroxidase staining only in the nuclei of those cells illuminated in the presence of riboflavin or methylene blue. The intensity of staining reflects the amount of free cytosine residues (Fig. 1*a* and 2). Illumination of the cells in the absence of photosensitizers or cells exposed to methylene blue or riboflavin in the dark failed to demonstrate significant staining (Fig. 1*b*). The specificity of the photochemical reaction was confirmed by demonstrating minimal peroxidase-positive material in cells treated with anti-T. Under these conditions less than 10% of the cells are killed as shown by trypan blue staining.

DISCUSSION

Large numbers of newborn infants are systematically exposed to an environmental agent with DNA-modifying activity. No procedure is presently available which can be used clinically to accurately identify and quantitate alterations in the genetic material of infants receiving phototherapy. Many of the techniques

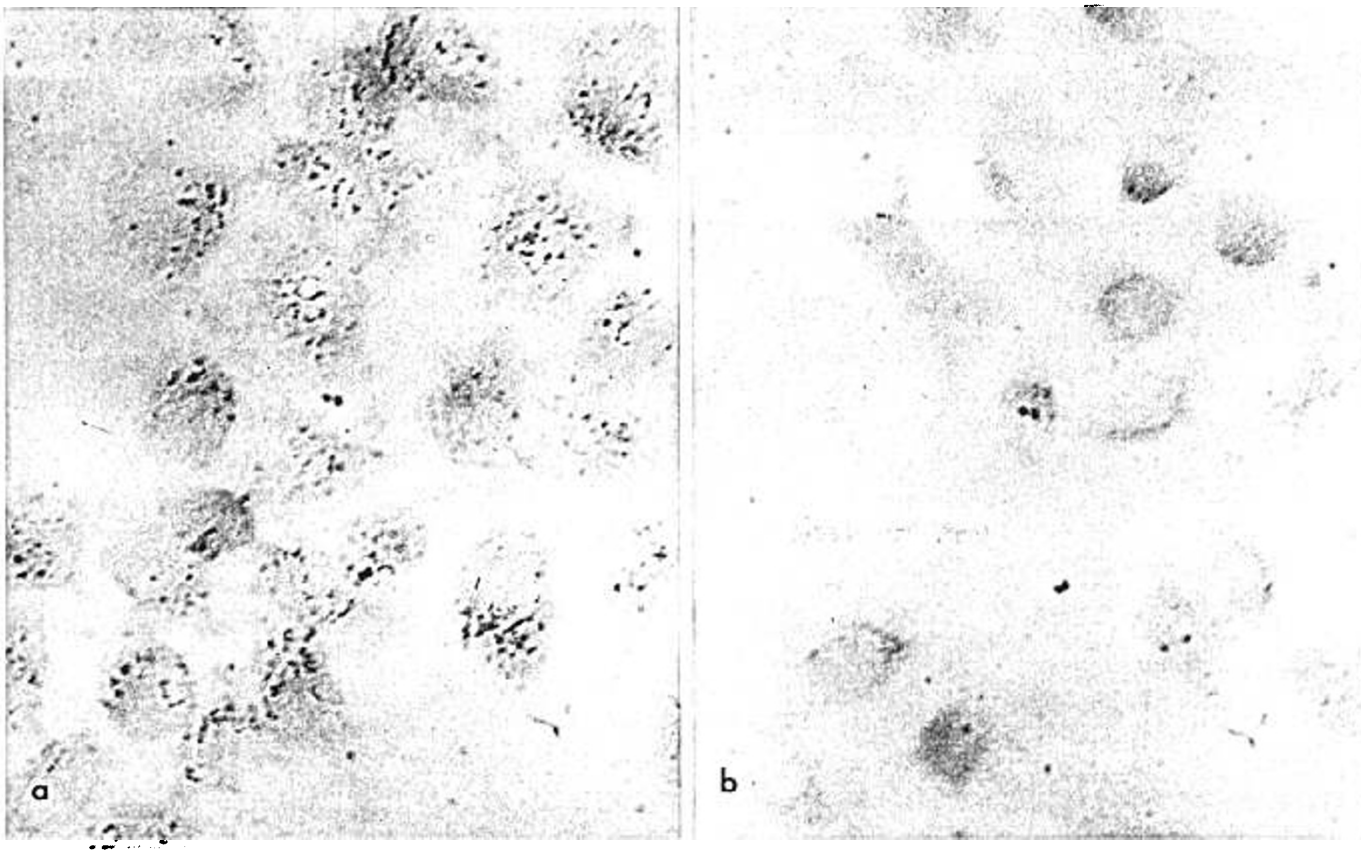

Fig. 1. *a:* peroxidase-positive staining in nuclei of cells grown in monolayers with 50 μg/ml riboflavin and 1 hr of light as described in "Materials and Methods." *b:* control cells treated with 50 μg/ml riboflavin in the dark.

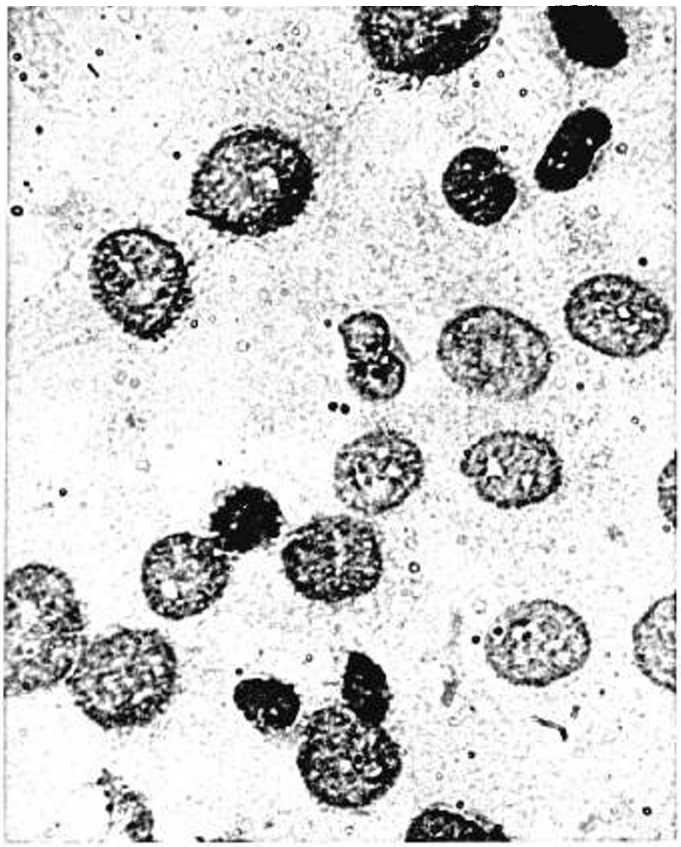

Fig. 2. Peroxidase-positive staining in nuclei of cells grown in monolayers treated with 10 μg/ml methylene blue and 1 hr of light as described in "Materials and Methods."

utilized to study the effects of photosensitizing agents on DNA *in vitro* are complex and do not lend themselves to routine use in a clinical setting.

Antinucleoside antibodies have been developed which specifically react with the purine and pyrimidine group of the immunizing antigen. These antibodies, which react with free base residues, are capable of reacting with single stranded, denatured, or partially denatured DNA. The immunochemical specificities of these reactions have been shown by complement fixation, precipitation, and radioimmunological techniques (3, 6). Photosensitizing agents like methylene blue, in the presence of oxygen and visible light, generate singlet oxygen which selectively destroys the guanine moiety of the DNA molecule (7). Garro *et al.* (3) and others (11) documented the methylene blue photodegradation of guanosine in isolated DNA using cytosine-specific antinucleoside antibody which reacted with the unpaired cytosine residues in the treated DNA. We have previously documented the light-activated methylene blue induced photooxidation of guanosine in the DNA of living human cells utilizing fluorescein-tagged antibody specific for the unpaired cytosine residues of the intracellular DNA (4). Because of occasional difficulties in recognizing fluorescence and the lack of permanence in the treated preparations, we felt that the fluorescent technique was unsatisfactory for routine clinical use.

The peroxidase staining procedure described in this report provides a permanent record which can be easily interpreted by a laboratory technician. Utilizing this procedure we were able to document the methylene blue-induced photooxidation of the guanosine moiety of the DNA in human cells in tissue culture. Moreover, the same technique was used to detect free cytosine residues in human cells exposed to phototherapy lights in the presence of low concentrations of riboflavin. The presence of these unpaired bases confirms our earlier investigations on the effect of light-activated riboflavin, on isolated DNA and the DNA of human cells in tissue culture (8). The present report underscores our earlier recommendations that riboflavin supplementation as an adjunct to phototherapy for hyperbilirubinemia requires thorough evaluation before its use can become routine (8).

The ease of preparation and interpretation of the antinucleoside-peroxidase staining procedure encourages us to further modify this technique to facilitate its use in clinical pediatrics for identifying and quantitating alterations in the genetic material of infants exposed to high intensity illumination or phototherapy.

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