0022-202X/82/7805-0460$02.00/0
THE JOURNAL OF INVESTIGATIVE DERMATOLOGY, 78:460–463, 1982

Vol. 78, No. 6
Printed in U.S.A.

In Vitro Assay for Phototoxic Chemicals

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The photosensitizing potential of chemicals known to produce photosensitivity in humans was compared to chemicals not considered to be photosensitizers in an *in vitro* assay. The assay involved exposure of human lymphoid cells to UVA (320–400 nm), and in some cases UVB (280–320 nm) radiation, in the presence of the chemicals and the assessment of phototoxicity as measured by the incorporation of ^{3}[H]-thymidine into nuclear DNA. All known photosensitizers tested were found to be phototoxic, while the nonphotosensitizing agents, with the exception of retinoic acid, were not phototoxic. Peripheral blood mononuclear cells were compared to a T lymphoblastoid cell line as target cells; the latter were superior in terms of convenience, cost and reproducibility of results. This test system has potential as a predictive assay for detecting additional phototoxic chemicals.

Photosensitivity due to environmental and therapeutic contact with chemicals has attracted clinical and research interest but still remains a poorly understood area of clinical photobiology. Progress has perhaps been hindered by the lack of a universally-accepted, convenient and reliable *in vitro* test for assaying the photosensitizing capacity of chemicals. Thus it is not always possible to confirm that a chemical is the agent responsible for a phototoxic or photoallergic reaction, nor is it possible to reliably predict which chemicals might be photosensitizers.

Many attempts have been made to develop a test of the photosensitizing activity of chemicals. *In vivo* tests have involved observations of the interaction of chemicals and nonionizing radiation with the skin of living humans [1–4] and of experimental animals [4–16]. *In vitro* tests have been used to study this interaction with mammalian cell lines [17,18], red cells [4,19,20], microorganisms [14,15,21,22], and biochemical reactions [23]. No one test has emerged from these studies as being clearly superior to all others and each has had one or more deficiencies. The most common problem encountered has been that of obtaining negative results despite strong clinical evidence that the agent involved produced photosensitivity reactions in humans. Other characteristics of the test systems have also limited their usefulness. The participation of humans as the target for a predictive assay of the photosensitizing potential of chemicals imposes ethical and financial limitations on the test. Similarly, the use of animals is expensive, time-consuming and demands specialized facilities. Furthermore, phototoxic responses observed in animal skin are often quantitatively and qualitatively quite different from the comparable responses in human skin.

The ideal test system for evaluating chemicals as photosensitizers should be simple, involve readily-available methodology and equipment, be inexpensive and sensitive. To meet these requirements an *in vitro* assay is required which is free of the problem of false-negative results. The present study was undertaken to determine whether lymphoid cells could be employed as a substrate target in an *in vitro* test and whether inhibition of tritiated thymidine incorporation would serve as an indicator of phototoxicity. Phototoxicity is used here in its broadest sense of photo-induced toxicity, the chromophore and pathogenesis of the toxicity being unknown. Chemical phototoxicity for the purposes of this study is defined as an alteration of cell function by an interaction between a chemical and nonionizing radiation; implied in this definition is the premise that such alteration cannot be detected following exposure of cells to the same doses of chemical or radiation alone. Phototoxicity was selected as the end-point for the test because it has been claimed that all photo-allergens are also phototoxic [24]. If this is correct, it should be possible in this test to detect photosensitizers *in vitro* with the present test regardless of whether they act *in vivo* via a toxic or immunologic mechanism.

MATERIALS AND METHODS

Target Cells

Two sources of lymphoid cells were used:

a. Peripheral blood mononuclear (PBM) cells isolated on Ficoll-Hypaque gradients from blood obtained from normal volunteer subjects. Informed consent was obtained for the procedure.

b. A virus-transformed lymphoblastoid (TL) cell line (#CCRF-8-5B2) derived from T lymphocytes of a normal volunteer. These cells were kindly supplied by Dr. H. Lazarus of the Sidney Farber Cancer Institute, Boston, Massachusetts.

Sources of Radiation

UVA radiation was provided by passing the refractively-collected radiant output of a 1.0 kW Xe arc lamp through 10 cm of circulating solution (40 g $CuSO_4$ and 40 g $CoSO_4$ per liter of distilled water). The spectral irradiance of the system was essentially limited to the 320–400 nm waveband with a peak at 365 nm (Fig 1). UVB radiation was provided by the output of two FS40 T12 fluorescent sunlamps (Westinghouse) passed through 5 cm of Backstrom fluid (80 g $CoSO_4$ and 250 g $NiSO_4$ per liter of distilled water) and two 2-mm thickness plates of Corning Pyrex 774 glass (Fig 2). This filter system eliminated all wavelengths below 280 nm.

The spectral irradiances of the sources were measured by a cosine-corrected UV spectroradiometer system (International Light, Inc., Danvers, MA; IL 700). Measurements of the irradiance of the UVA source were made for the integrated 320–400 nm band and of the UVB source for the integrated 280–320 nm waveband. For UVA radiation an IL SEE 400 detector, with a peak sensitivity at 360 and half-power points at 330 and 370 nm, was used. For UVB radiation an IL SEE 240 detector with a peak at 310 nm and half-power points at 290 and 320 nm was used. The irradiance was uniform to within ±10% over the entire field of exposure of each of the sources.

Radiation Doses and Test Chemicals

In preliminary studies a dose-response curve was determined for the effect of radiation alone on the target cells to permit selection of suitable radiation doses for use in combination with chemicals in the

Manuscript received June 17, 1981; accepted for publication November 9, 1981.

This work was supported in part by the Arthur O. and Gullan M. Wellman Foundation, and by National Institutes of Health Grant AM 25924-02.

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Abbreviations:
HBSS: Hank's buffered salt solution
PBM: peripheral blood mononuclear cells
TL: transformed lymphoblastoid

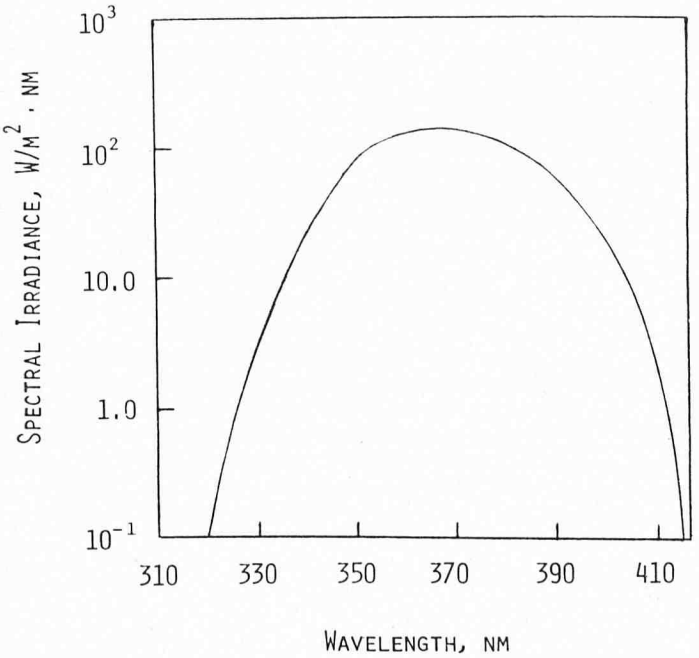

FIG 1. Spectral irradiance of the UVA radiation source.

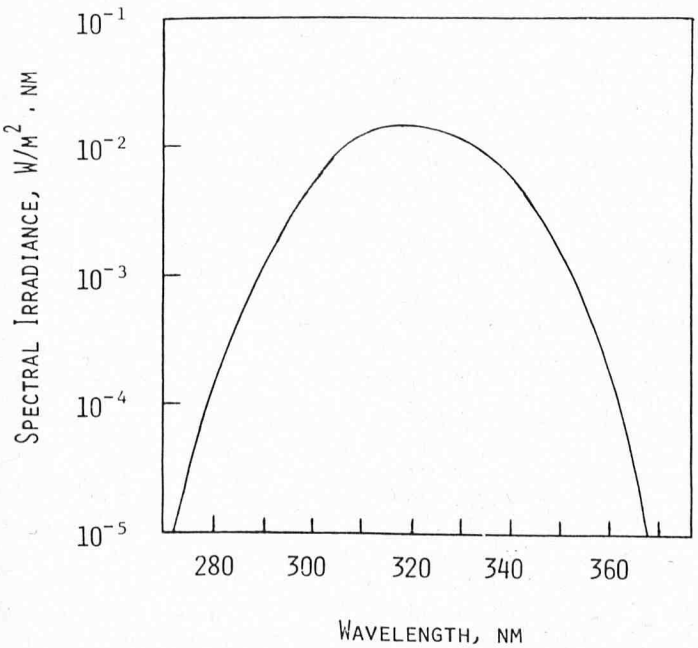

FIG 2. Spectral irradiance of the UVB radiation source.

study. The UVA radiation doses used with PBM cells and the TL cells for all tests were 10^3, 10^4 and 10^5 J/m^2. The test doses of UVB radiation were 10, 20, and 30 J/m^2 for the PBM cells and 5, 10 and 20 J/m^2 for the TL cells.

Eleven chemicals reported to be photosensitizers in humans [25–27] were studied and the results were compared with those obtained with 4 chemicals not considered to be photosensitizers. In addition, retinoic acid was tested because although this agent has not been reported to be a photosensitizer in humans, it has been found to potentiate photocarcinogens in mice [28,29]. The test concentrations of systemic drugs were selected on the basis of the mean serum levels found in humans following the ingestion of therapeutic doses of the drugs. Three doses were tested: a dose equal to the mean serum level, and doses which were 10-fold greater and 10-fold smaller than the mean serum level. For known topical photosensitizers, doses of 1, 10 and 100 μg/ml were first tested to define a range in which an effect was found; thereafter, a narrower range was selected on the basis of the initial results. The doses of chemicals tested were not toxic alone with lymphoid cells. The chemicals were either dissolved in ethanol or acetone and then diluted in Hank's buffered salt solution (HBSS) or were dissolved directly in HBSS depending on the reported solubility of each chemical [30].

Radiation Procedure and Assay

Cells were washed 3 times in HBSS (GIBCO, Grand Island, NY) without phenol red, and were suspended in that solution at a concentration of 2×10^6/ml. Three-milliliter samples of the cell suspension were dispensed into 3.5 cm Falcon plastic Petri dishes (Fisher Scientific, Boston, MA); test chemicals were added to appropriate dishes and all samples were incubated at 37°C in an atmosphere of 5% CO_2 for 15 min. Samples were then irradiated on a nonreflecting surface with the lids removed. Each test included control samples of cells exposed to radiation without prior treatment with chemical, cells treated with chemical but not exposed to radiation, and cells not exposed to chemical or radiation. After completion of the exposures, cells were washed, resuspended in R.P.M.I. 1640 (GIBCO) with 20% fetal calf serum (GIBCO) and antibiotics (penicillin 200 I.U./ml and streptomycin 200 μg/ml) (GIBCO). Cultures of 2×10^5 cells were established in round-bottom microtiter plates (Fisher Scientific). All sets of PBM cell cultures were established in pairs to include cultures not stimulated with mitogen and cultures stimulated by the addition of 0.25 μg/culture of phytohemagglutinin (PHA) (Ha 17, Burroughs Wellcome, Beckenham, England). Cultures of TL cells were not stimulated. Cultures for all conditions were made in triplicate. After PBM and TL cultures were incubated at 37°C in an atmosphere of 5% CO_2 for 66 and 42 hr, respectively, 0.5 μCi of ^{3}[H]-thymidine (sp. ac. 6.7 Ci/mM; New England Nuclear, Boston, MA) was added to each culture and the plates were incubated for a further 6 hr. The cells were then disrupted, the contents were collected on glass fiber filter strips and washed freely using an automated harvester (MASH II, Microbiological Associates, Walkersville, MD). The dried filter papers were suspended in scintillation fluid and the radioactivity measured in a Beckman LS 150 liquid scintillation spectrometer. The mean of the radioactive counts per minute (cpm) of the 3 samples for each condition was calculated, and the means for irradiated cultures were then expressed as a percentage of the control cultures which were not exposed to radiation or chemicals. For evaluation of the phototoxic activity of the chemicals the analysis was restricted to the results obtained with doses of the agents that gave a phototoxic effect with each of the 3 doses of radiation. The method of least squares [31] was then used to estimate the radiation dose required to reduce ^{3}[H]-thymidine incorporation to 50% of that in control cultures. PBM cells from at least 3 volunteers were used for testing each chemical. Tests with the TL cells were performed once only for each chemical.

RESULTS

An exposure to more than 6×10^5 J/m^2 UVA radiation was required to produce a 50% reduction of the mitogen-stimulated incorporation of ^{3}[H]-thymidine by PBM cells (Table I). It can be seen that 8 of 10 known photosensitizers, and one chemical (retinoic acid) not reported to be a photosensitizer, reduced the dose of UVA radiation required to produce a phototoxic effect equal to that achieved with radiation alone. The 4 control chemicals not reported to have any interaction with nonionizing radiation gave negative results and did not enhance the phototoxicity of radiation alone. Even allowing for differences in the concentrations of the various chemicals, methoxsalen, demethylchlortetracycline, and chlorpromazine were the most potent photosensitizers in this system. Two known photosensitizers, hexachlorophene and sulphanilamide, did not show any evidence of phototoxicity with UVA radiation. However, in tests in which these agents were combined with UVB radiation both were found to be photosensitizers (Table II).

Four photosensitizing chemicals and the nonphotosensitizers were also tested using the TL cell line as target cells (Table III). The results are similar to those obtained with PBM cells in that each of the known photosensitizers was phototoxic while the control chemicals were not. In comparison to PBM cells, the TL cell line was more sensitive to both UVA and UVB radiation. The reproducibility of results obtained with the two types of target cells was compared. PBM cells from 3 individuals were exposed to 10, 20 and 40 J/m^2 of UVB radiation, while on 3 separate days the TL cell line was also exposed to these doses

TABLE I. *Comparison of the effect on tritiated thymidine incorporation of exposure of peripheral blood mononuclear cells to UVA radiation in presence of chemicals reported to be photosensitizers (*) and other chemicals not considered to be photosensitizers*[a]

	Concentration of chemical (μg/ml)	Dose of UVA radiation ($J/m^2 \times 10^5$) required to reduce ^{3}[H]-thymidine incorporation to 50% of control
UVA radiation		>6.0
UVA radiation plus		
* Methoxsalen	1.0	0.0019
	0.1	0.08
* Chlorpromazine	1.0	0.1
* Demethylchlortetracycline	20.0	0.09
	2.0	0.14
Retinoic acid	3.0	0.21
* Hydrochlorothiazide	1.0	0.46
* Tetrachlorosalicylanilide	1.0	0.48
* 6-Methylcoumarin	10.0	0.63
* Nalidixic acid	30.0	0.32
* Musk ambrette	20.0	0.92
* Chlorpropamide	25.0	4.0
* Hexachlorophene	1.0	>6.0
* Sulfanilamide	40.0	>6.0
Ethanol	50.0	>6.0
Acetylsalicylic acid	100.0	>6.0
Caffeine	1.0	>6.0
Acetominophen	10.0	>6.0

[a] All cells were stimulated with PHA but control cells were not exposed to chemical or radiation.

TABLE II. *Effect on tritiated thymidine incorporation of exposure of peripheral blood mononuclear cells to UVB radiation in the presence and absence of chemicals*[a]

	Concentration of chemical (μg/ml)	Dose of UVB radiation (J/m^2) required to reduce ^{3}[H]thymidine incorporation to 50% of control
UVB radiation alone		40.4
UVB radiation plus		
Sulfanilamide	40.0	11.0
Hexachlorophene	1.0	17.8

[a] All cells were stimulated with PHA but control cells were not exposed to chemical or radiation.

of radiation. The ranges of doses required to reduce ^{3}[H]-thymidine incorporation by these cells by 50% were: PBM cells, 26.7 to 54.1 J/m^2; and TL cells, 20.6 to 26.8 J/m^2.

DISCUSSION

Eleven chemicals reported to be photosensitizers in humans were tested for phototoxicity with UVA and UVB radiation in an *in vitro* assay using lymphoid cells as a target. Nine of the chemicals were phototoxic in combination with UVA radiation and the two that were not phototoxic with that waveband were found to be phototoxic with UVB radiation. Four chemicals not reported to be photosensitizers were not phototoxic *in vitro* in combination with either UVA or UVB radiation. Retinoic acid, which is not reported to be a photosensitizer, but does potentiate experimental photocarcinogenesis and inhibits UVB-induced alterations of DNA synthesis in mouse skin [28,29,32], was found to be phototoxic in combination with UVA radiation.

Thus this test system appears to have potential as a predictive assay for screening chemicals for their photosensitizing potential and may have some advantages in comparison with previously reported assay systems. Since the test is an *in vitro* procedure it avoids the ethical considerations inherent in *in vivo* screening of chemicals in humans and the financial, time and space constraints of *in vivo* testing in animals. Lymphoid

TABLE III. *Effect of exposure of T lymphoblastoid cells to radiation in the presence and absence of chemicals*[a]

	Concentration of chemical (μg/ml)	Dose of radiation (J/m^2) required to reduce ^{3}[H]thymidine incorporation to 50% of control
UVA alone		2.2×10^5
UVA plus		
Methoxsalen	1.0	2.86×10^2
Chlorpromazine	1.0	7.3×10^3
Acetylsalicylic acid	100.0	$>2.2 \times 10^5$
Acetominophen	10.0	$>2.2 \times 10^5$
Alcohol	50.0	$>2.2 \times 10^5$
Caffeine	1.0	$>2.2 \times 10^5$
UVB alone		24.0
UVB plus		
Sulfanilamide	40.0	5.1
Hexachlorophene	1.0	10.6

[a] Control cells were not exposed to radiation or chemicals.

cells are nucleated and therefore do not have the obvious limitations of red cells as targets for detecting DNA-interacting photosensitizers. Lymphoid cells are of course not unique in their sensitivity to radiation and other mammalian cells may give similar results. However, lymphocytes are readily available and many laboratories are equipped to undertake the procedures outlined in this study.

Several modifications of the test system might improve its potential as a predictive assay. Although human peripheral blood mononuclear cells were successfully employed as the target for testing most of the chemicals, there are some disadvantages to their use. Considerable interpersonal variation exists in the sensitivity of lymphocytes to a phototoxic insult. Use of a transformed T lymphoblastoid cell line appears to overcome this problem and obviates the need to use PBM cells from several donors for tests with each chemical. Once the transformed cell line is established and an adequate supply of cells has been accumulated, there are the added advantages of ease and lower cost, as the target cells do not have to be separated from blood but are merely harvested and volunteers do not have to be reimbursed for donating blood.

The spectral irradiance of the radiation sources is another important consideration. It is generally considered that the action spectrum for most photosensitizers is in the UVA waveband. Our results confirmed that observation for most photosensitizers, but in 2 instances UVA radiation failed to induce phototoxic effects whereas UVB radiation did produce this effect. Some of the false-negative results obtained in other assay systems for phototoxicity may have resulted from a failure to use the appropriate source of radiation. For adequate testing of chemicals, it is essential to use both UVA and UVB radiation; possibly visible radiation should also be studied. Two modifications that were not explored in this study should be considered. A photoproduct of a chemical may be a photosensitizer while the original chemical is not; alternatively, the original chemical may be a much less potent photosensitizer. Prior exposure of chemicals to solar radiation or solar-simulated radiation would be useful for detecting such alterations. Secondly, a chemical may require metabolic activation or alteration before it acts as a photosensitizer. Provision could be made for such activation by, for example, incubating the chemical with a liver homogenate as is done in the Ames' test [33]. However, there do not appear to be any reports of nonphotosensitizing chemicals that acquire photosensitizing potential after photochemical or metabolic alteration; therefore, these 2 modifications may not be necessary.

Phototoxicity was used as the indicator of photosensitivity in this study and the success of the test in detecting all photosensitizing chemicals is founded on the premise that all photoallergens are also phototoxins. Photoallergens such as tetrachlorosalicylanilide and 6-methyl coumarin were found to be photo-

toxic in the study, but this may not be a universal finding. For example, *in vivo* studies in animals and man, in which erythema was used to assess phototoxicity, found that hexachlorophene [34], musk ambrette [35] and 6-methyl coumarin [26] were not phototoxic. Erythema is only one indicator of phototoxicity and it is possible that cell damage might occur *in vivo*, perhaps selectively resulting in a photoallergic response, without inducing the pathway leading to erythema. *In vitro* studies must therefore be interpreted with caution and comparisons between cellular phototoxicity and clinical phototoxicity as judged by the development of erythema may not always be valid. It should also be emphasized that any *in vitro* test for photosensitizers can only be regarded as a screening test. Chemicals that produce phototoxicity *in vitro* must be tested further in experimental animals, and possibly humans, to fully determine their photochemical interactions. The results obtained with retinoic acid in this study provide an example of this requirement for further study. Retinoic acid was phototoxic in our system but was not phototoxic in terms of producing erythema in mice in combination with UVB radiation [36] or UVA radiation (P.D. Forbes, personal communication). The photosensitizing potential of retinoic acid deserves further study.

The present study is only a preliminary examination of some of the parameters that must be considered in designing an *in vitro* test for photosensitizing chemicals. Much more work is required to fully define all the important variables in such a test. However, our results indicate that it may be possible to avoid a major problem associated with previously reported *in vitro* tests of phototoxicity, namely that of false-negative results, and still have the advantages of reduced cost and time required, as well as the convenience associated with an *in vitro* test.

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