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Effect of Near-Ultraviolet and Visible Light on Mammalian Cells in Culture II. Formation of Toxic Photoproducts in Tissue Culture Medium by Blacklight*

(human cells/cell lethality/photodynamic action/riboflavin-tryptophan-tyrosine)

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ABSTRACT **Near-ultraviolet radiation was found to be lethal for mammalian cells in Dulbecco's modified Eagle's medium without serum or phenol red. Irradiation of the cells with near-ultraviolet light while the cells were in phosphate-buffered-saline abolished the lethal effect. When only the medium was irradiated followed by the addition of unirradiated cells and serum, the cells were still killed. The photoactive components of the medium for this effect were riboflavin, tryptophan, and tyrosine. When riboflavin was deleted from the medium being irradiated and added later, almost no killing was detected. Irradiation of salt solution of riboflavin and tryptophan or riboflavin and tyrosine, resulted in cell killing. Little or no killing resulted when riboflavin, tryptophan, or tyrosine was irradiated singly.**

The formation of photoproducts toxic for mammalian cells appears to involve photodynamic action. Experiments utilizing Dulbecco's or similar media without proper controls may produce anomalous results from light illuminating the laboratory.

A previous report from this laboratory showed that near-ultraviolet (near-UV) radiation (blacklight) was lethal for mammalian cells in tissue-culture medium (1). Other studies on the effect of near-UV and visible light on mammalian cells in culture have been reported (2–4). We previously raised the possibility that components of the tissue-culture medium [Dulbecco's modified Eagle's medium (5)] might be serving as chromophores in the production of photoproducts toxic for mammalian cells in culture. The formation of photoproducts toxic for recombinationless bacteria was demonstrated by killing of the bacteria when added to media that had previously been exposed to blacklight (6). The ingredient responsible for the formation of photoproducts toxic to the bacterial cells was found to be tryptophan (7). Similarly, Zigman *et al.* have found that near-UV irradiation of tryptophan produces photoproducts which bind to proteins of human eye lens *in vitro* (8) and alter their chemistry and function adversely.

Previously we reported that when human D98/AH_2, mouse 3T6, and Chinese hamster V79 cells in Dulbecco's modified Eagle's medium (MEM) without phenol red were exposed to black light, the cells were killed (1). We speculated that toxic photoproducts could possibly be formed in the medium, which, when taken up by the cells, lead to lethality. We report here that the lethal effect of blacklight on mammalian cells at the same low doses used previously is due almost entirely to the effect on three medium components: riboflavin, tryptophan, and tyrosine.

MATERIALS AND METHODS

Cell Culture. The cells used were human D98/AH_2 (9), mouse 3T6 (10) and Chinese hamster V79 clone 753-B-3M (11). For routine growth of cells, Dulbecco's modified Eagle's medium (MEM) was used. It was supplemented with 10% calf serum but without addition of antibiotics. The cells were grown under 10% CO_2 at 37°.

Irradiation of Cells. The light sources used in this study were two General Electric F15T8 BLB integral filter blacklight tubes with emission in the 300- to 420-nm range peaking at 365 nm (12). Light exposure was carried out in covered Falcon 3002 60-mm dishes placed approximately 20 cm below the light source. The lids of these dishes exclude light below 300 nm (6). Exposure intensity was 4 $\mu W/mm^2$ at the cell surface, as measured by a Blak-ray meter J-221 (Ultraviolet Products, Inc.). Temperature was monitored and regulated to avoid any effect due to heating by the light source. The cells were trypsinized and plated in medium containing serum but no phenol red, 18 hr before exposure to blacklight. Twenty minutes before irradiation, the medium was removed and replaced with either 3.5 ml of phosphate-buffered saline containing NaCl, KCl, Na_2HPO_4, and KH_2PO_4; or MEM free of both phenol red and serum. The dishes were placed in the exposure chamber under 10% CO_2 for 20 min before the light was turned on. At various intervals, dishes were removed from the light and kept in the dark in the same chamber. After the last dishes were removed from the light, serum was added to all dishes.

Irradiation of Medium or Medium Components. Exposure conditions were similar to those of the last section except that no cells were present and only the aqueous solutions of medium components were exposed. The basic mixture (BaM) employed, to which other components were selectively added, contained NaCl, KCl, $CaCl_2$, $MgSO_4$, NaH_2PO_4, $Fe(NO_3)_3$, glucose, inositol, cysteine, and glutamine. For the determination of the medium components responsible for photoproduct formation, other ingredients were added to BaM: (*1*) amino-acid solution (AA) containing arginine, isoleucine, leucine, lysine, methionine, threonine, valine, glycine, and serine; (*2*) vitamin solution (Vit) containing nicotinamide, thiamine, choline, D-pantothenic acid and pyridoxine; (*3*) one or more of the following compounds: histidine, phenylalanine, tryptophan, tyrosine, and riboflavin. After irradiation, the missing MEM ingredients and serum were added to the dishes fol-

Abbreviations: MEM, Duebecco's modified Eagle's medium; BLB, BLB blacklight; the components of BaM (basic mixture), AA (amino acid solution) and Vit (vitamin solution) are given in Materials and Methods.

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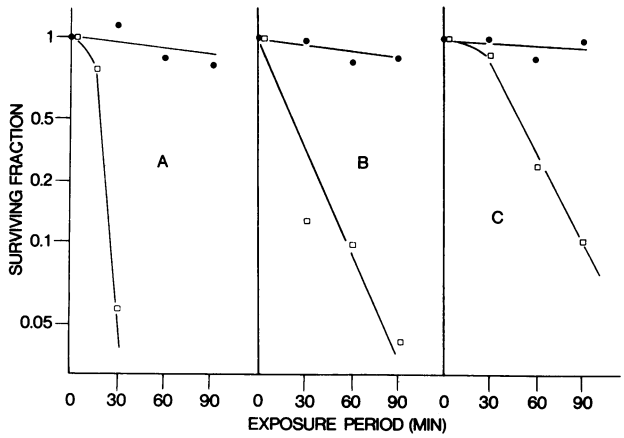

FIG. 1. Irradiation of cells in MEM (□) or phosphate-buffered saline (●). Surviving fraction is the fraction of cells capable of forming clones with the zero time value set equal to 1. (A) D98/AH_2 cells, (B) 3T6 cells, and (C) V79 cells.

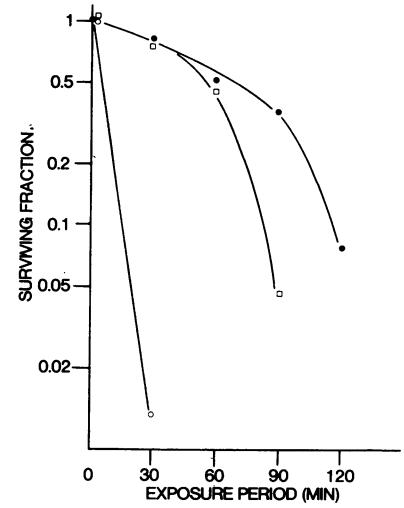

FIG. 2 Irradiation of MEM without phenol red followed by addition of 250 D98/AH_2 cells (○), 120 3T6 cells (□), or 120 V79 cells (●) per 60-mm dish. Surviving fraction is defined as in Fig. 1.

lowed by the addition of the cells. The concentrations of all the ingredients present during irradiation and subsequent cell growth were within 5% of those normally present in MEM.

Determination of Lethal Effects. The lethal effect of blacklight was ascertained by two different procedures. With the first procedure, cells exposed to blacklight in phosphate-buffered saline or medium and those added to irradiated medium were incubated in the dark at 37° for 7–10 days. Clones that were formed were stained with hematoxylin and counted. This method measured the amount of reproductive death of the cells. The other method measured the amount of physiological death by dye exclusion. At intervals after the exposure procedure, the cells were stained with trypan blue. Only those cells which were physiologically viable excluded the dye. Previously we found good correlation between physiological and reproductive death (1); however, both methods were utilized in some of the experiments to further confirm the lethal effects.

RESULTS

Our first experiment differs slightly from the earlier experiments (1), where cells in MEM without phenol red were exposed to BLB blacklight. There the exposed medium was changed to fresh MEM, serum was added and cells incubated in a dark incubator. Here, no post-irradiation medium change was performed. When 250 D98/AH_2, 120 3T6, or 120 V79 cells in MEM (without phenol red or serum) per 60-mm dish were exposed to BLB, the cells were killed (Fig. 1) at a more rapid rate than reported previously. For human D98/AH_2 cells, 90% of the cells lost the ability to form clones within 25 min as compared to the 30 min reported earlier. In the case of mouse 3T6 and Chinese hamster V79 cells, the loss occurred by 50 and 80 min as compared to the earlier report of 90 and 120 min, respectively. This suggests the possibility that one or more photoproducts might be present in the medium which were lethal for the cells and which, when not removed, continued to kill the cells.

When the medium bathing the cells was removed and replaced with phosphate-buffered saline before the dishes were exposed to blacklight, most of the cell killing was prevented (Fig. 1). The plating efficiencies of D98/AH_2 and 3T6 were reduced slightly, whereas no appreciable loss occurred with V79 cells (Fig. 1).

We can conclude that either toxic photoproducts were formed in the medium which killed the cells, or that some essential components of the medium were inactivated by the light. The latter alternative can be ruled out. In the experiments reported previously (1), the cells were exposed to BLB followed by replacement of irradiated medium with fresh medium, and the cells were still killed. Furthermore, we have now found (unpublished) that adding additional amounts of unirradiated riboflavin, tryptophan, and tyrosine to already irradiated solutions confers no protection against the cell killing.

The conclusion that toxic photoproducts were formed in the medium led to the next experiment. The prediction drawn from this conclusion was that, if the medium were exposed to BLB, toxic photoproducts should be formed which would cause cell killing, even if cells never exposed to BLB were added after the exposure period. Dishes containing MEM without phenol red or serum were exposed to BLB, and serum and cells were then added. Fig. 2 shows that human D98/AH_2, mouse 3T6, and Chinese hamster V79 cells were killed in the irradiated medium, even though the cells were not exposed to BLB. For D98/AH_2 cells, no clones were formed after the cells were inoculated into medium which had been exposed to BLB for longer than 30 min. An approximately 80-min exposure period reduced the plating efficiency of 3T6 cells by 90%. The V79 cells were slightly more resistant, requiring 120 min for the same amount of reduction.

The next experiments were designed to single out any components of the medium that may have formed the lethal photo-products. Human D98/AH_2 cells were used. Dishes containing BaM and various medium components were exposed to BLB for 90 min. After irradiation, the cells, serum, and the MEM components not present during exposure were added to the dishes. After 8 hr of incubation in a dark incubator at 37°, the medium was removed and trypan blue was added. Those cells which were viable and excluded the dye were counted.

Table 1 shows that when histidine, tyrosine, tryptophan, phenylalanine, Vit, or AA were singly present in BaM during irradiation, no photoproducts leading to cell killing were

TABLE 1. *Effects of irradiated MEM components on human D98/AH₂ cells*

Components irradiated	Dye-excluding cells (% of unirradiated BaM value)
MEM	0
BaM	109
MEM less riboflavin	84
BaM + Vit	114
BaM + AA	89
BaM + histidine	116
BaM + tyrosine	91
BaM + tryptophan	114
BaM + phenylalanine	91
Unirradiated controls	
MEM	111
BaM1	100

Various combinations of MEM components in 60-mm dishes were irradiated with blacklight (BLB, 4 μW/mm²) for 90 min. Serum, the missing components, and 120 D98/AH₂ cells were then added per dish. The cells were stained with trypan blue 8 hr later and the number of physiologically viable cells excluding the dye was counted.

formed. Only those dishes with MEM (less phenol red) showed any appreciable cell killing. The most startling effect was noted with dishes containing all ingredients except riboflavin. Omission of the riboflavin abolished the lethal blacklight effect.

Medium that was otherwise complete, with and without riboflavin, were next irradiated. After irradiation, riboflavin was added to those dishes containing no riboflavin and then serum and D98/AH₂ cells were added to all the dishes. The results are shown in Fig. 3. At least 90% of the cells were killed within a 30-min period in the medium containing riboflavin during irradiation. More killing occurred when the exposure period was increased. No killing was found, however, in those dishes containing all the medium components except

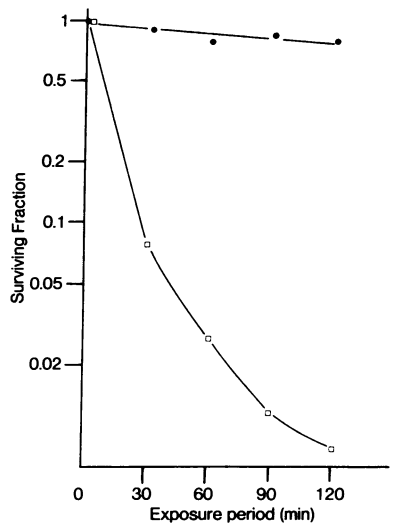

FIG. 3. Irradiation of MEM minus phenol red with riboflavin (□) or without riboflavin (●) followed by addition of 120 D98/AH₂ cells. Surviving fraction is the fraction of cells capable of excluding the dye trypan blue with the zero time value set equal to 1.

TABLE 2. *Effects of irradiated MEM components on human D98/AH₂ cells*

Components irradiated in BaM solution	A Relative plating efficiency (% of unirradiated MEM value)	B Dye excluding cells (% of unirradiated MEM value)
BaM only	92	98
Rib	103	93
Trp	102	N.D.
Tyr	91	N.D.
Trp + Tyr	102	N.D.
Rib + Vit	97	71
Rib + AA	103	95
Rib + His	108	96
Rib + Phe	96	84
Rib + Trp	0	0
Rib + Tyr	16	5.8
Unirradiated controls		
MEM	100	100
Others*	92–108	N.D.

Various combinations of MEM components in 60-mm dishes were irradiated with blacklight (BLB, 4 μW/mm²) for 90 min. Serum, the missing components, and 100 (A) or 1000 (B) D98/AH₂ cells were added per dish. In column A, the clones were stained and counted after the cells were incubated in the dark for 7 days. In column B, the cells were stained with trypan blue 8 hr later, and the number of physiologically viable cells excluding the dye was counted. N.D., not done.

* Same combinations as in irradiated dishes but kept in the dark.

riboflavin. This further confirmed the conclusion that the presence of riboflavin was necessary to achieve the killing effect by blacklight. The next experiment was designed to test whether the presence of other medium components in addition to riboflavin is required as well.

Dishes containing BaM and riboflavin plus various other components were exposed to BLB followed by the addition of the missing components, serum and cells. Column A of Table 2 shows that when BaM with riboflavin alone was exposed to BLB, no toxic photoproducts leading to the reproductive death of the cells were detectable. The most efficient combination was when both riboflavin and tryptophan were present. When riboflavin and tyrosine were present, a significant amount of cell killing was detected, but the amount was less than with the riboflavin–tryptophan combination.

In a parallel experiment, physiological death was measured after exposure of medium components to BLB and addition of cells. Again the riboflavin–tryptophan combination was the most effective, followed by riboflavin-tyrosine (Table 2, Column B). The other dishes, including the dishes with riboflavin, tryptophan, or tyrosine present singly, or tryptohan and tyrosine present together, showed little or no killing effect (Table 2).

DISCUSSION

An earlier report established that blacklight was lethal for human and other mammalian cells in tissue-culture medium (1). We have shown here that this lethal effect is still present when only the medium is irradiated and the cells are added later. We have also demonstrated that this lethal effect occurs

only when certain components of tissue-culture medium are present during irradiation and is not dependent on any other medium ingredients.

The presence of both riboflavin and tryptophan during irradiation resulted in the highest amount of cell killing. The combination of riboflavin and tyrosine also resulted in cell death, although less than the riboflavin–tryptophan combination. The presence of only one of the above three or of any other components of the tissue-culture medium during irradiation yielded little or no lethal effect. From the results obtained here and from photochemical and photobiological studies reported by others, it seems most likely that the reaction involves the riboflavin-sensitized oxidation of tryptophan and tyrosine.

There are many reports of the photooxidation of both tryptophan and tyrosine, among other organic compounds, by near-UV and visible light in the presence of riboflavin. Galston was one of the first to note the catalytic effect of riboflavin on the oxidation of indoleacetic acid, tryptophan, and other indole compounds in the presence of visible light (13–15). Since then, riboflavin and its analogues have been shown to be effective sensitizers in the oxidation of not only free tryptophan and tyrosine (16–18), but also of many other organic compounds. Numerous papers and reviews on such examples of photodynamic action have appeared which catalogue the reactions and describe possible mechanisms (19–23). The actual measured rate expressions, for the oxidation of tryptophan, are quite complex and may express several competing mechanisms for oxygen uptake (17, 21). Preliminary findings (unpublished) indicate that when solutions containing riboflavin, tryptophan, and tyrosine, in MEM concentrations, are exposed to blacklight in an atmosphere lacking oxygen, the cell killing effect is greatly reduced. This result further implicates a mechanism of photodynamic action (22). It has also been shown that histidine is readily oxidized using riboflavin as a sensitizer (15, 18, 22). Since histidine is included in our medium we expected to find some effect with it. The lack of any toxic effect seems to indicate that not only are no photoproducts of histidine toxic, but also that there is no appreciable depletion of histidine due to the photochemical reaction.

The near-UV region utilized in this experiment has been shown to be responsible for many unique effects. In bacteria, near-UV has been found to be mutagenic (26–29). The ability of DNA to transform bacterial cells was decreased by near-UV (30, 31). Recombinationless bacteria were killed when added to media previously exposed to light (6). Tryptophan without the presence of riboflavin was found to be the ingredient responsible for the formation of photoproducts toxic to the mutant bacterial cells (7), even though the absorption spectrum of the amino acid shows almost no absorption above 300 nm. The explanation for this anomaly apparently is that one of the photoproducts initially formed in very low concentrations is actually a photosensitizer for further oxidation of tryptophan (32). The sensitizer has been identified as *N*-formyl kynurenine (32). The dose needed for this effect is much greater than the doses used in our experiments. No such reaction has been shown to occur with tyrosine. Eisenstark recently found that tryptophan photoproduct formed during black light epxosure was mutagenic and influenced the genetic recombination process in bacteria (29). Tyrell *et al.* have shown that 365-nm radiation destroys photoreactivating enzyme isolated from yeast and also prevents *in vivo* photoreactivation of *Escherichia coli* (33).

A better understanding of the effect of irradiation by light in the near-UV region on mammalian cells is needed. We have found that the near-UV emission of visible-fluorescent tubes can cause the formation of photoproducts which kill mammalian cells in culture (unpublished). Our finding that photoproducts toxic for mammalian cells are formed when riboflavin and tryptophan or riboflavin and tyrosine are exposed to near-UV radiation, further emphasizes the fact that considerable caution should be exercised in experiments involving the irradiation of mammalian cells with visible fluorescent light or blacklight. Puck and Kao devised a technique utilizing BrdU and visible fluorescent light for the selection of auxotrophic Chinese hamster mutant cells after treatment with mutagens (34). Chu *et al.* (3) reported that such selective techniques when used with near-UV irradiation had highly mutagenic action. A number of other laboratories are investigating the effects of visible fluorescent light and blacklight on mammalian cells after the incorporation of BrdU into the cell nucleic acid (35–37).

Even when BrdU is absent, unexpected biological effects have been found in experiments performed with or near fluorescent lights or blacklights. Fogel found that visible fluorescent light increased the amount of polyoma virus induction 9-fold (37). Todd *et al.* (2) found that marsupial PtK cells in Eagle's minimal essential medium were killed when exposed to the blacklight radiation used for photoreactivation. When the culture medium was changed to F-12 (38), the killing was reduced to 10–50%. They attributed the protection to the decreased amount of phenol red in F-12. Our results suggest that the protection is most likely a result of the greatly reduced amount of riboflavin, tryptophan, and tyrosine present in F-12 (0.037, 2.0, and 5.4 mg, respectively, in F-12 as compared to 0.1, 10, and 36 mg/liter in Eagle's medium).

Another example of the caution necessary in interpreting experimental results is present in work done by Warburg *et al.* They reported that 10 μg/ml of riboflavin added to the culture medium inhibited the fermentation and growth of mouse ascites tumor cells (39). In a later paper they corrected this to say that the inhibition was not present when the experiment was performed in the dark (40). Furthermore, when the riboflavin was added to the culture medium and irradiated with 452-nm light and cells were then added after irradiation, the inhibition still took place.

An experiment by Lichtenstein and Goldman (41) showed that the conflict of reports on the inhibition, by riboflavin, of the uptake of methotrexate by L1210 mouse leukemia cells was due to a photochemical reaction that resulted when solutions containing both chemicals were inadevertantly exposed to light.

It is uncertain as to how these observations may apply to tumor growth in whole animals. In a recent review by Rivlin (42), riboflavin was shown to be implicated in many forms of cancer. However, almost all of the research cited in that review was on the nutritional aspects of riboflavin as a coenzyme.

It is difficult at present to ascertain the possible effects blacklight may have on humans. However, the results reported here further emphasize the necessity for additional studies. The possible mutagenic and carcinogenic action of

300–420 nm of radiation also needs to be carefully examined. Sunburn and skin cancer are common problems associated with excessive exposure to sunlight, which contains a significant amount of its radiation in the near-UV range. The results of this report indicate that the possible deleterious effect of near-UV irradiation may not be localized at the epidermis level. The toxic photoproducts which might be formed could circulate to other parts of the body, perhaps with adverse effects.

At least one type of cancer, that of the bladder, has been linked with high levels of production of tryptophan metabolites (43). It could not be stated without ambiguity whether the abnormal metabolites were the cause or merely the effect of the diseased state. The possibility that these metabolites were of photochemical origin has not been investigated. Warburg has stated that patients on high B-vitamin diets should exercise caution by wearing sunglasses in direct sunlight (40). Zigman has indicated that such caution should be exercised by all (8). We feel that, especially for those who are ingesting large amounts of riboflavin, similar caution concerning all exposed portions of the body might be in order.

These effects cause us to agree with the reservations of Kosterbauder and Sandvordeker concerning the administration of riboflavin to enhance the phototherapy treatment of neonatal hyperbilirubinemia (44). It is possible that riboflavin may not be the most suitable photosensitizer for the breakdown of bilirubin because of the photochemical action of riboflavin on other biological molecules such as tryptophan and tyrosine found free or present in enzymes. A search should be continued for a more suitable sensitizer which would act selectively on bilirubin.

Potentially harmful metabolites would not necessarily have to be formed endogenously. Many food items are deliberately irradiated with blacklight during the processing stage (12). Photoproducts could be formed which, when ingested, would also be circulated to all parts of the body. These light-mediated phenomena deserve further consideration, especially the study of long-term effects on biological systems.

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