The effects of ultraviolet irradiation on the pigment cells of the PET/Wmr mouse epidermis

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THE EFFECTS OF ULTRAVIOLET IRRADIATION ON
THE PIGMENT CELLS OF THE PET/WmR MOUSE EPIDERMIS

by

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I wish to express my gratitude to the following members of the Department of Biology at the University of Richmond: Dr. Willie M. Reams, Jr. for his guidance and supervision not only of this thesis, but for the two year period I was enrolled in the Graduate School; Dr. David W. Towle and Dr. Wilton R. Tenney for their reading of the manuscript and their helpful comments.

Thanks are expressed to my fellow student, Mr. John T. Earnhardt, for his assistance in counting and supplying me with some of my controls.

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Finally, I greatly appreciate the patience of all those individuals and groups who seemed to get a little less than all my attention. This understanding has made it somewhat easier for me to fulfill all my obligations.
ABSTRACT

Epidermal melanocytes in mammals are known to occur in a variety of shapes, sizes, and colors. The basic melanocyte morphology is dependent upon genetics, but there are a number of environmental factors that can cause modification.

In the epidermis of PET/Wmr mice, the population of melanocytes reaches a peak and begins to decline during the first post-natal week, and has disappeared within four weeks. Dispersed among the large, highly DOPA-reactive melanocytes are miniature melanocytes. These miniature melanocytes are weakly DOPA-reactive and appear early in the post-natal period. The miniature melanocyte population is relatively constant and these miniature cells are among the last to disappear.

The melanocytes of the epidermis in the PET/Wmr mouse were subjected to 3 different wavelengths of UV irradiation. Melanocyte counts of the experimental animals were compared with similar counts of control, non-irradiated animals in the first post-natal week.

The evidence indicates that these weakly DOPA-reactive, miniature melanocytes remain in fairly constant numbers during the first post-natal week, although UV irradiation did provoke a very clear increase in the numbers of the larger, highly DOPA-reactive melanocytes. That miniature melanocytes persisted throughout the experimental treatments suggest that they may represent a separate form of melanocyte.
INTRODUCTION

The PET strain of mice was discovered in 1958 at the Medical College of Virginia, Richmond, Virginia as a result of a cross between inbred C$_3$H and black mice of an unknown breed obtained from a local pet shop. The progeny of the mating were animals with Pigmented Extraepidermal Tissues (PET) and the mice have been inbred since 1958 to give the present PET/Nmr strain (Reams, 1963). Melanocytes of the present strain are largely restricted to the skin and to certain muscles of the hind limbs (Reams and Mayer, 1961). All the melanocytes of the mouse are derived exclusively from the neural crest (Rawles, 1947).

A melanoblast is the precursor of a melanocyte (Fitzpatrick, et al., 1971; Silver, et al., 1969). Reams (1963) says that once a melanoblast has been established in a tissue it has two modes of expression. In the first, which is typical of pigment cells of the skin, a melanoblast will undergo morphogenesis to produce a cell with branches of a dendritic type. The cell will ultimately produce melanin, becoming a true melanocyte with melanized melanosomes. In the second mode of expression, a melanoblast may begin melanogenesis while still unbranched and undergoing cell division, followed later by production of branches.

Reams (1956) also noted these cell types exhibit an orderly sequence in time of appearance. The unbranched DOPA-negative melanoblast is
the first to appear, followed by either an unbranched melanocyte or a branched melanoblast. Markert and Silvers (1955) reported on the melanocytes in 50 genotypes of mice and their results placed the primary responsibility for melanoblast differentiation on the tissue environment. DOPA is used to detect prospective melanocytes—cells just at the onset of synthesis of melanosomes (melanin granules). DOPA stains only those cells that have already begun to synthesize melanin and thus it fails to reveal the presence of melanoblasts in any of the tissue areas.

Ultraviolet irradiation has been shown to provoke changes in pigmentation. Rovee and Reams (1964) found that hyperpigmentation in the ventral skin of PET mice followed exposure to ultraviolet radiation, substantiating the findings of Quevedo and McTague (1963) for the foot skin. Rovee and Reams concluded that most of the pigment cells which appeared arose from a latent population already present but invisible in the normal PET mouse. This conclusion is contrary to the hypothesis that the increase in pigment cell number might be due to mitotic activity. Although no mitotic figures had been observed in the PET mouse pigment cells (Reams, 1966), it is possible that limited mitotic activity may occur.

Reams and Schaeffer (1968) found dermal melanocytes are responsive to X-rays and that X-irradiation could provoke appearance of melanocytes in the normally melanocyte deficient epidermis. It was found also that it was possible to reach a high limit of melanocyte density. These results supported the suggestion (Rovee and Reams, 1964) that increased pigment cell numbers are a result of activation of resident, latent pigment cells.
It has been suggested that UV irradiation of skin causes an increase in tyrosinase activity. Kawamura, et al., (1971), postulated that tyrosinase in human skin exists in a partially inhibited form. Normally, non-irradiated melanocytes are unable to oxidize tyrosine to form melanin, but upon UV irradiation of human skin incubated with tyrosine, the melanocytes were able to form new melanin.

Activation of the tyrosinase reaction to provoke hyperpigmentation of skin was thought (Fitzpatrick, 1971) to include oxidation of tyrosine to DOPA (3, 4 dihydroxyphenylalanine) by photochemical action and the acceleration of tyrosine ---- DOPA ---- melanin reaction by the presence of trace amounts of DOPA thus formed (see appendix). Kawamura, et al., (1971) doubts that the increased melanogenic activity is due to any photochemical reaction. Rather this activity is due to the synthesis of new tyrosinase in the proliferating melanocytes and also an increase in melanogenesis of melanocytes already present and engaged in the synthesis of melanin.

The purpose of the present study was to determine what effect UV treatment might have on the numbers of various forms of pigment cells in the epidermis of the PET/wmr mouse. It was desired to determine whether the response of the miniature melanocytes in relation to that of typical melanocytes to ultraviolet irradiation would give insight into possible morphogenetic relationships.
MATERIALS AND METHODS

Newborn mice were obtained from a stock colony of PET/dmr mice and three animals were killed each day for control data up to the first post-natal week. For the experimental series, mice were irradiated with ultraviolet light (UVL) approximately 24 hours after birth and every 24 hour period thereafter for one week. Three animals were killed following each of the UVL treatments.

Three different wavelengths of UVL were used in the experimental work: a short wave length of 250 nm (SUV); an intermediate of 300 nm (IUV); and a long wave length of 360 nm (LUV).

In the SUV and LUV work the exposure time for all experimental trials was two minutes, fifteen seconds with a 6 cm distance between the animal and the light source (Rovee and Reams, 1964). In the IUV work, it was determined from a Westinghouse calibration chart that a dosage equivalent to that of the SUV and LUV could be obtained with the distance between mouse and lamp at 15 cm and the time of exposure of 4 minutes. In order to immobilize the mice during irradiation, their feet and belly were stuck to double-sided masking tape which was attached to the table.

Upon completion of an UVL treatment, three animals were killed by decapitation and sections of the dorsal skin removed. The pieces of skin were placed dermis side down on a piece of filter paper to prevent
curling. The skin was then placed in a solution of 2N NaBr at 38 C until the epidermis could be stripped from the dermis.

The epidermis was placed in 5% formalin for a 20 to 30 minute period. It was then washed for 5 minutes in distilled water and placed in a previously prepared solution of DOPA for 1 hour at 38 C. After 1 hour, fresh DOPA replaced the original DOPA solution and the skin was incubated for three more hours. Following this, the skin was again washed in distilled water and placed in formalin overnight. Following the plucking of hair follicles, the epidermis was washed in distilled water and dehydrated with increasing concentrations of alcohol and then placed in xylene. Slides were prepared by mounting the epidermis, dermis side up, in balsam.

The DOPA solution used was made as follows: a 0.1 M stock solution of each of KH₂PO₄ (F.W. 136.09) and Na₂HPO₄ (F.W. 141.96) was made. Then 20 parts of M/10 KH₂PO₄ was added to 80 parts M/10 Na₂HPO₄ to give a buffer solution of pH 7.4. The DOPA-solution was prepared by adding 10 mg L-DOPA (L Dihydroxyphenylalanine) to each 10 ml of 7.4 phosphate buffer. The DOPA-solution was made just prior to use in order to assure maximum activity.

The non-irradiated control mice were killed and the skin handled in the same manner as the experimentals.

Results were obtained from the prepared epidermis by counting both the control and experimental pieces for melanocytes. Counts of the melanocytes were done using an ocular grid and a mechanical hand counter. The grid in all counts enclosed an area 0.49 mm² and from these counts the melanocyte density per 1 mm² was computed.
Special attention was given in the counting for the appearance of the miniature melanocytes and their numbers were kept separately. Differences in the appearance of a typical and a miniature melanocyte may be seen in Figures 5 and 6. A miniature melanocyte was accepted as a weakly DOPA-reactive cell with a nucleus less than half that of a typical melanocyte. The typical melanocyte was very strongly DOPA-reactive and, though varying in size, was noticeably larger than the miniature melanocytes.

Counts of the pigment cell numbers were made using five different areas for each piece of epidermis. These areas usually included counts from each of the four corners of the piece and one area in the center. Whereas this method may have been satisfactory from a randomness pattern, it by no means was always satisfactory due to variations caused by spotting patterns. Although the PET/Wmr mice are highly inbred, non-agouti black animals, there appears to be considerable regional variation in pigment cell densities of the dorsal epidermis. These areas were found in both control and experimental animals. Data were calculated as the average of the 5 areas from the samples of epidermis from each of three mice of each radiation dosage.
RESULTS

The results with the SUV and the IUV irradiation show that there is a substantial increase in the number of typical melanocytes of experimental animals as compared with the control animals (Table 1). Experimental animals received dosages of UVL during the first post-natal week and the control animals were those animals which received no dosages of UVL.

The results with the LUV as compared to controls showed little effect, as anticipated (see Pathak, et al., 1970) and as recently confirmed by Willis, et al., (1973).

The number of typical melanocytes in the irradiated animals is clearly rising with daily doses of UVL in the range of the SUV and the IUV. And clearly in the control animals by day 4 there is a drop in the typical melanocyte number which continues to the end of the first post-natal week.

The IUV series shows large numbers of typical melanocytes by the 7th day of irradiation. This number (426 per mm$^2$) contrasts with the number of such cells in the control animals (76 per mm$^2$).

Clearly the irradiation of both SUV and IUV treatments of the mouse epidermis has increased the numbers of typical melanocytes by the end of the first post-natal week. This is in contrast to the figure for the control animals which show the greatest concentration of melanocytes
on day 3 and thereafter gradually tapers off (Table 1).

The number of miniature melanocytes ranges from 5-13 per mm² in control animals during the first post-natal week, 5-16 per mm² in the SUV condition, 6-20 per mm² in the IUV condition, and 5-13 per mm² in the LUV condition (Table 2). Although the miniature melanocytes did show an increase in population, it was quite small as compared to the typical melanocytes in all cases.

Using a t-test for ungrouped data and a Wang calculator, the significance of the experimental data was found. It was found that the counts for the typical melanocytes were of significant value when compared with the control animals for the IUV and SUV conditions. This was found using the 15 raw counts of the experimental as compared with the 15 counts of the experimental. In 6 out of the 7 cases calculated, the values for the typical melanocytes were of a statistical significance.

Using the t-test for the miniature melanocyte numbers it was found that there was no statistical significance to the variations in the numbers of the miniature cells between the experimentals and controls.

Table 3 is a comparison of the typical and miniature melanocyte numbers on day 7 of the first post-natal week for the conditions of control and irradiated animals.

Figures 1, 2, 3, and 4 summarize in graphical for the counts of typical and miniature melanocytes for the experimental and control PET/Wmr mouse epidermis.
DISCUSSION

Typical melanocytes are larger, more highly DOPA-reactive cells, while the miniature cells by comparison are weakly DOPA-reactive and have a much smaller-sized nucleus. The miniature cells are among the first pigment cells to appear in the first post-natal week in PET/7mr mice and they are among the last to disappear. The miniature cells occur in relatively constant numbers during the first post-natal week. The typical melanocytes, however, reach a peak number and then drop off considerably in the first post-natal week.

A very clear difference in the morphology of the miniature and typical melanocyte can be seen by viewing Figures 5 and 6. A miniature cell was counted as a cell that had less than one-half the typical nucleus size. Also Figure 8 shows a melanocyte field where differences in size of the melanocytes may also be noted.

Breathnach (1958) noted the characteristics of a newly pigmented area of a freckle scar. He noted the appearance of small melanocytes scattered among the large freckle melanocytes and reported that these small cells differ from those in the rest of the pale area in being even smaller and less strongly DOPA-reactive. Further, he felt that these two types of cells "bred true" and transmitted inherently different capacities for melanogenesis. He concluded that because the two different types of melanocytes bred true (old epidermis forming
new epidermis to fill in scar area) then two distinct families of this race of cell may be present in epidermis of freckled subjects.

The problem remains, though, as to the nature of the miniature melanocytes seen in normal mouse epidermis. Are they only immature, typical melanocytes or are they in fact, as Breathnach (1958) suggests, a distinct cell type?

There has been considerable work with UV effects on the epidermis. All information points to indirect evidence that in young mice the melanocytes which later lose their melanogenic activity comprise the same population in adult mice which can be activated by appropriate treatments (Rovee, 1965).

Quevedo and Smith (1965) believe that the UV may alter the area surrounding epidermal melanocytes in such a way that the dendritic responses could be an indirect result of the change caused by the UV. Quevedo and McTague (1963) have mentioned the possibility that any increase in melanogenesis may not be a direct response of the melanocytes to UV treatment, but rather an indirect response dependent upon appropriate cues from neighboring epidermal cells (shown by Reams and Schaeffer, 1968 a and b; see also Quevedo, et al., 1966). Although the mechanism of pigment cell increase following UV irradiation may be mitotic based on the occasional appearance of juxtaposed melanocytes (Figures 7, a and b), Sato and Kawada (1972) regarded the mechanism as one where the UV treatment activated an increase in the number of melanocytes by activation of pigment formation in amelanogenic melanocytes. The pigment cell increase based solely on mitosis seems unlikely because of the cell turn-over rate (about 36 hours). The increase in cell numbers is too rapid for mitotic activity to be
a likely explanation.

Quevedo, et al., (1969) found that the treatment of the buttock area of adult Caucasian males with UV produced hyperpigmentation at all ages. Quevedo found in non-irradiated skin a great variation in DOPA-reactivity as well as in melanocyte numbers with advancing age similar to my findings with the PET/Mmr mice. On the other hand, he found a more uniform DOPA-reactivity in the epidermal melanocytes of irradiated skin and a significant increase in numbers of melanocytes at all ages of subjects irradiated.

Pathak, et al., (1970) demonstrated with guinea pigs that UV radiation will promote a significant increase in DNA synthesis in the 300 nm range. For the SUV it was observed that this wavelength evoked a much smaller increase in DNA synthesis and the LUV evoked no increase in DNA synthesis.

If a miniature melanocyte is an immature version of a typical melanocyte, then this DNA activation observed by Pathak should convert the miniature melanocyte into a typical sized melanocyte. Hence, the 300 nm UV was chosen because of a possible induction of an increased DNA effect that might provoke changes in the condition of the pigment cells. Differences in UVL wavelengths may serve as one explanation for the increased numbers of typical and miniature melanocytes with the IUV as compared with the SUV. As expected the LUV did not have any noticeable effect on the pigment cell numbers in either the typical or the miniature case. In the present study the important aspect is the comparison between typical and miniature melanocytes in the IUV results and the controls.

With the influence of the UVL used in the present study strong
enough to promote the increase in typical melanocytes by day 7 in the IUV by roughly a 26 fold increase from day 1 to day 7 and it might be reasonable to expect a pronounced change in the miniature melanocytes. As Table 2 reflects, the increase in miniature melanocytes is actually only a 4 fold increase for the same conditions as above. As there was no decrease in number of miniature melanocytes with the larger increase in typical melanocytes, then it would appear that the UVL has had little or no effect on the conversion of the miniature melanocytes into typical form.

In as much as the data do not lend support to the concept that the miniature melanocytes are a phase in the formation of typical melanocytes, it may be presumed that the miniature melanocytes represent a cell type distinct from that of the typical melanocytes (Reams and Howard, 1973).
LITERATURE CITED


TABLE 1. AVERAGE NUMBERS OF TYPICAL MELANOCYTES PER mm² IN THE PET MOUSE EPIDERMIS

<table>
<thead>
<tr>
<th>DAY</th>
<th>300 nm (IUV)</th>
<th>250 nm (SUV)</th>
<th>360 nm (LUV)</th>
<th>CONTROL</th>
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<tr>
<td>0--Newborn</td>
<td>11.6</td>
<td>11.2</td>
<td>12.0</td>
<td>13.9</td>
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<td>1</td>
<td>16.2</td>
<td>15.4</td>
<td>14.6</td>
<td>17.4</td>
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<td>42.7</td>
<td>107.2</td>
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<td>3</td>
<td>172.2</td>
<td>163.3</td>
<td>158.0</td>
<td>234.6</td>
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<td>177.8</td>
<td>168.3</td>
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<td>222.4</td>
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<td>76.1</td>
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<td>250 nm (SUV)</td>
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<td>16.2</td>
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TABLE 3. COMPARISON OF AVERAGE MELANOCYTE NUMBERS PER mm²
FOR THE SEVENTH (7) DAY OF THE POST-NATAL PERIOD

<table>
<thead>
<tr>
<th>CONDITION</th>
<th>TYPICAL MELANOCYTES</th>
<th>MINIATURE MELANOCYTES</th>
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<tr>
<td>300 nm (IU)</td>
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<td>20.0</td>
</tr>
<tr>
<td>250 nm (SU)</td>
<td>300.0</td>
<td>16.2</td>
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<tr>
<td>360 nm (LU)</td>
<td>26.3</td>
<td>6.5</td>
</tr>
<tr>
<td>CONTROL</td>
<td>76.1</td>
<td>6.5</td>
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FIGURE 1. GRAPH SHOWING RELATIVE NUMBERS OF TYPICAL AND MINIATURE MELANOCYTES IN PET/wmr MOUSE EPIDERMIS UPON TREATMENT WITH 300 nm UVL.
FIGURE 1

300 nm UV TREATMENT

MELANOCYTES PER \text{mm}^2

POST-NATAL DAYS

- \text{typical}
- \text{miniature}

N
FIGURE 2. GRAPH SHOWING RELATIVE NUMBERS OF TYPICAL AND MINIATURE MELANOCYTES IN PET/Almr MOUSE EPIDERMIS UPON TREATMENT WITH 250 nm UVL.
FIGURE 2
250 nm UV TREATMENT

MELANOCYTES PER mm²

POST-NATAL DAYS

○ typical
○ miniature
FIGURE 3. GRAPH SHOWING RELATIVE NUMBERS OF TYPICAL AND MINIATURE MELANOCYTES IN PET/Nmr MOUSE EPIDERMIS (CONTROL, NON-IRRADIATED).
FIGURE 4. GRAPH SHOWING RELATIVE NUMBERS OF TYPICAL AND MINIATURE MELANOCYTES IN F1ET/HeaR MOUSE EPIDERMIS UPON TREATMENT WITH 360 nm UVL.
Figure 4
360 nm UV treatment

Typical

Miniature

Melanocytes per mm²
FIGURE 5. 5 DAY PET/Wnr EPIDERMIS, DOPA 300 nm TREATED -- 45X

A MINIATURE MELANOCYTE

B TYPICAL MELANOCYTE
FIGURE 6. 2 DAY PET/WIHR EPIDERMIS, DOPA CONTROL -- 45X

A MINIATURE MELANOCYTE

B TYPICAL MELANOCYTE
FIGURE 7 a. POSSIBLE MITOSIS OF MELANOCYTE IN Ptf/^mt EPIDERMIS -- 100 X
FIGURE 7 b. POSSIBLE MITOSIS OF MELANOCYTE IN PET/wmr EPIDERMIS -- 100 X
FIGURE 8. 4 DAY PET/Wmr EPIDERMIS, DOPA CONTROL -- 45 X

A  MINIATURE MELANOCYTE

B  TYPICAL MELANOCYTE
APPENDIX

The current scheme of the metabolic pathway of tyrosine to melanin (based on the studies of Hempel, Swan, and the Nicolaus group—from Fitzpatrick, et al., 1971).

\[ \text{DOPA} \rightarrow \text{TYROSINASE} \rightarrow \text{MELANIN} \]

5, 6 Dihydroxyindole