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CLINICAL AND EXPERIMENTAL ASPECTS OF LONG-WAVE ULTRAVIOLET (UVA) IRRADIATION OF HUMAN SKIN

by

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This thesis is based on the following papers, referred to henceforth by their Roman numerals.

- I. Beitner H, Wennersten G. Clinical aspects of the immediate pigment darkening (IPD) reaction in normal individuals. *Acta Derm Venereol* 1980; 60:323-326.
- II. Beitner H, Ringborg U, Wennersten G and Lagerlöf B. Further evidence for increased light sensitivity in patients with malignant melanoma. *Br J Dermatol* 1981; 104:289-294.
- III. Beitner H, Wennersten G. The immediate action of longwave ultraviolet radiation (UVA) on suprabasal melanocytes in human skin: A transmission electron microscopical study. *Acta Derm Venereol* 1983; 63:328-334.
- IV. Beitner H, Wennersten G. A qualitative and quantitative transmission electron microscopic study of the immediate pigment darkening reaction. *Photodermatol* 1985; 2:273-278.
- V. Beitner H. The effect of high dose long-wave ultraviolet radiation (UVA) on epidermal melanocytes in human skin: A transmission electron microscopic study. *Photodermatol*. In print.

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ABBREVIATIONS

UV	-	ultraviolet light
UVA	-	long-wave ultraviolet light
UVB	-	middle-wave ultraviolet light
UVC	-	short-wave ultraviolet light
TEM	-	transmission electron microscope
J	-	joule
MM	-	malignant melanoma
SSM	-	superficial spreading melanoma
NM	-	nodular melanoma
LMM	-	lentigo maligna melanoma
MED	-	minimal erythema dose
IPD	-	immediate pigment darkening reaction
ANOVA	-	analysis of variance
PUVA	-	Psoralen + UVA

INTRODUCTION

Our increasing knowledge of physics, biology and electronics has gradually enabled us to clarify the nature and biological effects of solar radiation. The science of photobiology has found application in different fields of technology, biology and medicine, and is divided into 14 subspeciality research areas. One of these areas is photomedicine. The rapid advances in photochemotherapy in particular have created a growing interest in the biological properties of the radiation most commonly used, long-wave ultraviolet (UVA) light.

The ultraviolet spectrum

Isaac Newton was the first to demonstrate that visible light contained a spectrum of colours from red to violet (1). Newton however failed to find any dark regions within the solar emission spectrum. In 1779 Carl Scheele (2) reported on the blackening effect of white light on silver chloride crystals. In an experiment designed to quantify the effectiveness of various colours on the silver chloride crystals, Johan Ritter noticed that radiation beyond the violet was the most effective (3). This radiation became known as ultraviolet radiation. Based on investigations mainly by Coblenz et al (4, 5) the Second International Light Congress in Copenhagen divided the ultraviolet spectrum into three regions according to the physical and biological properties of the radiation. Wavelengths ranging from 200 - 290 nano meters (nm) was termed short-wave ultraviolet radiation, abbreviated to UVC; the range from 290 - 320 nm was designated middle-wave ultraviolet radiation, UVB; and the band closest to visible light, 320 - 400 nm, was named long-wave ultraviolet radiation, UVA. Solar radiation shorter than 200 nm is absorbed by the air and the radiation below 290 nm is absorbed by the ozone layer in the stratosphere. The ultraviolet light can be characterized as non-ionizing electromagnetic radiation. The radiation consists of energy packages called photons. The energy content of the radiation has an inverse relation to the wavelength. Thus, shorter wavelengths have a larger energy content than longer bands.

Penetration and absorption of UVA in the skin

The optical properties of the skin are complex and no precise theoretical model of the path of radiation within the tissue exists. The penetration of skin tissue layers is affected by direct reflection, scattering, absorption and direct transmission through the layers (6). Direct reflection occurs at

the boundaries of a given layer, the highest level being observed at the air - stratum corneum interface. Because of the uneven skin surface the reflection becomes diffuse. Scattering represents a nonabsorptive change of radiation pathway due to molecules, particles, organelles and cells within a layer of the skin. A strong inverse relationship exists between scattering and wavelength. This is probably one of the reasons why radiation of shorter wavelengths does not penetrate the skin as deeply as radiation of longer wavelengths. When radiation is absorbed in a layer this might lead to a photochemical reaction. Transmission of UV radiation in human skin is dependent on epidermal thickness and concentration and distribution of absorbing molecules, so-called chromophores. Examples of chromophores in human skin are melanin, DNA, amino acids, caroten and urocanic acid. The major UV-absorbing chromophore is the brown pigment melanin. Increasing melanin concentration means decreasing UVA-transmittance. Thus, in negro skin about 5 to 15% of UVA radiation reaches the basal layer of the epidermis. In Caucasian skin 50% of incident UVA radiation is transmitted through the stratum corneum and absorbed in the epidermis. Approximately 35 to 50% is transmitted through the epidermis and absorbed in the dermis (7, 8). Thus, it is reasonable to assume, that at least in Caucasians, UVA can affect both epidermal and dermal cells and structures.

Effects of UVA on human skin

In 1900 the Danish physician and Nobel laureate Niels Finsen, seeking UV rays that would affect lupus vulgaris with minimal side effects, made the first observations of UVA effects on skin (9). Jüngling (10), using sunlight and mercury lamp filtered through a glass window, showed that pigmentation and erythema could be produced by radiation above 320 nm. Making use of monochromatic light Hausser and Vahle demonstrated that the dose necessary

to induce erythema with radiation of 366 nm was approximately 1000 times larger than with 297 nm (11). They also thought that erythema was necessary in order to evoke pigmentation but Peemöller was able to show that UVA caused pigmentation without erythema (12). In 1927 Gutman (13) observed a gray-brown pigmentation immediately after UVA irradiation, a reaction which became known as the immediate pigment darkening reaction (IPD). The IPD and other effects of UVA and visible light were investigated in great detail independently by Hausser (14) and Henschke & Schulze (15). They used both glass-filtered lamps and monochromatic radiation. The action spectrum for IPD was 300 - 420 nm, with a maximum at 340 nm. The IPD was observed approximately 2 minutes after the start of radiation and maximal pigmentation was reached within one hour. They also observed the absence of IPD in skin exposed to pronounced external pressure during radiation, an indication that this reaction was oxygen dependent.

The histology of skin exposed to UVA was examined with light microscopy by Miescher (16). He noticed that the inflammatory and cell toxic reaction observed after short-wave UV-irradiation was absent when the skin was irradiated by UVA only. The principal injury caused by UVA was to the dermal capillaries with necrosis of the endothelial cells. Pathak et al (17, 18) showed in 1962 that UVA and visible light can initiate the formation of new melanin, if the proper amount of energy is used. It was simultaneously observed that the irradiation caused a delayed pigmentation that remained for at least 60 days.

Pathak and Stratton (19) investigated the biophysical effect of UVA on skin. They found that UVA and visible light radiation produced free radicals in pigmented skin and that the IPD represents oxidation of the melanin polymer, which appears to take place through the formation of melanin-free radicals.

In recent years the erythemogenic and pigmentary effects of UVA in human skin have been reinvestigated. These studies have produced somewhat conflicting results. Kaidbey and Kligman (20) reported that maximal erythema and pigmentation was observed immediately after exposure. Pigmentation faded rapidly with threshold doses of 4 J/cm^2 , but with larger doses (18 J/cm^2) IPD gave way without fading to delayed pigmentation. Gilchrest et al found maximal erythema 8 - 15 hours after UVA exposure (21). Kaqetsu et al reported in 1985 that UVA-induced erythema and pigmentation changes are irradiance dependent. Higher threshold doses of UVA were needed to develop erythema and pigmentation at the lowest irradiance than at the highest. In this investigation maximal erythema was also observed without latency after UVA exposure (22).

Whether or not UVA has carcinogenic properties remains to be clarified. Available data indicate that UVA administered in doses equal in erythemogenic effect to UVB or given continuously without any rest period may be carcinogenic (23). Staberg et al found a rise in the incidence of skin tumors in hairless mice preirradiated with a combination of UVB and UVA, and then exposed to suberythemogenic doses of UVA (24). Further investigations are needed.

The epidermal melanocyte

The pigmentation of human skin is produced by melanocytes. This ectodermally derived cell has its origin in the neural crest (25, 26) and migrates through the dermis to be present at the dermal-epidermal junction area in the 10th fetal week (27). Apart from in the epidermis, melanocytes can also be found in the eye, in hair follicles, around blood vessels, in peripheral nerves and in the lining of the coelomic cavity. In the human

epidermis there are on average 1560 melanocytes/mm². The density of melanocytes varies from site to site. However, there are no significant sexual or racial differences (28, 29, 30, 31).

During the late 1950s Birbeck et al (32), Clark & Hibbs (33) and others described the ultrastructure of the human melanocyte. In these early transmission electron microscopic (TEM) works the site of intracellular formation of the melanin-containing pigment granules was discussed. Investigators like DuBay et al (34) favoured the concept that melanin formation took place in the mitochondria but subsequently increasing amounts of evidence have been gathered supporting the view that melanin granules are formed in the so-called Golgi zone of the melanocyte (35, 36). In 1961 Seiji and associates (37) were able to demonstrate that in B₁₆ mouse melanoma cells transplanted to C-57 strain of mice (combining centrifugation, enzyme assays and transmission electron microscopy) melanin granules contained most of the tyrosinase activity. These granules, named melanosomes, were distinct cytoplasmic constituents of the melanin-forming cell. The premelanosomes formed in the Golgi zone localized in the perikaryon of the melanocyte, were transported to the periphery of the dendrites of the melanocytes. Microfilaments and microtubules were found to play a prominent role in this intracellular transport (38). During this transfer the premelanosomes gradually develop into mature, fully melanized melanosomes. This process of maturing is classified in four stages (I - IV). The size of stage IV melanosomes is genetically controlled and varies from race to race. There seems to be a good correlation between dark skin colour and single large melanosomes and light colour and aggregated small melanosomes (39). It is estimated that a melanocyte with its dendrites supplies 36 keratinocytes with melanosomes. This constellation of melanocytes and keratinocytes is known as the epidermal melanin unit (40). The transfer of melanosomes from melanocyte to keratinocyte is a phagocytotic process (41). In the keratinocyte small melano-

somes are aggregated as melanosomal complexes surrounded by a phagosomal membrane (42). In dark complexioned races melanosomes are dispersed as single organelles. In the cytoplasm of the keratinocyte the melanosomes are gradually degraded, and vanish through the repulsion process of keratinocytes in the stratum corneum (43, 44).

Light sensitivity and malignant melanoma

The etiology of malignant melanoma (MM) is multifaceted and complex. Sunlight has been considered a major risk factor in the etiology of MM (45, 46), mainly based on the inverse relationship between geographical latitude and incidence of MM (47, 48). The highest incidence rates are reported from countries at lower latitudes with Caucasian population of fair complexion (49, 50, 51). As early as 1896 Matas (52) reported on the low incidence of MM in American negroes. Black patients have a greater predisposition to develop acral melanomas in less pigmented skin areas and the same relationship has been observed in Japanese and Chinese patients (53). Epidemiologic data from Israel also show different incidence rates among Jews of different ethnic origin, with the lowest figures among those of darker complexion (54). Thus, differences in degree of pigmentation and incidence of MM indicate that individual light sensitivity might be of etiological importance.

AIMS OF THE STUDY

The aims of the present study were

- To investigate the IPD and its clinical characteristics in normal healthy individuals in relation to skin type and photodermatological parameters
- to evaluate light sensitivity as an etiological factor in MM
- to examine the immediate effect of UVA in non-erythematous doses on epidermal melanocytes in the normal skin of Caucasian individuals with skin type II and III
- to study keratinocytes and epidermal melanocytes with TEM before and immediately after UVA irradiation in individuals with a significant IPD (skin type III) compared with those with a cramped IPD (skin type II)
- to follow the effect of high doses of UVA in epidermal melanocytes in healthy subjects belonging to skin type III with normal IPD by TEM

MATERIALS AND METHODS

A. Clinical studies (I, II)

Subjects (I, II)

130 consecutive cases of MM were studied. 73 healthy Caucasian individuals without any known pathological reaction to solar irradiation were used as controls. The melanoma group consisted of 63 men and 67 females. Mean age at diagnosis for the different melanoma types are specified in Paper II, Table 2.

The control group consisted of 28 (I) and 29 (II) men respectively and 44 females, with the following age distribution: men 39 ± 18 (range 22 - 76) years; females 37 ± 11 (range 22 - 62).

Classification of skin type (I, II, III, IV and V)

Normal reactions to solar radiation were evaluated for all individuals, the criteria (55, 56, 57) being based on the history of the usual reaction to the first hour of full sun exposure in early summer in Sweden. The degree of erythema and pigmentation usually obtained was assessed as follows: Skin type I-always burn, never tan; II-always burn, sometimes tan; III-sometimes burn, always tan; and IV-never burn, always tan.

Histopathological classification of MM (II)

Depending on the size of the skin biopsy, one to several pieces of the malignant melanoma were prepared for histology and stained with haematoxylin and eosin according to routine procedures. In some cases Masson's stain for pigment was used. The MM:s were classified in the following groups: superficial spreading malignant melanoma (SSM), nodular melanoma (NM) and lentigo maligna melanoma (LMM) (58).

Light source (I, II, III and IV)

Light testing in the first four experiments was carried out with an Osram high pressure xenon arc lamp (XBO 150 W) in a Zeiss microscope lamp housing with a quartz collector to produce a round, uniformly bright spot, 1.5 cm in diameter, at an exposure distance of 15 cm from the filter holder to the skin. For estimation of minimal erythema dose (MED) the lamp was equipped with a Schott WG 295 filter giving a sun spectrum type radiation (59). In order to provide UVA and visible radiation, suitable for IPD inducement, the lamp was also equipped with a 3 mm ordinary glass filter and a KG I heat protective filter. The measurement procedure of the relative spectral irradiance is described in detail in Paper III (60, 61). Absence of measurable amounts of irradiation below 320 nm, when the lamp was equipped with the filter combination of Schott WG 295, 3 mm glass and KG I, is demonstrated in Fig. 1, Paper III.

Light testing procedure (I, II)

Testing for threshold doses of MED was done on the untanned part of the mid

back. The skin was exposed to 6 - 32 seconds of UVB in a series of increasing exposures with 2-second increments (62, 63). Minimum perceptible erythema was assessed 24 hours after exposure.

IPD was tested on the untanned part of the gluteal region. Continuous UVA radiation was administered and the time lapse before the appearance of a significant, clearly visible, gray-brown IPD was registered, from which the corresponding threshold dose in J/cm^2 was calculated.

All testing took place during the winter months, with an interval of at least four months after sun bathing.

B. Experimental studies (III, IV and V)

Subjects (III, IV and V)

Ten healthy Caucasian volunteers, 8 females and 2 men, 23 to 60 years old, participated in the studies. In studies III and IV, three individuals belonging to skin type II, with a cramped IPD and two subjects with skin type III with low IPD threshold values were included. Details about light testing data are presented in Paper III, Table I. Only subjects with normal IPD with skin type III were included in the last investigation.

Light source (V)

The light source used in experiments III and IV has previously been described in "light source" part A.

In experiment V irradiation was carried out with a glass-filtered UVASUN 2000 lamp (Mutzhass International AG, Switzerland). Relative spectral irradiance expressed as a percentage of the total UV-emission was measured with a spectro-radiometer H 20 UV-V built on a calibrated monochromator (Jobin Yvon, Longjumeau, France). The spectral irradiance curve (V, Fig. 1) demonstrates the absence of measurable amounts of irradiation below 320 nm. The intensity of the lamp with this filter at a distance of 20 cm was determined using an 88 XLA radiometer (Photodyne Inc., California, USA) to 27.8 mW/cm² (64).

Light testing procedure (III, IV)

IPD and MED were tested as described above. With the XBO 150 W lamp equipped as for IPD testing, the untanned part of the gluteal region was irradiated with 0.45, 0.9, 1.75, 3.6, 7.25 and 14.5 J/cm² of UVA. This corresponds to 1, 2, 4, 8, 16 and 32 minutes of irradiation time.

Light testing procedure (V)

Irradiation was carried out with the UVASUN 2000 lamp at an exposure distance of 20 cm to the untanned part of the gluteal region. The skin was covered with a black lightproof photopaper in which a hole of 1.5 cm in diameter had been cut. The center of the lamp was aimed at this uncovered part of the skin, which was irradiated with 50, 100 and 150 J/cm² corresponding to 30, 60 and 90 minutes of irradiation time.

Transmission electron microscopy (III, IV and V)

Punch biopsies, 2 mm in diameter, were taken from the gluteal region before irradiation and within 2 minutes of UVA exposure. The specimens were directly transferred to small UVA opaque containers at a temperature of +4°C where they were fixed in 2% glutaraldehyde in 0.1 M sucrose buffer, pH 7.4 (300 mOsm)(65). The tissues were post fixed in 1% osmium-tetroxide, stained with uranyl acetate and, after dehydration through graded concentrations of ethanol and propylene oxide, embedded in Epon (III, IV) or Agar 100 (V). Sections (600 Å) were cut on an LKB ultratome. The thin sections were mounted on one hole copper slate grids, 2 x 1 mm, post stained with uranyl acetate and lead citrate and viewed in a JEOL 100 C TEM at 60 kV. All consecutive basal epidermal melanocytes - identified by the presence of premelanosomes, melanosomes and by the absence of tonofilaments, desmosomes and Langerhans' granules - sectioned through their nuclei, were photographed. Surrounding basal keratinocytes were evaluated in Paper IV. The qualitative judgement of the electron micrographs was made before classification according to degree of UVA exposure.

Observed qualitative changes were quantified and evaluated statistically with Student's t-test (IV) or chi-square test (V) (60).

Morphometry (IV)

Paper IV investigated the number of melanosomes in the keratinocytes surrounding the basal melanocytes and the number of melanosomes in the perikaryon of the melanocytes before and after 14.5 J/cm² of UVA. This was done in one subject with no visible IPD and skin type II and one belonging to skin type III with an IPD threshold dose of 2.7 J/cm². The longitudinal and transverse melanosome diameters in basal keratinocytes chosen at random

were also measured together with the number of keratinocytes with capping phenomenon (melanosome complexes predominantly distributed above the keratinocyte nucleus). Further details are presented in Paper IV.

RESULTS

A. Clinical studies

Light testing (I)

In the control material of normal individuals of Caucasian origin only skin types II, III and IV were represented. MED and IPD threshold doses for the different skin types are summerized in Table I.

Table I.

Skin type	Number of Individuals	MED (s) (mean value $\pm$ S.D.)	IPD (J/cm ²)
II	9	10.7 $\pm$ 6.2	≥ 9.0
III	56	15.3 $\pm$ 7.6	3.8 $\pm$ 2.3
IV	7	18.6 $\pm$ 4.1	2.8 $\pm$ 1.9

Tested with analysis of variance (ANOVA) the mean MED value for individuals with skin type II was significantly lower than for those with skin type III ($p < 0.05$) and skin type IV ($p < 0.01$). The difference between skin type III and IV was not significant.

IPD threshold values revealed a similar pattern. The difference between skin types II and III, and II and IV, was statistically significant, $p < 0.05$ and $p < 0.01$ respectively, but this was not the case between skin types III and

IV. Among the nine individuals with skin type II, only one showed a positive IPD at 9 J/cm². Four subjects were subsequently retested with higher UVA doses and two were then positive, with IPD seen at 12 and 14 J/cm²; the other two individuals were still negative at the maximum exposure of 16 J/cm².

In skin type III (n = 56) no significant correlations were found between sex or age and MED or IPD doses. Furthermore, no significant relationship, either negative or positive, was found between the individual's MED and IPD. The squared correlation coefficients (r²) were 0.00 for men and 0.08 for women. Expressed in a different way - the probability of no significant correlation between IPD and MED was 100% for men and 92% for women.

Histopathological classification of MM (II)

The distribution in relative and total numbers among the different tumor and skin types are presented in Table II.

Table II.

Tumor type	Skin type I	%	Skin type II	%	Skin type III	%	Skin type IV	%	Total	%
SSM	2	1.5	24	18.5	59	45.4	11	8.5	96	73.8
NM			4	3.1	12	9.2	1	0.8	17	13.1
LMM					3	2.3	2	1.5	5	3.9
Not classified					9	6.9	3	2.3	12	9.2
TOTAL	2	1.5	28	21.5	83	63.9	17	13.1	130	

The mean age for LMM patients at diagnoses was 67.6 ± 11.7 years which was significantly higher than for SSM ($p < 0.01$) and NM ($p < 0.05$), when calculated with t-test. The mean ages for SSM and NM at diagnosis were 50.9 and 51.7 years respectively. Patients belonging to skin type IV showed the highest mean age, 61.5 ± 9.5 years. The higher mean age for tumor occurrence in skin type IV proved to be statistically significant, when assessed by ANOVA, $p < 0.01$. This difference remained even when the patients with LMM were excluded, $p < 0.05$.

Light test data of MM patients and controls (II)

Total mean MED for MM patients was in general significantly lower (t-test; $p < 0.01$) than for controls (Table III).

Most patients ($n = 83$) and controls ($n = 57$) belonged to skin type III. The difference in mean MED value for this skin type (Table III), when patients and controls were compared, was significant (t-test; $p < 0.01$). Except for LMM no significant difference between tumor types was found.

Table III. Mean MED value $\pm$ S.D. (seconds) in relation to tumor and skin type compared with controls.

	Skin type	Skin type II	Skin type III	Skin type IV	Total
SSM	13.0	11.0	11.8	14.4	11.9 ± 5.1
NM		10.0	12.8	18.0	12.5 ± 5.7
LMM			4.7	21.0	17.2 ± 9.5
TOTAL	13.0 ± 7.0	10.9 ± 3.9	12.1 ± 5.3	15.1 ± 6.4	12.3 ± 5.2
CONTROLS (mean value)		10.7 ± 6.2	15.1 ± 7.7	18.6 ± 4.1	14.8 ± 7.3

Mean IPD values displayed no significant differences between MM-group and controls. The difference when each skin type for the two categories were compared were small (Table IV).

Table IV.

Skin type	Mean IPD threshold doses $\pm$ S.D., J/cm ²	
	Controls	MM patients
II	≥ 9.0	≥ 9.0
III	3.8 ± 2.3	3.6 ± 5.7
IV	2.8 ± 1.9	3.5 ± 5.5

MED and IPD were not correlated to age and sex when assessed for the largest group of controls, skin type III. In the largest melanoma group, SSM skin type III, no significant correlation was found between age and MED. The squared correlation coefficient for men was 0.19 and for females 0.08.

B. Experimental studies

Light test data

MED in seconds varied among the five subjects who participated in experiments III and IV, between 6 and 10 s. IPD was absent up to a maximal dose of 20 J/cm² in the three subjects who belonged to skin type II. The

remaining two individuals belonging to skin type III showed similar low IPD threshold doses of 2.7 J/cm^2 . The five subjects included in Paper V, all demonstrated a clearly visible erythema in direct connection to UVA exposure at 50 J/cm^2 . IPD was observed after 3 - 5 J/cm^2 of irradiation. Exposure to these erythema-producing doses of UVA caused a delayed tanning that was visible 3 months afterwards.

INTRACELLULAR OBSERVATIONS IN MELANOCYTES

Low dose UVA (III)

Before UVA-exposure

The epidermal melanocytes had in general a large oval nucleus and narrow surrounding cytoplasm. In the cytoplasm intermediate filaments were clearly visible (III, Fig. 4 b). Cytoplasmic organelles such as Golgi apparatus were poorly developed. Mature stage IV melanosomes were rare or absent in skin type II melanocytes. Mitochondria were congested and had intact inner membranes.

After UVA-exposure

In skin type II multiple endo/exocytotic vesicles, swollen mitochondria with partial to total dissolution of the inner membranes and large solitary vacuoles were seen after 3.6 J/cm^2 (III, Fig. 2). After 14 J/cm^2 formation of numerous small vesicles associated with enlarged Golgi complexes appeared

in the perikaryon in part of the epidermal melanocytes (III, Fig. 3). In skin type III melanocytes after maximum suberythemic irradiation also appeared essentially unaltered (III, Fig. 5).

High dose UVA (V)

In order to investigate whether the difference in melanocyte reaction to UVA irradiation in those with skin type II and cramped IPD and skin type III with normal IPD threshold values, was dose dependent, 288 melanocytes evenly distributed among the five volunteers with skin type III and normal IPD were investigated before and after erythema-producing UVA doses.

Before UVA-exposure

The general impression of non-irradiated melanocytes was similar to the observations made in Paper III. In 9/75 (12%) a single change from this pattern was observed. Seven of these cells had swollen mitochondria with dissolved cristae. Two cells showed a combination of swollen mitochondria, increased endo/exocytotic activity and one enlarged Golgi apparatus (Table V).

After UVA-exposure

The changes observed in the epidermal melanocytes after irradiation of 50 - 150 J/cm² were: enlarged Golgi apparatus with associated vesicles predominantly of the coated variant, swollen mitochondria with ruptured cristae (V, Fig. 3 a and b), marked endo/exocytotic activity (V, Fig. 4) and cells

with two nuclei (V, Fig. 5). Thus, the morphological changes were of the same kind as previously observed in skin type II at lower UVA doses. Tested with chi-square according to the following formula:

$$\chi^2 = \sum \frac{(O_n - E_n)^2}{E_n} ; \quad (O_n = \text{Observed values}, E_n = \text{Expected values})$$

each of these signs of morphological change in the melanocytes before and after irradiation were statistically significant (chi-square = 13.2 - 32.0; $p < 0.01 - 0.001$). As shown in Fig. 1 the relative number of observed changes in each individual varies, but the accumulated data from the five subjects demonstrates a sharp increase in observed changes at 50 J/cm² and then tends to level out between 100 and 150 J/cm².

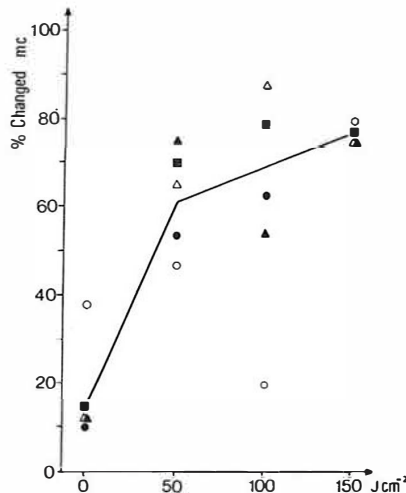


Fig. 1. The relative distribution of melanocytes (mc) with one or more observed changes in each of the five subjects included in the study. Each subject is indicated by an individual symbol. The line represents the mean value.

Irradiated cells show a significant increase in one or more of the ultra-structural changes mentioned above (chi-square = 64.9; $p < 0.001$). Melanocytes with a combination of three or more of these changes were significantly (chi-square = 15.7; $p < 0.01$) more common among irradiated cells (table V).

Table V. Epidermal melanocytes with observed enlarged Golgi apparatus with associated vesicles, swollen mitochondria with ruptured cristae, endocytosis and cells with two nuclei.

UVA J/cm ²	Number of cells with changes	(%)	Number of cells with changes	(%)
0	11/75	(14.7)	2/75	(2.7)
50	49/80	(61.3)	12/80	(15.0)
100	46/67	(68.7)	19/67	(28.4)
150	51/66	(77.3)	18/66	(27.3)
TOTAL	157/288	(54.5)	51/288	(17.7)
Chi-square	64.9 ($p < 0.001$)		15.7 ($p < 0.01$)	

Morphometric measurements (IV)

Morphometry was carried out in order to follow the flow of stage IV melanosomes before and after UVA irradiation in one subject with IPD, skin type III, and one without IPD, skin type II, between epidermal melanocytes

and surrounding keratinocytes. The total number of melanosomes counted by four independent observers were 12 624. In skin type II no significant difference in the number of melanosomes was observed after 14.5 J/cm² of UVA irradiation, either in the perikaryon of melanocytes or in the basal keratinocytes. In skin type III the mean value and standard deviation of melanocytes per area unit was 48.8 ± 7.89 before irradiation. After exposure the corresponding mean value was 37.8 ± 2.95 . This decrease was not, however, statistically significant. The corresponding number of melanosomes in the keratinocytes remained essentially unaltered (IV, Table II).

The difference in longitudinal and transverse diameters of the keratinocyte melanosomes before and after irradiation was not significant (IV, Table III). In sections from biopsies with IPD, capping was observed in 57.5% of basal keratinocytes before and 57.2% after irradiation.

INTRACELLULAR OBSERVATIONS IN KERATINOCYTES (IV)

Qualitative observations on keratinocytes (IV)

In the keratinocytes no qualitative changes of the ultrastructure or the distribution of cell organelles have been observed due to UVA irradiation.

DISCUSSION

Skin typing was introduced by Fitzpatrick in 1975 (55). It is a practical method for characterizing the individual reaction pattern to sun exposure. The method has proven useful in clinical practice as a parameter in light tests and for estimation of start doses in photochemotherapy. Although the precise mechanism underlying the MED reaction is still unknown, the reaction itself is considered to be a well established parameter for the investigation and assessment of individual light sensitivity. The possibility of using IPD as a parameter in the investigation of light sensitivity had previously not been sufficiently explored. These investigations started with a study concerning the relationship between skin type, MED and IPD (I). When skin types were compared with each other, individuals with skin type II in general had a low MED dose and a high IPD threshold value. This pattern is consistent with a history of increased light sensitivity. Absent or high IPD threshold values were seen for all individuals in skin type II. In skin types III and IV all subjects demonstrated IPD at significantly lower UVA doses. Within the largest group, skin type III, no correlation could be observed in individuals between IPD and MED. Thus, IPD and MED have to be considered separate phenomena.

We have used the increased knowledge gained from this investigation to include the IPD as an important parameter in our panel of light tests. Start doses in our practice of photochemotherapy are based on skin typing. In situations when doubts arise about the skin type to which a subject belongs IPD can be used for refinement of the skin typing. Individuals with absent or cramped IPD are then regarded as belonging to skin types I or II. Furthermore, the IPD has a practical advantage over MED, in that the former

is usually clearly visible after a few minutes, in contrast to MED, that has to be read after 24 hours. Thus, the decision time is shortened, and the safety of the patient is enhanced.

The investigations were continued by comparing light test data from 130 consecutive melanoma patients with 73 controls. The purpose was to quantify possible differences in light sensitivity in the two categories. The proportion of the main clinical and histological types of melanoma (Table II) was similar to that observed in the majority of previously reported studies of MM (67). Thus, the patients in the study (II) probably constituted a representative sample of MM patients. Patients with LMM showed a significantly higher mean age at diagnosis than SSM and NM. Light test data were also different. All LMM patients belonged to skin types III and IV. LMM patients also demonstrated normal MED values, which serves as further evidence that this form of MM represents a biologically separate entity. Overall the melanoma group had lower MED values. When the largest group, skin type III with 57 patients and 83 controls, was compared mean MED was significantly lower in MM patients. It must however be stressed that individual MED values varied within each skin type in both groups. As among controls IPD was absent or difficult to develop in skin types I and II and IPD did not differ between controls and patients within each skin type. Thus, IPD is not a parameter that discriminates between healthy individuals and MM patients. However, as a consequence of the increased proportion of skin types I and II in MM, which has been confirmed by other investigators (68, 69), the number of MM-patients with absent or cramped IPD was increased (II). The inability to tan and increased light sensitivity probably constitute important risk factors among Caucasians developing melanoma (70).

The first TEM study (III) investigated the effect of UVA on the ultra-structure of melanocytes immediately after irradiation. The observed

intracellular changes with a combination of enlarged Golgi apparatus with associated coated vesicles, swollen mitochondria and increased endo/exocytotic activity was seen only in melanocytes from subjects belonging to skin type II and lacking IPD. Melanocytes in subjects with skin Type III and IPD remained essentially unaltered. This difference further emphasizes the importance of characterizing the light sensitivity profile of individuals included in this kind of experiment. No erythema was observed in the skin after the maximal UVA irradiation of 20 J/cm² used at the present study. This is consistent with earlier studies in which UVA-induced MED in fair-skinned Caucasians have been found to be in the range 29 - 36 J/cm² (71). The results of the experiment indicated a connection between individual pigmentation capacity and UVA dose. The combination of the presence of IPD and the absence of intracellular changes could indicate the possibility that one function of the IPD might be to absorb extensive UV light, thus reducing the dose reaching the melanocytes and underlying structures. However, the observed difference might also be due to a difference in the number of preformed melanosomes in the keratinocytes, that is the presence of a melanin filter. In experiment No. III a clearly visible difference in the number of melanosomes in the keratinocytes was seen at low magnification between individuals with skin type II and those with skin type III. As a consequence of this observation the material was further penetrated for quantitative estimations.

The mechanism of the IPD is still not fully understood and remains to some extent controversial. Based on morphological, light microscopical, biophysical, biochemical and TEM studies (13, 14, 18, 72, 73, 74) the IPD, is considered in current photobiological literature (75, 76, 77) to consist of the following events: a) photooxidation of preformed melanin (78) and the creation of unstable semiquinone-like free radicals (18); b) a changed distribution pattern of preformed melanosomes in basal and suprabasal keratino-

cytes (74); c) a changed distribution pattern of microtubules from the perinuclear area of the melanocytes to the periphery, which leads to the establishment of prominent dendritic processes (72, 73); d) movement of melanosomes from the perikaryon to the dendrites of the melanocytes (72, 73) and e) an increased transfer of melanosomes to the basal keratinocytes. The results from our morphometric study do not support the concept that IPD is the result of major morphological changes either in epidermal melanocytes or in the surrounding keratinocytes. Thus, I believe that the IPD should be considered a passive photochemical reaction occurring in preformed melanosomes. This view is supported in a recent reevaluation of the IPD mechanism (79) and seems to be gaining increasing acceptance (80, 81, 82). The concept of IPD as a purely photochemical reaction is also the main reason for my not adopting the more recent synonym "immediate tanning-IT". According to my opinion IT is associated with the morphological change concept (83).

The impression of a quantitative difference in number of preformed melanosomes between those individuals with and those without IPD was confirmed by the morphometric study (IV). The number of preformed melanosomes was 10 times higher in the melanocytes and the keratinocytes of the subjects showing IPD. The limited number of individuals investigated does not allow the drawing of general conclusions about this difference between skin types II and III. On the basis of the available knowledge about the IPD it seems plausible to suggest that the absent or cramped IPD in skin types I and II could be due to the presence of too few preformed melanosomes.

Since the question arose as to whether the absence of intracellular changes in the melanocytes of individuals with IPD was dose dependent, an investigation with high erythema-producing doses of UVA was carried out (V).

The changes observed in the skin type III individuals after UVA irradiation

were of the same nature as those previously found in skin type II melanocytes after irradiation with suberythemic doses of UVA (III). In this study (V) cells with two nuclei were seen after 150 J/cm² of UVA (V, Fig. 5). The significance of this finding was difficult to evaluate. As serial sectioning was not performed it could not be ascertained whether the nuclei profiles were from one or two nuclei. The irradiation time was 1.5 hours, which seems to be a rather short period of time for assuming that this represents a postmitotic event due to the UVA irradiation. The perikaryon of these cells was much larger than that seen in "normal" melanocytes. One possible explanation is that these cells are so-called giant melanocytes (84), which have been observed after UV irradiation.

In both irradiated and non-irradiated melanocytes ultrastructural deviations could be found. The most commonly observed alteration was the swelling and partial to total dissolution of inner membranes and cristae of the mitochondria. This phenomenon was not observed in adjacent keratinocytes. The difference indicates that the histotechnical preparation causes a greater injury in the mitochondria of the melanocytes. This could be due to a different metabolic state in these different cell types. The significant increase in irradiated melanocytes suggests that the UVA irradiation induces a changed metabolic state in the cell, which is more vulnerable to the mitochondria of the melanocytes. A possible mechanism for this alteration could be that increased amounts of lactate is accumulated in the melanocytes during the ischemic phase that occurs before the fixation of the cell is completed (85). As a result the intracellular pH level is decreased which can cause swelling of mitochondria (86). Another possibility is that the shape of the mitochondria changes with different metabolic activity levels in the cell (86). Thus, the observed change in the appearance of the mitochondria could be of physiological nature.

The most striking change in melanocytes was the combination of an enlarged Golgi zone with several Golgi apparatuses with long discs and with numerous associated vesicles, predominantly of the coated variant - so-called primary lysosomes, that significantly increased after UVA exposure (III, Fig. 3 and V, Fig. 3 a and 3 b). Similar changes have previously been observed in psoriatic patients after PUVA treatment (87). Since these changes could not be related to the amount delivered of UVA, it was assumed that they were caused by the photochemotherapy. The results of our investigations (III and V) show that UVA alone can induce this pattern of changed activity in part of the melanocytes. These observations can, thus, serve as an ultrastructural basis for further studies of prospective intracellular changes in the melanocytes due to PUVA.

The enlarged Golgi zone combined with swollen mitochondria and the increased endo/exocytotic activity are characteristic of other cell types with a mainly secretory function (88) and thus can be considered as a physiological reaction, caused by the UVA-radiation.

In the light of previous knowledge, it was somewhat surprising to find all these changes indicative of altered melanocyte activity already approximately 30 minutes after the start of irradiation. They probably represent the onset of melanosomal differentiation resulting in delayed tanning 18 - 24 hours later. This has been demonstrated in C 57 Black mouse melanocytes (89).

The two major protective systems of the skin against electromagnetic radiation are the thickening of the stratum corneum (90, 91) and the layer of melanin containing mature melanosomes in the keratinocytes, the melanin filter. This melanin barrier has proven to be the most important single factor in the protection of the skin (92, 93). The inherent epidermal melanin content of skin unexposed to UV-irradiation is termed constitutive

skin colour (83). As indicated previously the IPD probably reflects the level of constitutive pigmentation, because the presence of IPD seems to parallel the presence of constitutive pigmentation seen in skin types III and IV and vice versa in skin types I and II. It has been shown, that the melanin filter significantly diminishes the UV-radiation penetrating the epidermis and reaching the basal layer in which the melanocytes are situated. Thus, due to the filter effect the UVA-dose affecting the melanocytes is reduced and higher UVA doses are required to pass the threshold that activates the melanosomal differentiation in the melanocytes. This could be the mechanism behind what seems to be a dose dependent response in the melanocytes. It offers an explanation for the lack of ultrastructural alterations in the melanocytes of individuals with IPD and belonging to skin type III at lower UVA doses and why these signs of a changed activity in the melanocytes were induced after the dose increase.

The maximal UVA doses used in these experiments are high, but can be compared with doses obtained in real life. The UVA doses during the summermonths in Sweden are 14 - 18 J/cm² per hour. Sun tanning between 10 a.m. to 4 p.m. a nice summer day would result in a total UVA dose of 84 - 108 J/cm². In commercial sun tan beds the UVA dose per hour is in the range of 50 - 70 J/cm². The pigmentation obtained as a result from exposure to commercial fluorescent UVA lamps have by some investigators been claimed to be due to the low percentage of UVB emitted by this tubes. The results of this investigation demonstrates that melanocytes, independent of skin type, react promptly to UVA radiation if sufficient doses are used.

GENERAL SUMMARY

- The clinical characteristics of the IPD in 72 normal healthy Caucasians individuals and its relation to age, sex, skin type and MED were investigated. Individuals classified as skin type II were found to have a significantly lower mean MED and higher mean IPD than those with skin type III and IV. The IPD was absent in several subjects with skin type II under the experimental conditions used. Age or sex did not appear to be significantly related to MED and IPD. No significant correlation, either positive or negative was found between the MED and IPD, when investigated for the individuals in the largest group, skin type III. Thus, IPD and MED have to be considered separate phenomena.

- MED, IPD and skin typing were performed on 130 patients with malignant melanoma and on 73 controls. The tumors were histologically classified according to Clark. The tumors of 12 patients remained unclassified. The proportion of melanoma types was similar to that observed in previous studies of malignant melanoma. Patients belonging to skin type IV and patients with lentigo maligna melanoma had a significantly higher mean age at diagnosis. The IPD threshold value was significantly higher in skin type II than in skin types III and IV both in patients and controls. Thus, IPD is not a parameter that discriminates between healthy individuals and melanoma patients. When the largest group, skin type III with 57 patients and 83 controls, was compared mean MED was significantly lower in melanoma patients. Individual MED values varied within each skin type in both groups. In general these data indicate that light sensitivity in a wide sense is of etiological importance in malignant melanoma.

- The immediate effects of UVA in non-erythematous doses on epidermal melano-

cytes in normal unpigmented skin of healthy Caucasians with skin types II and III were investigated with TEM in order to detect possible morphological changes. In skin type III the melanocytes remained essentially unaltered. In skin type II multiple endo/exocytotic vesicles, swelling and partial to total dissolution of cristae in the mitochondria and numerous coated vesicles associated with an enlarged Golgi apparatus were observed after 3.6 to 14.5 J/cm² of UVA irradiation. No IPD was observed in any of the subjects with skin type II irradiated with UVA up to 20 J/cm².

- Qualitative observations of the keratinocytes were obtained from five subjects. No changes of the ultrastructure in the keratinocytes could be observed after 14.5 J/cm² of UVA radiation. A sample of one subject with IPD (skin type III) and one without (skin type II) was investigated with morphometry. The number of preformed melanosomes was 10 times higher in the melanocytes and in keratinocytes of the subject showing IPD as compared with the one who did not. No statistically significant difference was observed in either skin type regarding the number of mature (stage IV) melanosomes or redistribution of melanosomes in the keratinocytes after UVA irradiation. These results indicate that the IPD should mainly be considered a photochemical reaction occurring in preformed melanosomes. The absence of IPD in skin type II could be due to the presence of too few preformed melanosomes.

- Five healthy subjects with IPD (skin type III) were irradiated with 50, 100 and 150 J/cm² of UVA. TEM showed a significant increase in ultrastructural alterations of the same nature as described previously after PUVA therapy. The changes were interpreted as an increased secretory activity in a portion of the epidermal melanocytes. It is concluded that melanocytes, independent of skin type, react promptly to UVA radiation if sufficient doses are used.

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LEGENDS TO FIGURES

Fig. 1a. Relative spectral irradiance of the Osram high pressure xenon arc lamp (XBO 150W) equipped with a Schott WG 295 filter, a 3-mm ordinary glass-filter and a KG I heat-protective filter giving long-wave ultraviolet radiation.

Fig. 1b. Relative spectral irradiance curve of the glass-filtered UVASUN 2000 lamp. No measurable amounts of UV-radiation below 320 nm could be detected with the equipment used.

Fig. 2. Epidermal melanocyte before UVA exposure. Large oval nucleus with narrow cytoplasm. Multiple well preserved mitochondria ($\leftarrow$) and premelanosomes ($\leftarrow$). The Golgi zone (g) with vesicles ($\leftarrow$) is poorly developed. Bar = 1 μ m.

Fig. 3a. Melanocyte (mc) and part of the adjacent keratinocyte (kc). Dose 150 J/cm². Bar = 1 μ m. Frame = Fig. 3b.

Fig. 3b. High power electron-micrograph demonstrating numerous coated vesicles ($\leftarrow$) and premelanosomes ($\leftarrow$) associated with enlarged Golgi complexes (g). Swollen mitochondria with ruptured cristae ($\leftarrow$) and stage IV melanosomes (*) are seen. Invaginations of the cell membrane ($\leftarrow$) indicating endo/exocytoses. The basal lamina (b). Bar = 1 μ m.

Fig. 4. Numerous invaginations of the melanocyte cell membrane suggestive of a high endo/exocytotic activity. Dose 50 J/cm². Bar = 1 μ m.

Fig. 5. Electron-micrograph showing a melanocyte with a large perikaryon, 2 nuclei with nucleoles (n), centrosome (∇), swollen mitochondria ($\leftarrow$) and multiple stage IV melanosomes (*). Dose 150 J/cm². Bar = 2 μ m.

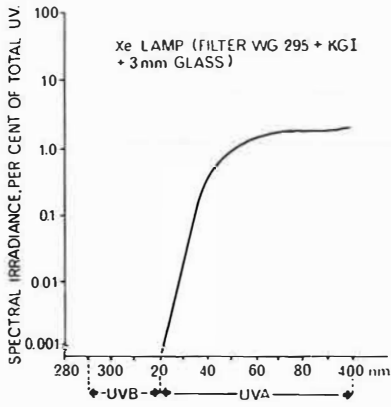


Fig. 1a.

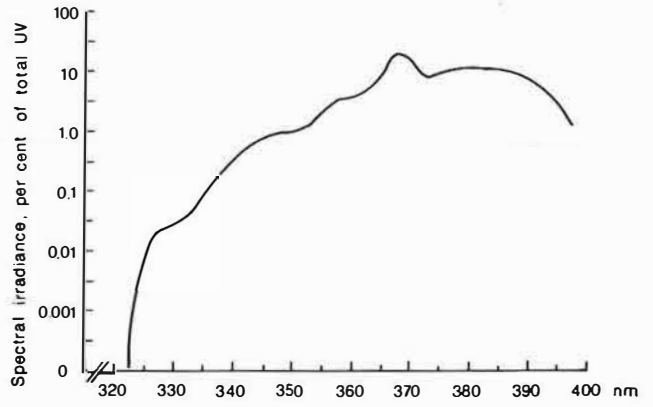


Fig. 1b.

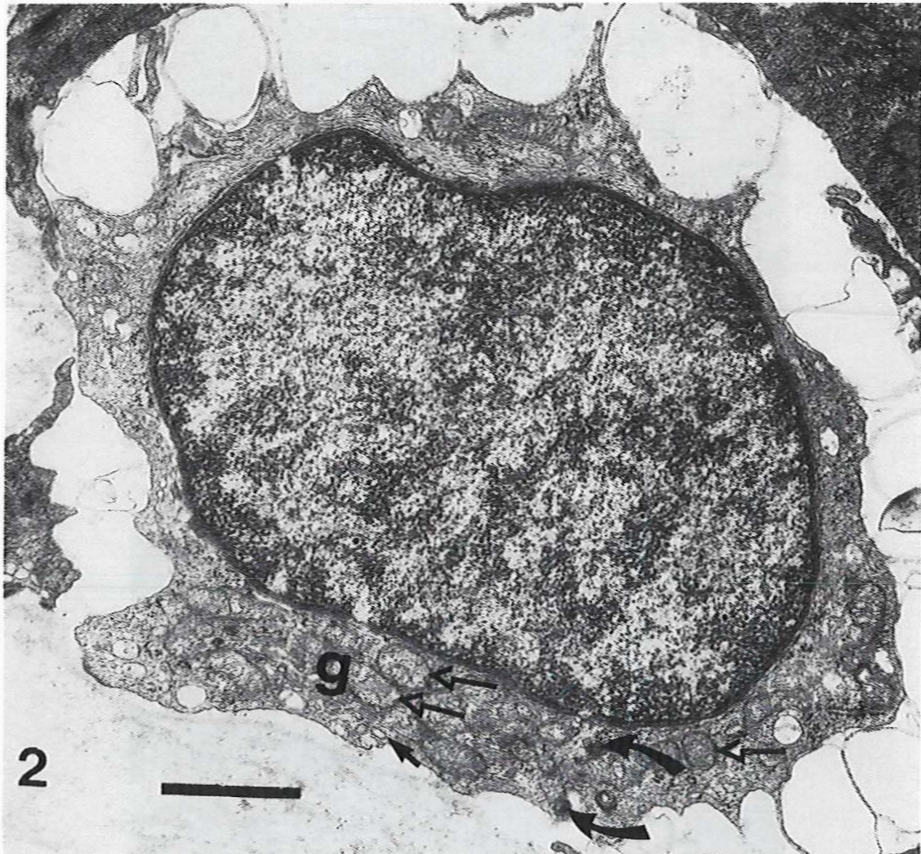


Fig. 2.

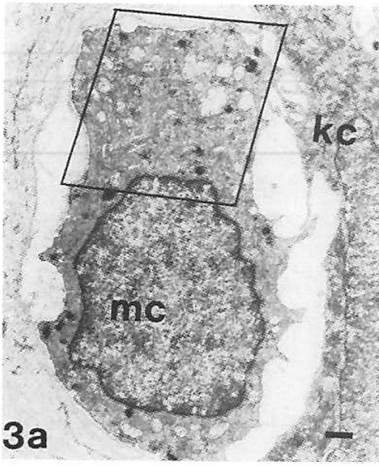


Fig. 3a.

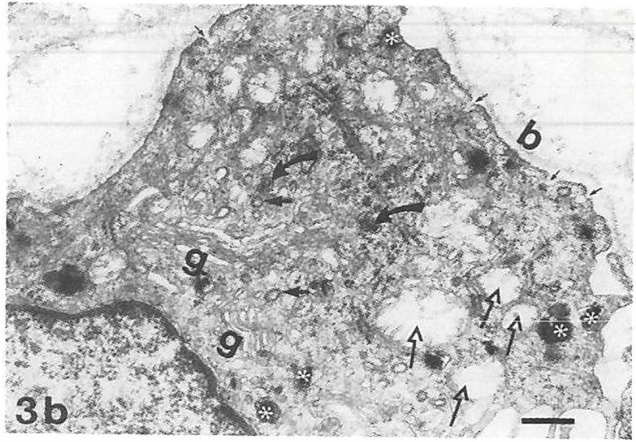


Fig. 3b.

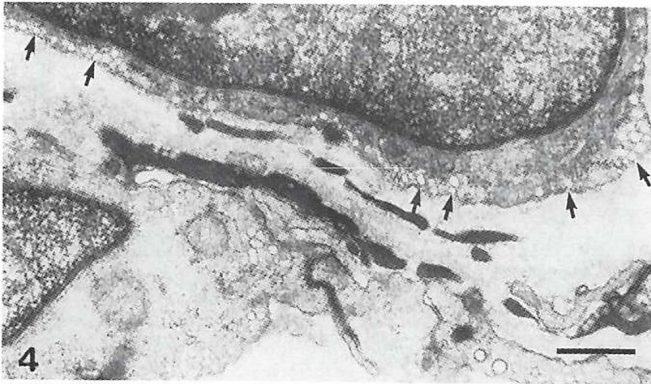


Fig. 4.

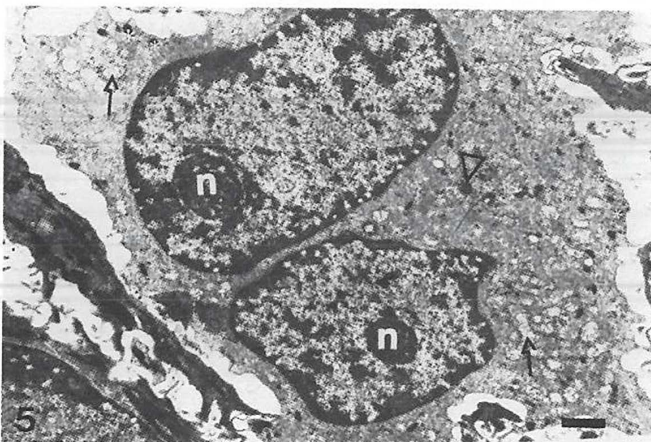


Fig. 5.