

# Vitamin A Exerts a Photoprotective Action in Skin by Absorbing Ultraviolet B Radiation

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**Retinyl esters, a storage form of vitamin A, concentrate in the epidermis, and absorb ultraviolet radiation with a maximum at 325 nm. We wondered whether these absorbing properties of retinyl esters might have a biologically relevant filter activity. We first used an *in vitro* model to assess the photoprotective properties of retinyl palmitate. We then applied topical retinyl palmitate on the back of hairless mice before exposing them to 1 J per cm<sup>2</sup> ultraviolet B, and assayed the levels of thymine dimers produced in epidermal DNA 2 h following ultraviolet B exposure. Finally, we applied topical retinyl palmitate or a sunscreen on the buttocks of human volunteers before exposing them to four minimal erythema doses of ultraviolet B; we assayed the levels of thymine dimers produced 2 h following ultraviolet B exposure, and determined the intensity of erythema**

**24 h after ultraviolet B. *In vitro*, retinyl palmitate was shown to be as efficient as the commercial filter octylmethoxycinnamate in preventing ultraviolet-induced fluorescence or photobleaching of fluorescent markers. The formation of thymine dimers in mouse epidermis was significantly inhibited by topical retinyl palmitate. In human subjects, topical retinyl palmitate was as efficient as a sun protection factor 20 sunscreen in preventing sunburn erythema as well as the formation of thymine dimers. These results demonstrate that epidermal retinyl esters have a biologically relevant filter activity and suggest, besides their pleomorphic biologic actions, a new role for vitamin A that concentrates in the epidermis. Key words: DNA damage/retinoids/sunscreening agents/ultraviolet rays. *J Invest Dermatol* 121:1163–1167, 2003**

**T**he skin contains sizable amounts of vitamin A (retinol). Retinol can be esterified by free fatty acids. Free and esterified retinol reach together about 1 nmol per g in both epidermis and dermis (Vahlquist, 1982; Vahlquist *et al*, 1982). Studies in mouse have shown that, in epidermis, esters of retinol account for approximately 90% of total vitamin A, the remaining 10% being retinol (Törmä *et al*, 1987; Sorg *et al*, 1999). In mouse, retinol concentrations decrease from the serum to the dermis, then to the epidermis, whereas the opposite is observed for retinyl esters (Sorg *et al*, 1999). Thus epidermis, like the liver, which stores most of body vitamin A, concentrates vitamin A in the form of retinyl esters.

Vitamin A (retinol and retinyl esters) strongly absorbs ultraviolet (UV) radiation between 300 and 350 nm, with a maximum at 325 nm, a wavelength range received from the sun at earth level. Thus it is not surprising that human or mouse epidermal vitamin A is destroyed by sun or UV exposure (Berne *et al*, 1984; Tang *et al*, 1994; Andersson *et al*, 1999; Sorg *et al*, 1999; 2002; Tran *et al*, 2001). The mouse epidermis can be loaded with large amounts of vitamin A following a topical application of retinol or retinaldehyde. This epidermal vitamin A originating from topical application is also very sensitive to the destructive action of UVA and UVB (Tran *et al*, 2001; Sorg *et al*, 2002).

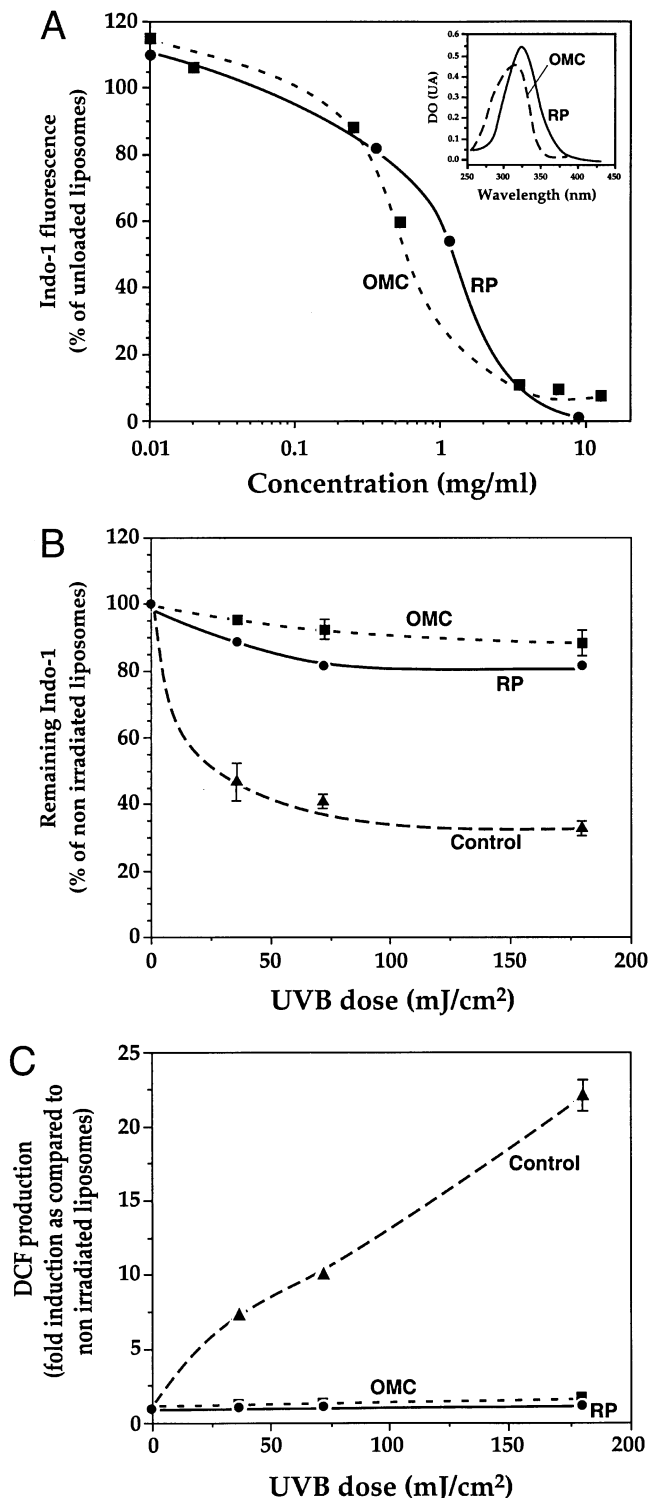
Besides vitamin A, UV radiation can be absorbed by many other chromophores in the skin; thus the major manifestations of the skin after acute UV irradiation are erythema, sunburn cell formation, and cyclobutane pyrimidine dimers, the latter being a direct index of DNA damage. DNA not only represents an important target for UV, but any damage to DNA can have deleterious consequences for the cells and the organ to which they belong. The cells can avoid the consequences of the destructive actions of UV by (1) preventing the interactions between UV and DNA and (2) detecting DNA modifications and repairing them before they can have biologic consequences (Zhou and Elledge, 2000; Rouse and Jackson, 2002).

The main strategies to prevent skin DNA damage consist in (1) limiting direct skin exposure to sunlight, (2) wearing protective clothes, and (3) using topical molecules that either act as UV filters or interfere with the biochemical reactions induced by UV. Among these molecules, sunscreens (Black *et al*, 1997; Ananthaswamy *et al*, 1998; Young *et al*, 2000), green tea polyphenols (Katiyar *et al*, 2000; 2001), and  $\alpha$ -tocopherol (Chen *et al*, 1997; McVean and Liebler, 1997; 1999) were shown to reduce erythema, sunburn cell formation, and pyrimidine dimers in mouse and human skin. DNA lesions can still be induced at a very high rate even in sunscreen-protected skin, however (Liardet *et al*, 2001), and some studies suggest that sunscreen use could be associated with an increase in melanoma incidence (Bigby, 1999; Autier, 2000). The fact that this is only due to increased exposure times by people who believe that they are well protected is still controversial (Autier *et al*, 1999; 2000), and toxic photoreactions involving sunscreen molecules are not excluded. Therefore the use of topical natural molecules that have photoprotective properties would represent a safe alternative to artificial sunscreens in the prevention of photocarcinogenesis.

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Abbreviations: DCFH, 2',7'-dichlorofluorescein; OMC, octylmethoxycinnamate.



**Figure 1. Retinyl palmitate has photoprotective properties in an *in vitro* model.** (A) Indo-1-encapsulated liposomes, the membranes of which contained increasing concentrations of OMC or retinyl palmitate (RP), were analyzed for Indo-1 fluorescence (excitation 330 nm; emission 405 nm). Insert shows the absorption spectrum of RP. (B) Indo-1-encapsulated liposomes, the membranes of which contained 6.5 mg per ml OMC or 10 mg per ml RP, were exposed to increasing UVB doses, and then Indo-1 was extracted and assayed by fluorometry. The graph shows the percentage of intact Indo-1 as a function of UVB dose. (C) DCFH-encapsulated liposomes, the membranes of which contained 6.5 mg per ml OMC or 10 mg per ml RP, were exposed to increasing UVB doses, and then DCF fluorescence was analyzed (excitation 485 nm; emission 530 nm). The results for OMC are reproduced from a previous publication (Tran *et al*, 2002).

The contribution of natural endogenous molecules in preventing the interactions between UV and DNA is therefore of major importance. In this study, we used an *in vitro* model (Tran *et al*, 2002) to assess the ability of retinyl palmitate – the predominant form of epidermal vitamin A – to prevent DNA damage and erythema induced by a single UVB exposure, due to its absorption properties. In this model, fluorescent markers (Indo-1 or 2',7'-dichlorofluorescein (DCFH)) are encapsulated into liposomes. Indo-1 becomes fluorescent when liposomes are exposed to a low intensity excitation wavelength, and when the liposome membranes contain molecules able to absorb the excitation wavelength the fluorescence is decreased in a concentration-dependent manner. If the intensity of the excitation beam is high enough, it can destroy Indo-1 by photobleaching. In this case, it is possible to assess the photoprotective action of absorbing molecules dispersed in liposome membranes. Finally, when DCFH is encapsulated into liposomes, it can be oxidized to DCF upon UVB excitation, and it is possible to assess the prevention of this photo-oxidation by absorbing molecules dispersed into liposome membranes. In this study, using this model, the photoprotective properties of retinyl palmitate were compared to those of octylmethoxycinnamate (OMC), a well-known UV filter contained in many sunscreens, which was shown to be efficient in the mentioned liposome model (Tran *et al*, 2002). We then applied topical retinyl palmitate on the skin of hairless mice or human subjects, before exposing them to UVB, and determined the amounts of thymine dimers in epidermal DNA. In human subjects, we also observed the UVB-induced erythema 24 h after UV exposure.

## MATERIALS AND METHODS

**Chemicals** Egg yolk dried L- $\alpha$  phosphatidylcholine 60% (lecithin), retinyl palmitate, and DCFH diacetate were purchased from Sigma Chemical (St Louis, MO). DCFH was produced by hydrolysis of DCFH diacetate (NaOH 0.1 mol per L, 30 min at room temperature) (LeBel and Bondy, 1990). Indo-1 was purchased from Molecular Probes (Leiden, The Netherlands). All solvents were from Merck (Darmstadt, Germany). OMC was a gift from Hoffmann-La Roche (Basel, Switzerland). Retinyl palmitate, as a 2% oil-in-water cream, and its vehicle were provided by Pierre Fabre Dermo-cosmetique. The sunscreen All Day 20+ (Louis Widmer, Rheinfelden, Switzerland) was bought in a drugstore; it contains the filter ethylhexylmethoxycinnamate and the sunscreen titanium dioxide, and has a sun protection factor (SPF) of 20.

***In vitro* experiments** Liposomes were prepared as previously described (Tran *et al*, 2002). Briefly, 30 mg lecithin and 15 mg cholesterol were dissolved in 2 ml chloroform in a glass tube, and then chloroform was evaporated under nitrogen flux. The film of lipids was then resuspended in phosphate buffer containing the fluorescent marker (Indo-1 6  $\mu$ mol per L, or DCFH 370  $\mu$ mol per L); the suspension was sonicated 30 s at 50 W and filtered through 0.45  $\mu$ m pores, and finally dialyzed through a 6000–8000 Da membrane in order to remove excess fluorescent markers. Irradiation of liposomes was performed using six TL 20 W/12 Philips tubes. The UVB and UVA fluxes were 3.7 mW per cm<sup>2</sup> and 0.5 mW per cm<sup>2</sup>, as determined by a digital radiometer Waldmann 585.100 recently calibrated, and the distance from the liposome suspension was 30 cm. The photoprotective properties of lipophilic agents were assessed as previously described (Tran *et al*, 2002).

**Treatments and irradiation of mice** Six adult female hairless mice were used. Three mice were treated on the back with retinyl palmitate 2%, and three others with its vehicle, once a day for 3 d, as previously described (Tran *et al*, 2001). Twenty-four hours after the last topical treatment, the mice received a single dose of 1 J per cm<sup>2</sup> UVB. This dose was chosen in accordance with a previous study showing the dose-dependent action of UVB on the photodestruction of mouse epidermal retinoids (Sorg *et al*, 1999). Two hours later, the mice were sacrificed, the skin was harvested, and epidermis was separated from dermis by heat (Tran *et al*, 2001).

**Human subjects** Six adult males participated in the study; their age ranged from 22 to 26 y (mean age 24  $\pm$  1). One subject was phototype I, one phototype II, and four were phototype III. All subjects were in good

health with no evidence of acute, chronic illness or cutaneous disease. They had no history of abnormal response to sunlight and did not take medication with known photosensitizing properties.

**Human protocol** The human protocol was approved by the Ethic Committee of the Hôpitaux Universitaires de Genève. Written consent was obtained from all participants in the study. Three visits per person were required. During the first visit the individual minimal erythema dose (MED) was determined by exposing the buttock skin to graded doses of UVB from a medical UVB lamp (TL 20 W/12; Phillips). The lowest dose resulting in uniform erythema over the irradiation site 24 h after irradiation was considered the MED. The five subjects with phototypes II–III had an MED of 74 mJ per cm<sup>2</sup>; the subject with phototype I had an MED of 55 mJ per cm<sup>2</sup>. On the first visit 20 mg per cm<sup>2</sup> vehicle, retinyl palmitate 2%, or the commercial sunscreen All Day with SPF 20 were applied to a 2 × 2 cm area of the buttock on an occlusive dressing to prevent dispersion and mixing of the creams. In previous studies on the metabolism of topical retinoids, this dose of 20 mg per cm<sup>2</sup> was shown to load the epidermis with high amounts of retinyl esters (Tran *et al.*, 2001; Sorg *et al.*, 2002). The occlusive dressing was left 3–4 h. Patients returned 24 h later for a second application. Thirty minutes later, excess product was eliminated using a compress, and the treated zones were exposed to 2 or 4 MED UVB (these UVB doses were absolute doses, and were not adjusted according to an SPF value). Skin punch biopsies 2 mm in diameter were taken at each site 30 min after the irradiation and snap-frozen immediately in order to assess DNA damage. Erythema was measured with a Minolta chromameter CR-300 (Minolta, Osaka, Japan) 24 h after the irradiation as described (Park *et al.*, 2002).

**Pyrimidine dimers** The spatial distribution of thymine dimers was visualized in mouse histologic slices using an antibody that binds to thymine dimers (clone KTM53, Kamiya, Seattle, WA). For quantitation of thymine dimers, DNA was extracted from mouse epidermis or the human punch biopsies (Gemmell and Akiyama, 1996), and then thymine dimers were detected by immuno dot blots using the mentioned antibody, as described by Smit *et al.* (2001). The quantitation of dot blots was performed by a densitometer from Molecular Dynamics (Redwood City, CA) and the software ImageQuant.

**Statistical analysis** Student's *t* test and ANOVA were performed to compare two or three series of data, respectively. Values significantly different from vehicle-treated skin were indicated: \*, *p* < 0.05; \*\*\*, *p* < 0.001.

## RESULTS

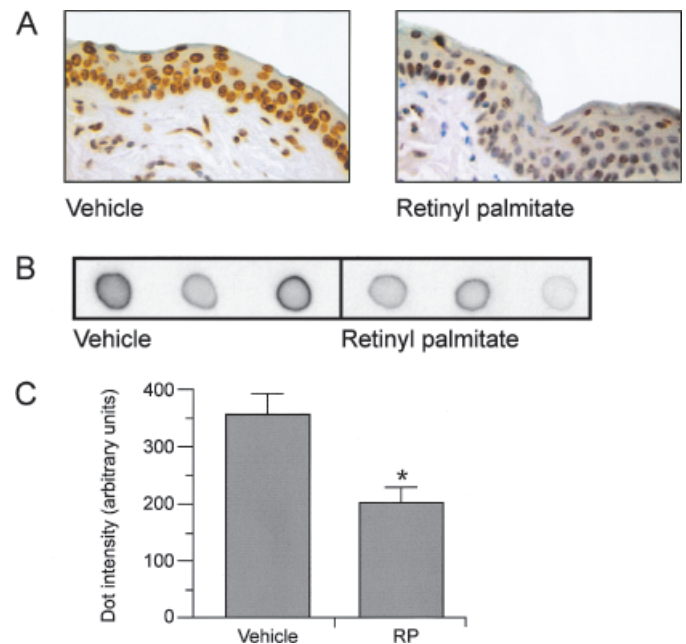
**Retinyl palmitate has photoprotective properties in an *in vitro* model** Using an *in vitro* model (Tran *et al.*, 2002), we evaluated the photoprotective properties of retinyl palmitate; OMC, which was shown to be an efficient UV filter in this model (Tran *et al.*, 2002), was used as a positive control. This model assesses the filter capacity of a lipophilic molecule contained in the membranes of liposomes, as well as its ability to prevent the photobleaching or the photooxidation of hydrophilic molecules encapsulated in the aqueous compartment of the liposomes (Tran *et al.*, 2002). In this model, retinyl palmitate decreased the fluorescence of Indo-1 in a concentration-dependent manner, indicating a filter capacity similar to that of the sunscreen OMC (Fig 1A). When liposomes containing Indo-1 or DCFH were exposed to increasing UVB doses, retinyl palmitate was as efficient as OMC in preventing Indo-1 photobleaching (Fig 1B) or DCFH oxidation (Fig 1C).

**Topical retinyl palmitate inhibits thymine dimer formation in hairless mice** Thymine dimer formation is a primary marker of DNA damage and represents a risk factor for the development of skin cancers. In order to assess the potential ability of retinyl palmitate to prevent a deleterious biologic action of UVB, we measured the amount of thymine dimers in DNA from the epidermis of mice that had been exposed to UVB following a 3 d topical treatment with retinyl palmitate 2% or its vehicle. Two hours after UVB, the density of epidermal nuclei stained for thymine dimers was lower in mice treated with retinyl palmitate 2% compared to those treated with vehicle

(Fig 2A). This was confirmed by assessing the density of thymine dimers per mass unit of DNA using immuno dot blots followed by densitometric analysis: the amounts of thymine dimers in 10 ng DNA was 43% lower in mice treated with retinyl palmitate 2% compared to its vehicle (Fig 2B, C), indicating that retinyl palmitate inhibited the formation of thymine dimers by filtering UVB.

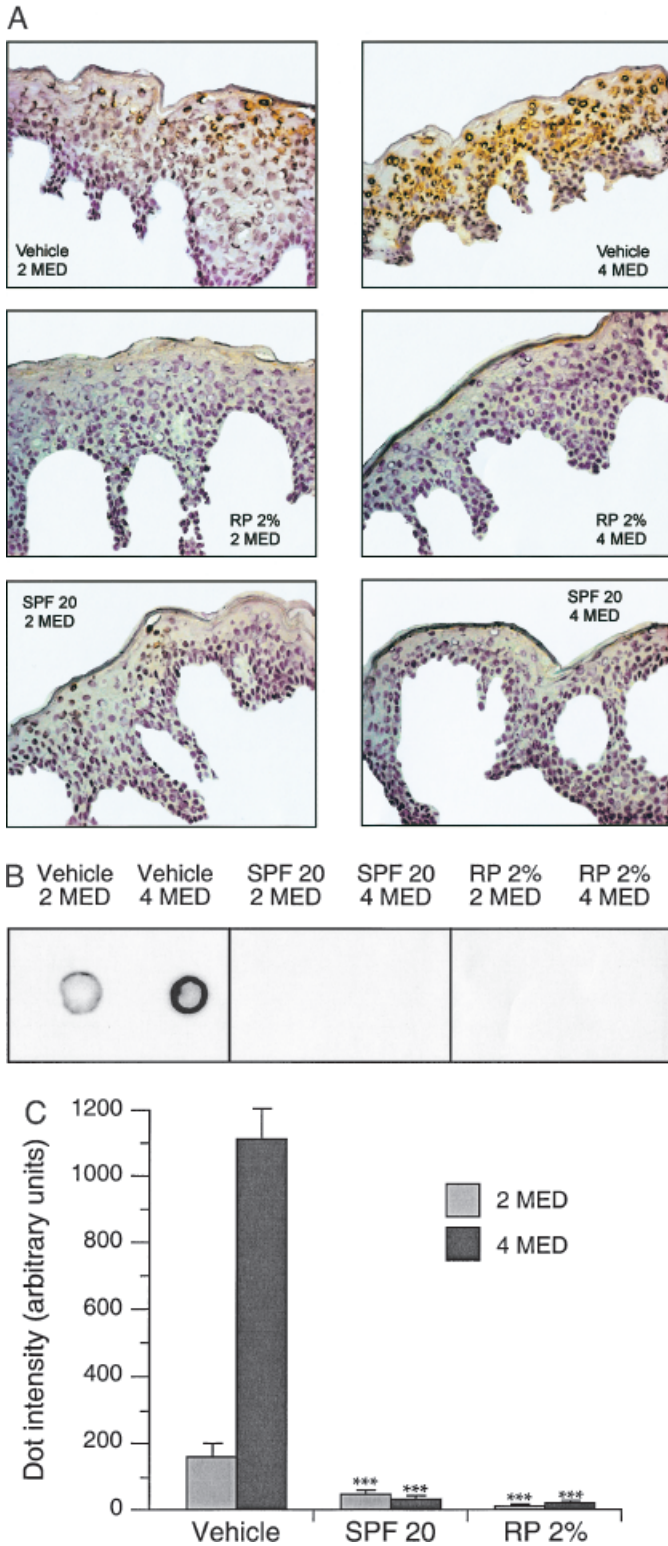
**Topical retinyl palmitate inhibits thymine dimer formation in human skin** We then wondered whether these results could be reproduced in human. We measured the amount of thymine dimers in the punch biopsies of volunteers who had been exposed to 2 or 4 MED UVB following a topical treatment with 20 mg per cm<sup>2</sup> retinyl palmitate 2%, commercial sunscreen with SPF 20, or retinyl palmitate vehicle. Thirty minutes after UV exposure, the density of epidermal nuclei stained for thymine dimers was lower in zones treated with either sunscreen or retinyl palmitate, compared to those treated with vehicle (Fig 3A). In DNA from zones treated with vehicle and exposed to 2 and 4 MED, two spots were clearly apparent, the one from the zone exposed to 4 MED being darker; this indicates that thymine dimers are produced in cutaneous DNA from zones exposed to 2 and 4 MED in a dose-dependent manner. In zones pretreated with SPF 20 sunscreen or retinyl palmitate, no spot was visible, although the densitometric analysis revealed a low amount of darker areas (Fig 3B, C). In other words, topical retinyl palmitate and the sunscreen used prevented the formation of thymine dimers in zones exposed to up to 4 MED.

**Topical retinyl palmitate inhibits UVB-induced erythema in human skin** The erythema observed 24 h after a single UVB



**Figure 2. Topical retinyl palmitate inhibits thymine dimer formation in hairless mice.** Hairless mice were treated with topical retinyl palmitate 2% or vehicle for 3 d before being exposed to 1 J per cm<sup>2</sup> UVB. Thymine dimers were assessed in epidermal sections by immunohistochemistry (A) and immuno dot blots (B) followed by densitometric analysis (C). (A) Histologic slices stained for thymine dimers from one mouse; positive nuclei are stained brown. (B) Dot blots showing the amounts of thymine dimers for each mouse. (C) Densitometric analysis of the dot blots, showing the mean pixel density ± SE of the three blots shown in (B). This experiment was repeated with another group of three mice, with similar results.



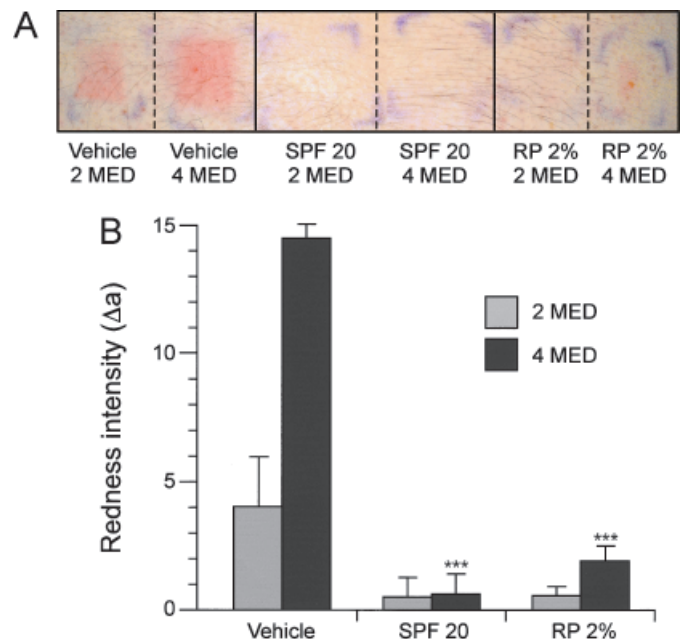


**Figure 3. Topical retinyl palmitate inhibits thymine dimer formation in human skin.** Delimited zones of the buttocks of human volunteers were treated with either retinyl palmitate (RP), its vehicle, or the sunscreen All Day (SPF 20); the same zones were treated again 24 h later with the same products, and then exposed 30 min later to 2 or 4 MED UVB. Two millimeter punch biopsies were removed 30 min after UVB irradiation and analyzed for thymine dimers. (A) Histologic slices stained for thymine dimers from one subject, showing positive nuclei in brown. (B) Immuno dot blots showing thymine dimers in DNA from skin biopsies. (C) Densitometric analysis of the dot blots, showing the mean pixel density  $\pm$  SE of the three blots shown in (B).

exposure is considered to be a marker of cutaneous injury and inflammation. This marker is used to determine the SPF of sunscreens. The skin of human volunteers was pretreated with 20 mg per cm<sup>2</sup> retinyl palmitate 2%, commercial sunscreen with SPF 20, or retinyl palmitate vehicle, and then exposed to either 2 or 4 MED UVB. Twenty-four hours following UVB exposure the intensity of erythema was assessed *in vivo* using a chromameter, and a picture of the erythema appearing on each exposed zone was taken. Both topical retinyl palmitate 2% and SPF 20 sunscreen strongly inhibited the intensity of erythema that developed following 4 MED UVB. In zones exposed to 2 MED UVB, due to the great variability of results, the downward trend is not statistically significant (Fig 4). We did not expose human subjects to higher UVB doses than 4 MED for ethical reasons. Although the retinyl palmitate concentration applied on the skin was higher than 2 mg per cm<sup>2</sup> – the concentration used for SPF determinations – Fig 4(B) suggests an approximate SPF value of 4 for topical retinyl palmitate.

DISCUSSION

Epidermal retinyl esters are considered as the storage form of vitamin A, as (1) this is the case in the liver where 99% of total vitamin A and derivatives are retinyl esters; (2) they account for 85%–90% of total epidermal vitamin A; (3) there is a retinyl ester gradient from the blood to the epidermis; and (4) they are precursors of the other endogenous vitamin A derivatives (Blomhoff *et al*, 1992; Napoli, 1996). Most of the epidermal retinyl esters are depleted by a single exposure to UVB (Sorg *et al*, 1999), indicating that retinyl esters strongly absorb radiation in the UVB range, which is in accordance with their absorption spectrum and extinction coefficient. This destruction is thus triggered by a direct effect of UVB on the retinyl ester molecules and not an indirect effect mediated by UVB-induced oxidative stress (Sorg *et al*, 2002). The energy absorbed by retinyl esters is no longer available to damage other chromophores such as DNA, flavins, or



**Figure 4. Topical retinyl palmitate inhibits UVB-induced erythema in human skin.** Delimited zones of the buttocks of human volunteers were treated and exposed to UVB as described in the legend to Fig 3. Twenty-four hours later, pictures were taken from the zones exposed to UVB for one subject (A), and the intensity of the observed erythema was analyzed with a chromameter for all six subjects (B).

NAD(P)H. We therefore wondered whether these absorbing properties of retinyl esters might have a biologically relevant filter activity. The action spectrum for the formation of thymine dimers – an index of DNA photodamage – has a maximum at 260 nm, corresponding to the maximum of the absorption spectrum of nucleic acids (Matsunaga *et al*, 1991). This is in the UVC range, a wavelength range with no biologic relevance, as UVC is filtered out by the atmosphere. Thymine dimers can still be produced at 300 nm, however, a wavelength received from the sun at the earth's surface. This explains why exposure of the skin to the sun or solar simulators produces epidermal thymine dimers (Clingen *et al*, 1995; Bykov *et al*, 1998). Similarly, the action spectrum for sun-induced erythema has a maximum at 300 nm (Anders *et al*, 1995). On the other hand, retinol and retinyl esters have an absorption maximum at 325 nm, but still absorb UV radiation at 300 nm. Thus it is conceivable that cutaneous retinyl esters, which concentrate in the epidermis, can absorb enough energy from the sun to decrease the formation of epidermal thymine dimers and erythema. The aim of this study was to assess the ability of epidermal retinyl palmitate, loaded in the skin after topical application, to decrease the formation of epidermal thymine dimers and erythema induced by UV radiation. The UV source we used consisted of TL 20 W/12 Phillips tubes (medical UVB), and was shown to be adequate to produce epidermal thymine dimers.

Before applying topical retinyl palmitate *in vivo*, we assessed its physical absorbing properties using an *in vitro* model (Tran *et al*, 2002). According to this model, retinyl palmitate was shown to be as efficient as OMC – a well-known molecule used in sunscreens – in preventing the photobleaching or the photooxidation of molecules encapsulated into liposomes. This indicates that retinyl palmitate, incorporated into phospholipid membranes, acts as a filter able to protect hydrophilic molecules from the destructive action of UVB. This *in vitro* filter effect was subsequently shown to have a biologic relevance *in vivo* both in mice and in human healthy volunteers, as demonstrated by the inhibition by topical retinyl palmitate of thymine dimer formation and erythema appearance following UVB exposure. As the endogenous cutaneous retinyl ester concentration ( $\approx 1 \mu\text{M}$ ) is much lower than that obtained after topical application ( $\approx 100 \mu\text{M}$ ), it is unlikely that endogenous cutaneous vitamin A could significantly prevent DNA damage by its filter property.

In conclusion, these data reveal a new potential role of epidermal retinyl esters, the storage form of epidermal vitamin A: thus, besides serving as precursors of the biologically active forms of epidermal vitamin A, which have antiphotocarcinogenic properties, epidermal retinyl esters also contribute to protecting DNA from UV damage. Retinyl esters are endogenous substances that can easily be loaded in high amounts in the epidermis by topical application; that they possess such an efficient filter action towards solar UV radiation compared to synthetic solar filters is of great interest, due to their lower toxicity, physiologic regulation, and other biologic properties relevant to the prevention of photocarcinogenesis.

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