

An action spectrum (290–320 nm) for TNF α protein in human skin *in vivo* suggests that basal-layer epidermal DNA is the chromophore

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Terrestrial solar UVB radiation (\approx 295–320 nm) readily induces cyclobutane pyrimidine dimers (CPDs) in human skin DNA that result in characteristic mutations associated with nonmelanoma skin cancer. The proinflammatory cytokine TNF α is important in mouse skin chemical carcinogenesis and is thought to also play a role in UVR-induced skin cancer by its immunomodulatory properties. There is some *in vitro* evidence that CPDs initiate the production of TNF α , and we tested this hypothesis by comparing the wavelength dependence (action spectrum) for TNF α protein induction in human skin *in vivo* with our earlier *in vivo* action spectra for CPD induction in four different epidermal layers of human skin. Normal volunteers ($n = 35$) were irradiated with physiologically relevant doses of monochromatic UVB (290–320 nm), and TNF α concentration was assessed, by high-sensitivity ELISA, in exudates from skin suction blisters raised 8 h after irradiation. An action spectrum, constructed from the slopes of the dose–response curves at the different wavelengths, showed maximal efficacy at 300 nm. An excellent match was observed for TNF α and the CPD action spectrum for cells in the lower basal epidermis. These data strongly suggest that UVB-induced photodamage to DNA in the epidermal basal layer is a major trigger for TNF α production. The TNF α may originate directly from the keratinocytes in this layer or inflammatory cells that are rapidly recruited into the upper dermis (e.g., neutrophils) as a consequence of DNA photodamage to basal-layer keratinocytes.

cytokine | DNA photodamage | photoimmunosuppression | cyclobutane pyrimidine dimer

Solar UV radiation (UVR) is known to have immunomodulatory effects in mice and humans (1, 2). The murine data unequivocally show that UVR-induced suppression of cell-mediated immunity is important in photocarcinogenesis and resistance to infectious agents. A single suberythemal exposure of solar simulated radiation (SSR) suppresses the induction phase of the contact hypersensitivity (CHS) response in skin cancer-prone human skin types I and II (2). In contrast, erythemal exposures were necessary for comparable levels of immunosuppression in skin types III and IV, which are less prone to skin cancer. The role of UVR-induced immunomodulation in humans remains unclear, but it is strongly suspected to play a role in human skin cancer (1) and photosensitivity disorders such as polymorphic light eruption (3). The immunomodulatory effects of UVR are mediated by the induction of molecular and cellular factors, including cytokines such as IL10 and TNF α (4, 5). We have shown that moderate single exposures of SSR readily induce high levels on TNF α mRNA (6) and protein (7) in human skin *in vivo*. Mouse studies also suggest that TNF α may play a role in chemical skin carcinogenesis (8) and photocarcinogenesis that is not related to its immunoregulatory properties (9).

An understanding of the mechanisms of UVR immunomodulation requires knowledge of the chromophores involved. There is a considerable body of evidence that stratum corneum uro-

canic acid (UCA) and epidermal DNA are major chromophores for the immunosuppressive effects of UVR in mouse studies *in vivo*, as measured, for example, by the suppression of the induction and elicitation phases of the CHS response. *In vitro* (10, 11) studies have provided evidence that DNA is an important chromophore for the induction of IL10 and TNF α protein and that this finding is mediated as a consequence of the formation of cyclobutane pyrimidine dimers (CPDs), which are readily induced in human skin *in vivo* by UVR (12, 13). To date, there has been no definitive evidence that DNA is a chromophore for TNF α in human skin *in vivo*.

Action spectra or wavelength-dependence studies are important because they may help to identify a chromophore and its location within the skin. Furthermore, action spectra also are important for hazard assessment, and therefore protection strategies, of specific parts of the solar UVR spectrum. This notion is important in sunscreen design because we have recently shown that the immune protection factor of a sunscreen on human skin may be considerably less than its sun protection factor, which is based on its ability to inhibit erythema (14). There are few action spectra data on the immunological effects of UVR. Mouse studies on the suppression of the CHS response implicate UVB in the solar UVR range (15). There are no comparable human data, and the role of UVA in the immunoregulatory effects of UVR remains uncertain (16). The aim of this study was to determine an action spectrum for the induction of TNF α protein in human skin *in vivo*. Our aim was to compare our TNF α data with our action spectra generated for CPD induction in different layers of human epidermis (13) to determine whether the CPD was the putative lesion for TNF α induction. Furthermore, the induction of TNF α is likely to reflect a human action spectrum for at least some of the immunological effects of UVR.

Results

Individual linear regression analyses of dose–response data at 290 nm ($n = 6$), 295 nm ($n = 3$), 300 nm ($n = 10$), 310 nm ($n = 8$), and 320 nm ($n = 8$) are shown in Fig. 1. In the interests of clarity, individual data points and r^2 values are given for 295 nm only because there were just three volunteers at this wavelength (Fig. 1*b*). The action spectrum for TNF α production is shown in Fig. 2*a*. This result also is plotted in Fig. 2*b*, after normalization at its peak of 300 nm, with previously published action spectra for CPD in four different layers of human epidermis of skin types I and II (13), which are also normalized at their 300-nm peak. The CPD action spectra also were based on slope analysis. The TNF α data were statistically compared with the CPD data in

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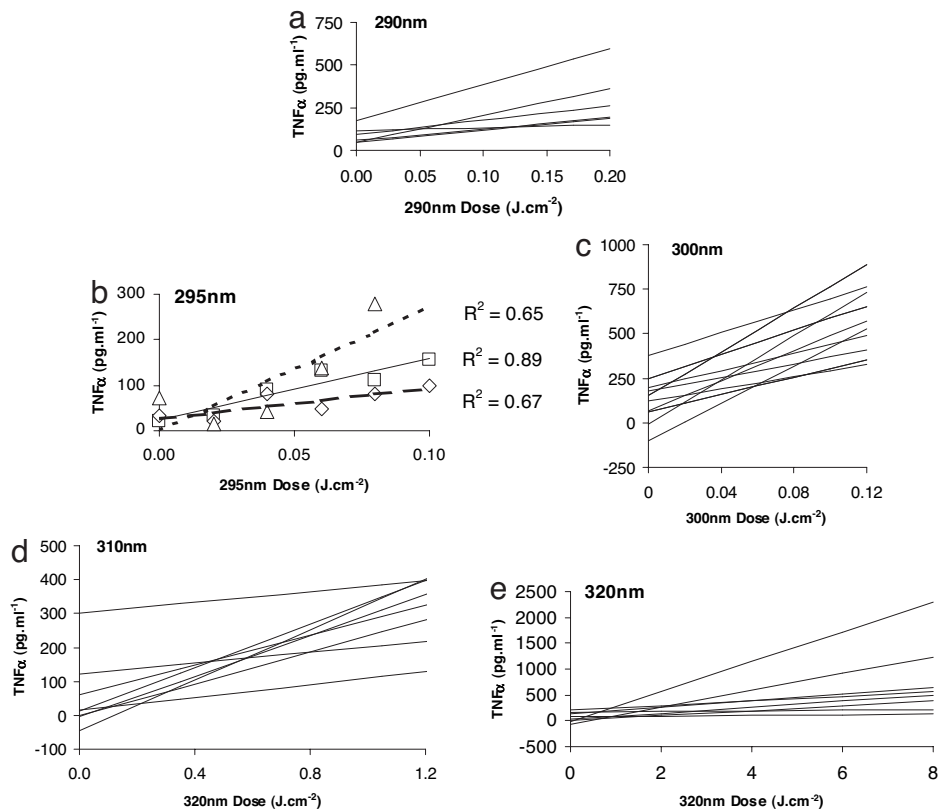


Fig. 1. Linear regression analyses. (a) 290 nm. (b) 295 nm. (c) 300 nm. (d) 310 nm. (e) 320 nm. Inclusion criteria for all volunteers included a 300-nm MED of either 0.028 or 0.040 J·cm⁻². Regressions are based on the mean TNF α value of two samples. (b) Individual data points and r^2 values for the three volunteers in this group.

each epidermal layer at 290 nm because Fig. 2*b* shows a difference between the CPD basal 8+ data and the other epidermal layers. This analysis showed no difference between the values of TNF α and the basal 8+ data ($P = 0.8$), whereas there were differences between TNF α and the other epidermal layers ($P = 0.01$ – 0.05). Thus, the TNF α spectrum shows the best match with the lower basal layer (8+ cell layers) spectrum. Fig. 2*b* also shows the action spectrum for quantitatively assessed erythema at 6 h from the same volunteers as the TNF α study, normalized at its peak value of 300 nm. The UVR dose value has been set to represent a just-perceptible minimal erythema dose (MED), where delta-erythema (DE) is 50 (arbitrary units).

Discussion

Two main initiating photochemical events have been proposed for the immunoregulatory effects of UVR: (i) the formation of CPDs, in which DNA is the chromophore; and (ii) the formation of *cis*-UCA from stratum corneum-bound *trans*-UCA, in which the latter is the chromophore. It is probable that both mechanisms play a role depending on the specific immunological pathway (17). Furthermore, there also is evidence that UVR-induced oxidative stress plays a role (18), mediated by as yet unknown chromophores. The aim of our study was to determine the action spectrum for TNF α protein, a proinflammatory cytokine known to have immunoregulatory properties either directly, by its induction of other cytokines such as IL8 (19), or by the stimulation of the migration of Langerhans cells from the epidermis (20, 21). It would have been interesting to determine the action spectrum for IL10, but our previous studies showed no increase in protein at 8 h after irradiation and a relatively modest increase at 15 h (7), which is not a convenient sampling time point for volunteer studies.

Extensive pilot studies showed that it was necessary to select volunteers carefully to ensure obtaining data that could be analyzed with relatively small numbers of volunteers. This finding is because our unpublished studies showed that TNF α release was inversely related to SSR MED; the lower the MED range, the more TNF α was released per unit physical dose (J·cm⁻²). However, MED was a better predictor of TNF α response than skin type because, although MED is inversely correlated with skin type, there is considerable MED overlap between skin types (22). Thus, volunteers were selected from a narrow MED range (two adjacent increments in the test series) at 300 nm because this level is the peak of the erythema action spectrum (13). Fig. 1 shows that at some wavelengths (e.g., 300 and 310 nm), the regression lines seem to fall into two distinct families that were not related to 300-nm MED at the wavelength of interest or skin type. It is possible that response depends on TNF α polymorphisms.

Young *et al.* (13) showed that action spectra for CPD induction in human skin *in vivo* varied at wavelengths <300 nm with the epidermal layer in which they were assessed. This finding was not surprising because epidermal DNA and stratum corneum UCA (both with maximal absorbance in the UVC region) offer much greater attenuation at these shorter UVB wavelengths, and, in effect, these molecules act as natural sunscreens for target chromophores beneath them. Comparisons of our action spectrum for TNF α and CPD induction (Fig. 2*b*) showed that the best match was between TNF α and deep basal layer CPD. We also compared our TNF α action spectrum with that for the photoisomerization of UCA in human skin (23), and the results are markedly different especially between 300 and 320 nm. Thus, based on human action spectroscopy *in vivo*, our data strongly suggest that DNA, specifically in the basal layer, is an important chromophore for TNF α and supports the *in vitro* mouse kera-

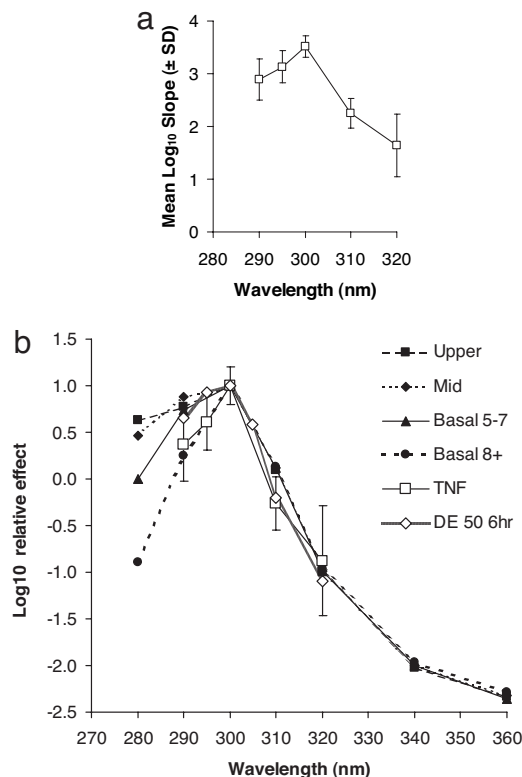


Fig. 2. Action spectra for TNF α , DNA photodamage, and erythema. (a) Action spectrum for TNF α induction \pm SD at 8 h. (b) TNF α spectrum (\pm SD) in comparison with action spectra for CPD in four different epidermal layers (upper, middle, and basal layers at two depths) at 0 h (13) and a just-perceptible erythema (DE = 50) measured by reflectance spectroscopy at 6 h in the current TNF α study volunteers. Note that (i) all data are normalized at 300 nm; (ii) TNF α was not measured at 305 nm because the manufacturer stopped production of the ELISA kits that we used; and (iii) basal 5–7 cells are found in layers 5–7, and basal 8+ is more than eight cell layers deep.

tinocyte studies of Kibitel *et al.* (11), in which T4 endonuclease V (T4N5)-enhanced CPD repair was shown to reduce TNF α protein production. A similar *in vivo* approach was made by Wolf *et al.* (24). However, these authors were not able to demonstrate any significant reduction of epidermal CPD by T4N5 or the presence of TNF α protein, although the T4N5 did reduce mRNA levels. Fig. 2b also shows that the 6-h erythema action spectrum is similar to the TNF α spectrum at wavelengths \geq 300 nm, but has a better fit with the DNA damage in the regions above the upper basal layer at the shorter wavelengths, as we previously reported for 24-h erythema (13). This finding suggests that TNF α and erythema share DNA as a common chromophore, but that the location of this chromophore may be important for different endpoints.

The main source of the TNF α in our studies is likely to be basal layer keratinocytes. However, dermal DNA photodamage also may be important to mast cells that store preformed TNF α (25) and that can be released by UVB exposure (26). However, this notion seems unlikely given the low cellular content of the dermis at the time of irradiation and the substantial amounts of TNF α that are recovered in suction blister fluid. It also is possible that early recruitment of dermal inflammatory cells, such as neutrophils (27, 28), may contribute to the TNF α detected 8–9 h after irradiation.

Given that DNA is the likely major chromophore for erythema (13) and TNF α induction, and there is evidence that CPDs are important in the suppression of CHS in mice (29), one might

expect the action spectra for erythema and suppression of the induction phase of CHS in humans to be similar. However, we recently concluded that these action spectra are different and that UVA is more immunosuppressive than it is erythemogenic in human skin (14). This finding suggests that other chromophores, such as UCA, also may be important, and this notion is supported by an action spectrum for the photoisomerization of *trans*- to *cis*-UCA in human skin *in vivo*, in which UVA had a more important role than it did for erythema (23). Some authors have suggested that *cis*-UCA can induce TNF α (30, 31), but others have not supported this theory (32–34). Our data support DNA, rather than UCA, as the main chromophore for TNF α . However, overall, the multiple individual events that contribute to functional measures of immunomodulation may well have different chromophores and, therefore, different action spectra (17).

In conclusion, our data provide human *in vivo* support for the pivotal role of DNA photodamage, especially CPD, in the release of TNF α by solar UVR. It is clear that DNA photodamage is important in the mutational effects (35) that give rise to nonmelanoma skin cancer. Such photodamage also may be important with regard to the immunological effects mediated by TNF α , which are thought to be related to skin cancer. Our data also add to the limited body of nonerythema human skin action spectra and provide a possible spectral weighting function for at least some of the immunological effects of UVR.

Materials and Methods

Monochromatic Radiation Spectra, Dosimetry, and Skin Irradiation.

Full details are described by Young *et al.* (13). We used monochromatic spectra at 290, 295, 300, 305, 310, and 320 nm from a monochromator with a grating blazed at 250 nm, which were delivered through a liquid light guide with a 5-mm exit diameter that made direct contact with the skin. A 5-nm full-width at half-maximum (FWHM) bandwidth (i.e., bandwidth at which 50% maximal irradiance occurs) was used for 290–310 nm and a 10-nm FWHM bandwidth was used for 320 nm; the latter also was filtered with 1-mm WG320 to attenuate any shorter wavelengths. The spectral maxima and their FWHM bandwidths were all verified spectroradiometrically before their use. These values are the same as those published by Young *et al.* (13), with the exception of the maxima of 295 and 305 nm, the shapes of which are similar to the other 5-nm FWHM spectra.

Volunteers and Study Protocol. Healthy male and female (1:1) volunteers ages 18–32 years, with mainly skin type I or II determined by detailed questionnaire and interview, were fully informed of all procedures and gave written informed consent before taking part in the studies, which were approved by the Ethics Committee of St. Thomas' Hospital and done in accordance with the declaration of Helsinki. The volunteers were screened on the basis of their just-perceptible MED at 300 nm by using a $\sqrt{2}$ dose series on previously unexposed buttock skin. They were recruited if their 300-nm MED was either 0.028 or 0.040 J·cm⁻² (60% and 40% of volunteers, respectively, and evenly represented in the different wavelength groups). These inclusion criteria were based on our studies that showed that TNF α release per unit physical dose of UVR was an inverse function of MED, rather than skin type. Dose–response studies were done with each wavelength ($n = 3$ –10) with a predetermined (by pilot studies) range of six physical UVR doses in duplicate, the highest being equivalent to approximately four MED depending on individual MEDs. The erythema response at each dose was quantified with a reflectance meter (Dia-Stron) at 6 h after irradiation, after which 5-mm suction blisters were raised (7) that took \approx 2 h. The exudate (5–25 μ l per blister) was collected at \approx 8 h after irradiation and stored at -30° C. Comparisons of SSR-induced TNF α mRNA at 6 and 24 h showed

higher levels at 6 h (6), whereas peak protein levels were seen at 15 h after three MED SSR, but a substantial rise was seen at 8 h (7). Suction blister fluid was analyzed in batches for TNF α by using high-sensitivity commercial ELISA kits (GE Healthcare).

Data Analysis. The statistical approach that we used was based on Matthews *et al.* (36), who used one summary measure of serial measurements per subject. We used the slope of linear regression analysis of mean TNF α concentration (pg/ml) from duplicate samples, versus UVR dose (J \cdot cm $^{-2}$), which was determined for each volunteer within a given wavelength group; >70% of r^2 values were >0.5, of which 40% were >0.75. Values <0.5 were evenly distributed across all wavelength subject groups. The mean r^2 of all 35 regressions was 0.6 (SD \pm 0.3), which was consistent with <25% of each individual measurement being random error. The mean log $_{10}$ slope \pm SD was plotted against wavelength to generate an action spectrum. This action spectrum was not quantum-corrected so that it could be directly compared with other nonquantum-corrected action spectra in the skin. The CPDs showed epidermal layer differences at wavelengths <300

nm (Fig. 2b). Therefore, comparisons of the TNF α and CPD data at the common wavelength of 290 nm were made by linear regression with dummy variables on the log of the slopes after normalization at 300 nm, correcting for unequal variance and repeated measurements per subject by Huber–White standard errors (37).

Erythema. Full details are given by Young *et al.* (13). Briefly, dose–response curves for erythema reflectance were generated as a logit function of log $_{10}$ UVR dose. These values were used to calculate the dose needed to achieve a given level of erythema at 6 h that was expressed as DE, which represented the difference between the test site and an adjacent nonirradiated control. Mean log $_{10}$ 1/dose was plotted against wavelength to generate an action spectrum.

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