Oral nicotinamide protects against ultraviolet radiation-induced immunosuppression in humans

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Introduction

Skin cancer is the most common malignancy in Caucasian populations (1) and is a major burden on public health systems (2). Ultraviolet (UV) radiation from sunlight is the prime cause of skin cancers, as it causes both DNA damage and immune suppression (3). The importance of a functioning immune system in skin cancer development is best illustrated in organ transplant recipients, whose chronic immunosuppression results in a dramatically increased skin cancer incidence (4). Even very low suberythemal doses of UV radiation can suppress skin immune responses (5), and numerous animal and human studies have established the central role of UV-induced immunosuppression in skin carcinogenesis (6).

More than 30 years ago, Kripke (7,8) showed that skin cancers, which are normally highly antigenic and readily rejected in animals with normal immunity, will progressively grow in animals whose immune responses have been suppressed by UV irradiation. In humans, susceptibility to UV immunosuppression correlates with skin cancer history, as patients with previous melanoma or non-melanoma skin cancers are more likely to demonstrate profound UV-induced suppression of skin immune responses than matched controls (9).

Abbreviations: DTH, delayed-type hypersensitivity; EI, erythema index; MED, minimal erythema dose; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; PARP, poly-adenosine diphosphate ribose polymerase; PPD, purified protein derivative; ssUV, solar-simulated ultraviolet; UV, ultraviolet.

Despite the use of sunscreens, which tend to confer less protection against immunosuppression than against sunburn (10), the incidence of skin cancer continues to rise (1). A variety of oral agents, such as botanical supplements (11,12) and omega-3 polysaturated fatty acids (13), have been shown to protect from UV-induced immunosuppression in mice, while oral omega-3 fatty acids can reduce sunburn sensitivity in humans (14). Nicotinamide, one of the principal forms of the vitamin B3 complex, has been shown to reduce UV-induced immunosuppression and carcinogenesis in mice when administered topically (15), while nicotinic acid, another form of vitamin B3 that is converted to both nicotinamide adenine dinucleotide (NAD) and nicotinamide in vivo (16), has been shown to be photoprotective in mice when given orally (17). We have previously found topical nicotinamide to be highly effective in preventing UV-induced immunosuppression in humans (18). Oral intervention with an inexpensive, easily accessible and safe supplement would provide an ideal chemopreventive adjunct to other forms of sun protection.

Foods rich in nicotinamide include meats, nuts, eggs and legumes and deficiency of nicotinamide (pellagra) is characterized by diarrhoea, dementia and a striking photosensitive dermatitis (19). Nicotinamide has been successfully used in the treatment of numerous dermatological disorders, including autoimmune blistering disorders, acne and rosacea (20). Adverse effects of nicotinamide have only been reported with extremely high oral doses (>6 g/day) and include nausea and vomiting, other gastrointestinal symptoms, headache, fatigue and dizziness; the vasodilatation and flushing seen with niacin and nicotinic acid are not observed with nicotinamide (21).

Delayed-type hypersensitivity (DTH) responses to recall antigens have previously been used in humans as models of UV-induced immunosuppression (22–24). We have shown that even suberythemal UV doses equivalent to ambient daily sun exposure can reliably suppress subsequent DTH responses to the recall antigen tuberculin purified protein derivative (PPD; Mantoux reactions) at irradiated sites (local immunosuppression) (18). This model enables the in vivo assessment of various agents for their ability to reduce or prevent UV-induced immunosuppression. The present studies used the Mantoux model of skin immunity in healthy Mantoux-positive volunteers who had previously been vaccinated with Bacille Calmette-Guerin. We have previously used this model to demonstrate immune protection by various topical agents, including isoflavonoids, capsaicin and melatonin (25–26) as well as 5% nicotinamide, which was by far the most effective (18). We now report our findings that oral nicotinamide is photoprotective in humans.

Materials and methods

Participants

Ethics approval was obtained for these studies from the University of Sydney and the Sydney South West Area Health Service Human Ethics Committee. Healthy adult Mantoux-positive volunteers, who had previously been vaccinated with Bacille Calmette-Guerin and were of Fitzpatrick’s skin types I–IV (27), were recruited from the Royal Prince Alfred Hospital staff, students and visitors. Volunteers were required to refrain from taking any vitamin supplements or immune modulating medication such as anti-inflammatory agents or corticosteroids and to avoid any sun exposure to their lower backs for at least 4 weeks prior to and during the study. All volunteers provided written informed consent.

Equipment

A 1000 W xenon-arc solar-simulator (Oriel, Stratford, CT) was used as the source of UV radiation in these experiments. This was filtered to attenuate visible and infrared wavelengths and remove UVC, thus producing a spectral output of solar-simulated ultraviolet (ssUV) closely resembling natural sunlight as published previously (28). The spectral irradiance of the solar simulator was measured with an OL754 spectroradiometer (Optronics, Orlando, FL)
calibrated against standard lamps. The spectroradiometer was used to calibrate a handheld IL-1350 broadband radiometer (International Light, Newburyport, MA), which was then used to rapidly monitor lamp output on a daily basis. Irradiation was performed with volunteers seated on an adjustable backless chair to allow their lower backs to be positioned 7.5 cm from the output beam. Black opaque vinyl irradiation templates were affixed to the back so that discrete 2 × 3 cm areas of skin could be selectively irradiated.

Mantoux testing
All volunteers had initial Mantoux testing performed 1 week prior to the experiments with tuberculin PPD (CSL Limited, Parkville Victoria, Australia) diluted to a standard volume of 0.05 ml in 0.9% normal saline solution and injected intradermally using 29 gauge needles. Three different doses of PPD were injected on one side of the lower back, in order to confirm Mantoux positivity and identify the dose of PPD that produced an appropriately sized reaction (induration of ~5 mm in diameter) for use in subsequent Mantoux tests. Mantoux reactions were assessed 72 h after injection. The Mantoux-induced erythema index (EI) was measured in triplicate at each site and also at an adjacent area of skin, using a portable reflectance spectrometer (Dia-stron, Andover, Hampshire, UK). The Mantoux-induced erythema (i.e. redness specifically caused by the DTH response) was calculated as the difference between the mean EI of the Mantoux test site and the mean EI of the adjacent skin.

In addition, all volunteers had their minimal erythema dose (MED) or sunburn threshold determined on a separate area of the back on the first day of irradiation. Ten 1 cm² areas of the upper back were exposed to graded ssUV doses and MED was determined 24 h later as the dose that caused just perceptible erythema.

Study protocol
These studies were designed as double-blinded, randomized, placebo-controlled crossover trials. During the first arm of each study, volunteers were randomly assigned to take either placebo or nicotinamide for 7 days. Placebo tablets contained lactose 35 mg (Stenlake Compounding Chemist, Sydney, New South Wales, Australia) and were visually matched to the nicotinamide 500 mg tablets (Nature’s Own, Virginia, Queensland, Australia). On the third day of tablet consumption, three 2 × 3 cm areas were irradiated with 1, 2 and 4 J/cm² ssUV, once daily for three consecutive days. After the final irradiation, Mantoux testing was performed at the irradiated sites and also at an adjacent unirradiated control site. The Mantoux reactions at all sites were then measured 72 h later as Mantoux-induced EI. After a 4 week washout period, the entire protocol was repeated on the contralateral back while the volunteer took the opposite tablets to those taken in arm 1. The first group of volunteers took only one tablet daily (i.e. 500 mg nicotinamide; ‘low dose’).

Measurement of blood NAD levels
A further five volunteers were recruited to measure erythrocyte NAD levels after ingesting a 500 mg nicotinamide tablet under fasting conditions. NAD levels were estimated using the method described by Jacobson et al. (29). The ‘nicin number’, defined as [NAD/nicotinamide adenine dinucleotide phosphate (NADP)] × 100%, was used to estimate nicin status. Blood was collected at baseline (before tablet consumption), 30, 60 and 90 min after tablet consumption. Whole blood was frozen in 0.1 ml aliquots to prevent NAD consumption by cells until ready for use. It was then solubilized by vortexing in 1 ml of ice-cold 1.0 M NaOH and then the pH was adjusted to 7.0 by the addition of 0.25 ml of 2.0 M H₂PO₄. To remove proteins from the sample by precipitation, an equal volume of ice-cold 1 M HClO₄ was added. After 10 min incubation at 4°C, the samples were centrifuged at 850g for 10 min. Supernatant (2 ml) was transferred to a new tube and adjusted to pH 7.0 by addition of 1 ml of 1 M KOH. The samples were centrifuged again to remove the KCIO₄ precipitate, followed by enzymatic cycling assays for NAD and NADP. Samples were mixed with NAD or NADP assay cocktails and absorbance was measured within an hour in a microplate reader at 600 nm. The NAD cocktail included 5 vols of NAD premix [ethanol 570 mM, bicine 114 mM, 3-(4,5-dimethylthiazole-2-yl)-2,5-bisphenyl tetrazolium bromide 0.48 mM, ethylene-diaminetetraacetic acid 4.8 mM and bovine serum albumin 1 mg/ml in final concentration], 1 vol of alcohol dehydrogenase (1 mg/ml) and 1 vol of 40 mM phenazine ethosulfate. The NADP cocktail comprised 5 vols of NADP premix [isocitrate 7.1 mM, phosphate buffer (pH 6.8) 76 mM, 3-(4,5-dimethylthiazole-2-yl)-2,5-bisphenyl tetrazolium bromide 0.48 mM, MgCl₂ 7 mM and bovine serum albumin 1 mg/ml], 0.28 vol of isocitrate dehydrogenase (10 mg/ml) and 1 vol of 40 mM phenazine ethosulfate.

Statistical analysis
In all experiments, each test site was compared with the unirradiated control site in the presence and absence of oral nicotinamide. Immunosuppression (AEI) was determined as the difference in the Mantoux-induced erythema of irradiated test sites to that of the unirradiated control site. Results are presented as mean ± SEM.

Statistical analysis was performed using Microsoft® Excel X for Mac® and SPSS 11 (Statistics Package for Social Sciences) for Mac®. Characteristics between two groups were compared using paired, two-tailed Student’s t-tests with Bonferroni corrections applied to all P values. In addition, repeated measures analysis of variance was used to analyze the significance level of differences in dose–response curves between nicotinamide interventions versus placebo. Results were considered significant if P < 0.05.

Results
Sixty-one volunteers were enrolled in and completed these studies (15 men and 15 women in the high-dose, 500 mg three time daily trial and 16 men and 15 women in the low-dose, 500 mg daily trial), with no adverse effects. Four volunteers completed arm 2 of the high-dose study after a 5 week washout (instead of 4 weeks). Removing this set of data from the results did not affect the overall outcomes and therefore they were included in the final analysis. The mean age of volunteers in both the high- and low-dose studies was 32 years (range 20–61 and 19–54, respectively). The three doses of ssUV used (1, 2 and 4 J/cm²) were equivalent to 20, 39 and 78% and 20, 38 and 77%, respectively, of the mean minimal erythema dose (MED) for the high-dose and low-dose volunteers. In the high-dose nicotinamide study, three volunteers had MEDs <4 J/cm² and four had MEDs equal to 4 J/cm² (the highest UV dose). In the low-dose study, there were three volunteers with MEDs <4 J/cm². Hence, the majority of volunteers did not experience erythema or tanning in response to irradiation, and omission of subjects who received erythemal UV doses from the analysis did not affect the final results (data not shown).

Oral nicotinamide did not alter susceptibility to sunburn
METH TESTING was performed on all volunteers during both arms of the study protocol. In this way, a comparison could be made between the MED recorded while volunteers were taking nicotinamide or placebo. The mean MED of volunteers on nicotinamide supplementation at 500 mg three times per day was 5.0 ± 0.2 J/cm² (placebo also 5.0 ± 0.2 J/cm²; P = 0.42) and for nicotinamide 500 mg daily was 5.3 ± 0.2 J/cm² (placebo 5.2 ± 0.2 J/cm²; P = 0.33). Furthermore, there was no significant difference in the MED of volunteers who took nicotinamide daily versus three times daily (P = 0.44). Hence, oral nicotinamide had no effect on the volunteers’ sunburn thresholds, suggesting that it does not act as sunscreen or prevent the molecular mechanisms that lead to sunburn.

Oral nicotinamide did not alter immune responses at unirradiated sites
The Mantoux-induced EI of DTH responses in unirradiated skin did not significantly vary between initial testing (before any tablets) and repeat testing in the presence of oral placebo or nicotinamide at either high or low dose. In the high-dose study, the mean initial EI was 103 ± 5.5, compared with 108 ± 5.5 on placebo and 103 ± 5.0 on nicotinamide. Similarly, in the low-dose study, the initial EI was 85.0 ± 5.8, compared with 86.6 ± 6.1 on placebo and 82.6 ± 3.6 on nicotinamide. Hence, oral nicotinamide had no intrinsic effects on the Mantoux reaction in the absence of UV exposure. These results also indicate that our low-dose UV irradiation protocol, which was able to locally suppress Mantoux responses, did not cause systemic immunosuppression (i.e. suppression of Mantoux reactions at adjacent, un-irradiated sites).

Oral nicotinamide reduces UV-induced immunosuppression
In the presence of placebo, exposure to three fixed doses of ssUV (1, 2 and 4 J/cm²/day) resulted in significant immunosuppression in a UV dose-dependent manner. In the first group of volunteers (high dose), in the presence of placebo, ssUV doses of 1, 2 and 4 J/cm²/day resulted in significant suppression of 16.7 ± 3.6, 42.9 ± 4.4 and 70.0 ± 5.0 erythema units (equivalent to immunosuppression of ~15, 40 and...
55%, respectively, n = 30; Figure 1A). Mantoux reactions at each of these UV-irradiated sites were significantly reduced compared with the unirradiated control (paired Student’s t-test; P < 0.001 for each UV dose). Oral nicotinamide supplementation at 500 mg three times per day for 7 days significantly reduced this immunosuppression to 6.6 ± 2.6 (not significantly different from the unirradiated control), 14.6 ± 3.7 (P < 0.01) and 34.0 ± 4.0 units (P < 0.01), equivalent to 7, 14 and 33% for the three doses of ssUV, respectively. These protective effects remained for the second group of volunteers taking low-dose nicotinamide (n = 31; Figure 1B). In the presence of placebo, ssUV doses of 1, 2 and 4 J/cm²/day caused immunosuppression of 13.3 ± 1.7, 24 ± 2.4 and 39.6 ± 3.4, respectively (14, 24 and 43%; all P < 0.001). This immunosuppression was significantly reduced to 6.6 ± 1.4, 12.1 ± 1.4 and 19.0 ± 2.2 (8, 14 and 22%, respectively) in the presence of oral nicotinamide 500 mg daily. Repeated measures of analysis of variance showed that the ΔEI with nicotinamide was significantly different to that with placebo (P < 0.001) for all three doses of ssUV and for each of the two doses of nicotinamide tested.

**Blood levels of NAD increased after nicotinamide dosing**

Mean blood NAD levels increased by 30% an hour after 500 mg nicotinamide consumption (Figure 2; P = 0.035). By 90 min, there was no significant difference from the baseline NAD level (P = 0.59), suggesting that NAD is rapidly taken up by the tissues.

**Susceptibility to ssUV immunosuppression correlated with MED but not age or gender**

The data were further analyzed, to assess risk factors for susceptibility to UV-induced immunosuppression, by combining the placebo arm results from the high- and low-dose studies to give a total of 59 volunteers (two volunteers participated in both studies and their latter results were thus excluded). Immunosuppression by each dose of ssUV (1, 2 and 4 J/cm²²) did not significantly correlate with age either in these 59 volunteers or when the data from each group were analyzed separately. There was, however, an inverse correlation between MED and immunosuppression by the highest ssUV dose (n = 59; r = −0.29; P < 0.05; linear regression analysis), indicating that volunteers with paler skin were slightly more susceptible to UV immunosuppression.

Using repeated measures analysis of variance without adjustment for potential confounding factors, there was no significant difference in immunosuppression between men and women. Linear regression analysis using a random intercept model to account for repeated measurement was then carried out by backward stepwise regression, using P = 0.05 for exclusion. The model included main effects for gender, UV dose (1, 2 and 4 J/cm²²), age and log-MED and all two-way interaction effects, but always including gender as a main effect. With this further analysis, there was still no significant effect of gender on susceptibility to immunosuppression.

**Discussion**

UV radiation-induced immunosuppression can occur with doses well below the sunburn threshold; in the present study, we found significant immunosuppression with daily suberythemal UV doses equivalent to <8 min of midday summer sunlight (30). Sunscreens may prevent sunburn while allowing immunosuppression (10), and the efficacy of sunscreens is further limited by suboptimal frequency and volume of application (31). There is also increasing concern about the adverse effects of sun avoidance and sun protection. Vitamin D deficiency, which is surprisingly frequent in the general population, is associated with increased risk of autoimmune, malignant and cardiovascular disease as well as rickets and osteoporosis (32). There is thus a need for adjunctive photoprotective agents, which would not compromise vitamin D status, could be taken orally to improve compliance and are non-toxic, inexpensive, free of patents and widely available.
E. Yiagemides et al.

This is the first report of oral nicotinamide providing immune protection against UV exposure in humans. We initially used a daily nicotinamide dose of 1500 mg because this is the dose most widely used to treat autoimmune blistering disorders (20). Although this dose is well tolerated in clinical practice and in our volunteers, a lower daily dose would be more appropriate, and more cost-effective, as a daily supplement for UV protection. We thus repeated our irradiation protocol in a second group, this time using a nicotinamide dose three times lower. Nicotinamide at 500 mg daily conferred a higher level of immune protection as the higher dose. As with topical nicotinamide (18), oral nicotinamide did not affect volunteers’ sunburn sensitivity, but rather provided a specific immune-protective effect. This protection against immunosuppression (33) but not sunburn (34) in human volunteers has also been reported with topical liposomes containing the DNA repair enzyme T4-endonuclease.

Oral nicotinamide supplementation at both high and low doses had immune-protective effects despite slight differences in the degree of UV-induced immunosuppression that occurred with placebo in the two groups. The degree of UV-induced immunosuppression during the placebo arms is, however, in keeping with the small variation seen in other human studies utilizing similar doses of ssUV (18,24–26). As reported previously, we found that volunteers with paler skin were slightly more susceptible to UV immunosuppression (18).

Gensler et al. (17) showed that oral niacin (nicotinic acid) reduced UV-induced immunosuppression and also carcinogenesis in mice, with demonstrated increases in skin NAD levels compared with control. Almost all of the nicotinamide in whole blood is within erythrocytes (29), which demonstrates that the oral nicotinamide was absorbed by the body, measurably increasing bioavailability. While we found an increase in blood NAD levels after oral dosing, how this correlates with skin NAD levels in humans is yet to be determined.

The mechanisms of nicotinamide chemoprotection are not yet fully understood, but probably involve its role in cellular energy metabolism and high-energy cellular processes such as DNA repair. We previously reported microarray analysis on human skin treated with topical nicotinamide (18). In this study, six healthy volunteers were irradiated with a single suberythemal dose of ssUV to the lower back. Discrete UV- and non-UV-exposed areas were then treated with 5% nicotinamide or vehicle and biopsied 24 h later. Gene set enrichment analysis identified differentially regulated gene sets; there was UV-induced down-regulation of genes involved in energy metabolism and anti-apoptotic pathways in skin treated with vehicle, but this was normalized in skin treated with nicotinamide.

Nicotinamide is the primary precursor of NAD, required for the manufacture of adenosine triphosphate in the citric acid cycle. Skin NAD content has been shown to be an important factor for cell survival following UV exposure (35), and NAD is the sole substrate of nuclear poly-adenosine diphosphate ribose polymerase (PARP). PARP is a multifunctional enzyme with roles in DNA repair, genomic stability and regulation of p53 gene expression (36). Human skin cells with reduced levels of NAD have a lower survival rate and higher genomic instability following exposure to UV (35), whereas increased intracellular NAD is associated with enhanced protection against photo-oxidative stress (37). UV exposure causes DNA damage, which is a key trigger for UV-induced immunosuppression (38) and that stimulates PARP activity, utilizing NAD (35). Overactivation of PARP can, however, deplete cellular adenosine triphosphate and cause cell death (36). Nicotinamide acts on this pathway both by increasing cellular NAD and hence energy levels (35) and also by directly inhibiting PARP (39). Hence, nicotinamide may act to enhance DNA repair following UV exposure by providing cells with adequate energy levels and preventing PARP overactivation. Nicotinamide is also a known inhibitor of SIRT1, an NAD+ dependent deacetylase that promotes DNA stability and is down-regulated by UV irradiation (40). Although nicotinamide has been shown to inhibit tumorigenesis in mice (15), future experiments should address the effects of nicotinamide on DNA photolesion formation and repair.

In conclusion, this study demonstrates that chemoprotection of the immune system from sunlight can be achieved with oral nicotinamide at daily supplemental doses in humans. Nicotinamide is an inexpensive, non-toxic and patent-free compound that holds promise for skin cancer chemoprevention.

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