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# Dependence of photocarcinogenesis and photoimmunosuppression in the hairless mouse on dietary polyunsaturated fat

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**Abstract**

A series of semi-purified diets containing 20% fat by weight, of increasing proportions (0, 5%, 10%, 15% or 20%) of polyunsaturated sunflower oil mixed with hydrogenated saturated cottonseed oil, was fed to groups of Skh:HR-1 hairless mice during induction and promotion of photocarcinogenesis. The photocarcinogenic response was of increasing severity as the polyunsaturated content of the mixed dietary fat was increased, whether measured as tumour incidence, tumour multiplicity, progression of benign tumours to squamous cell carcinoma, or reduced survival. At the termination of the study approximately 6 months following the completion of the 10-week chronic UV irradiation treatment, when most mice bore tumours, the contact hypersensitivity (CHS) reactions in those groups supporting the highest tumour loads (fed 15% or 20% polyunsaturated fat), were significantly suppressed in comparison with the mice bearing smaller tumour loads (fed 0, 5% or 10% polyunsaturated fat). When mice were exposed acutely to UV radiation (UVR), a diet of 20% saturated fat provided almost complete protection from the suppression of CHS, whereas feeding 20% polyunsaturated fat resulted in 57% suppression; the CHS of unirradiated mice was unaffected by the nature of the dietary fat. These results suggest that the enhancement of photocarcinogenesis by the dietary polyunsaturated fat component is mediated by an induced predisposition to persistent immunosuppression caused by the chronic UV irradiation, and supports the evidence for an immunological role in dietary fat modulation of photocarcinogenesis in mice.

*Keywords:* Photocarcinogenesis; Dietary fat; Immunosuppression; Contact hypersensitivity; Ultraviolet rays

## 1. Introduction

Experimental UVR-induced skin carcinogenesis has been shown to be sensitive to both the quantity and the type of dietary fat. The action of the dietary fat occurs at the post-initiation phase of tumour induction [5,29], and diets which provide high levels of polyunsaturated fats have been shown to exacerbate the photocarcinogenic outcome in mice. If the polyunsaturated fat intake is restricted to such a degree that essential fatty acid deficiency results, UVR-initiated tumours remain latent [29], but may be revealed by reconstituting the diet with polyunsaturated fat.

Tumour transplantation studies have implicated an immunosuppressed state as a prerequisite for the promotion or outgrowth of UVR-initiated tumours, in contrast to chemically- initiated tumours [11]. It has been suggested that the post-initiation modulation of photocarcinogenesis by dietary fat has an immunological component [10]. In support has been a recent examination of the progressive profiles of several relevant immune parameters during the photocarcino-

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genesis induction process, which has demonstrated that high dietary corn oil, in comparison with a low corn oil diet, increased the suppression of the delayed type hypersensitivity reaction by UVR, decreased the number of splenic T lymphocytes, increased the number of suppressor T lymphocytes, and exacerbated the impairment of the ability to reject transplanted UVR-induced tumours [4].

It is uncertain at present how the dietary fat might modulate the relevant immune function, which has been shown to involve a UV-specific impairment of T cell function induced by UVR in addition to its tumour-initiating effect. However there is strong evidence from murine models that dietary polyunsaturated fat levels regulate the substrate availability for prostanoid synthesis, and may cause immunologically relevant alterations in the cutaneous prostanoid array produced in response to UV irradiation [10].

To date studies of the effect of altered dietary fats on photocarcinogenesis have been limited to the feeding of single fat diets, which do not simulate the human dietary fat intake. In this study we examine the effect on photocarcinogenesis in Skh:HR hairless mice of a series of diets providing 20% by weight of total fat with a variable polyunsaturated fat content, the remaining complement being saturated fat. We have chosen sunflower oil (polyunsaturated) and catalytically hydrogenated cottonseed oil (saturated) as the two fats, as these are relevant to the Western diet and comprise the major margarine ingredients. In addition, to further probe the hypothesis that dietary fat acts by an immunological mechanism, we have assessed the T cell-mediated immune responsiveness while feeding different dietary fats, using the CHS reaction, both in mice exposed acutely to UVR or in mice exposed chronically to a cumulative carcinogenic dose of UVR, and examined the relationship of these responses to the photocarcinogenic outcome.

## 2. Materials and methods

### 2.1. Animals

Inbred female Skh:HR-1 hairless mice from the Department of Veterinary Pathology breeding colony, 10–21 weeks old, were evenly age-distributed into experimental groups and housed in wire-topped plastic boxes on vermiculite bedding (Boral Ltd., Camellha, N.S.W.). They were maintained at 25°C, with 12 h of lighting (GEC F40GO gold light which does not emit any UVR) alternating with 12 h of dark, and allowed free access to water and to stock mouse pellets (Labfeed, Northbridge NSW) when not being fed semi-purified diets.

### 2.2. UV irradiation

Simulated solar UVR (SSUV; 290–400 nm) was used to induce photocarcinogenesis and was provided by a bank of two sets of three 120 cm UVA tubes (Sylvania F40BL) flanking a single 120 cm UVB tube (Oliphant FL40SE), housed in a planar arrangement in a reflective batten and filtered through a layer of 0.125 mm cellulose acetate film (Kodacel; Eastman Chemical Products, Kingsport, TN, USA) to remove radiation below 290 nm. The filter was replaced every 5 h when photodegradation significantly reduced the transmission of the shorter wavelengths, as detected spectrophotometrically. The spectral characteristics of this source have been previously described [27]. UVB (280–320 nm) radiation, identified by others as the immunosuppressive waveband of solar UVR [9], was provided by a single unfiltered 120 cm UVB tube. Integrated irradiances for UVA (320–400 nm) and UVB were measured at the target distance (19 cm) using an International Light IL1700 radiometer with SEE 035 (UVA) and SEE 240 (UVB) detectors. The irradiances for SSUV were $4.13 \times 10^{-3}$ W/cm$^2$ UVA and $2.18 \times 10^{-4}$ W/cm$^2$ UVB, and for unfiltered UVB were $2.2 \times 10^{-3}$ W/cm$^2$ UVA and $2.3 \times 10^{-4}$ W/cm$^2$ UVB.

The minimal erythemal dose (MED) was determined from a graded series of irradiation doses, and was defined as the lowest exposure resulting in a statistically significant increase in the mid-dorsal skinfold thickness at 24h post-irradiation [26]. For SSUV, this was 1.96 kJ/m$^2$ UVB and 37.17 kJ/m$^2$ UVA (15 min exposure); for unfiltered UVB this was 1.04 kJ/m$^2$ UVB and 1.0 kJ/m$^2$ UVA (7.5 min exposure).

### 2.3. Semi-purified diets

The basic semi-purified powdered diet has been previously described [8,29]. The fats were kindly donated by Mr. R. Berry, Vegetable Oils Ltd., Mascot, N.S.W., without added antioxidant, and were stored at

2°C in the dark, the oil under nitrogen gas. Soybean protein isolate (Supro-500E, Protein Technologies International, St. Louis MO, USA) provided the protein source at 23% by weight of the diet, and sucrose the carbohydrate source at 45% by weight. Vitamins and minerals were added according to the American Institute of Nutrition standard mixtures AIN-76$^{TM}$ at 1% of diet [1], and crude fibre as finely ground wheaten straw. The diets were designed to provide 20% by weight of fat, comprising 0, 5%, 10%, 15% or 20% polyunsaturated sunflower oil, the balance being catalytically hydrogenated cottonseed oil (Diets 1–5, Table 1). This level of dietary fat, while high, reflects the recently assessed fat intake of the average North American diet [24]. The fatty acid composition of the fats was assayed by gas-liquid chromatography following methyl esterification as previously described [8].

Diets were mixed fortnightly, stored at 2°C and fed daily in small jars in weighed amounts providing 13.8 kcal (57.74 kJ) per mouse, throughout the entire experimental period. This caloric intake supports normal growth, and there was no difference in average body weight between mice fed the different diets.

The fat content of the soybean protein was determined gravimetrically by 3 consecutive extractions of 20 g samples into chloroform/methanol 2:1. The extracts were washed with deionised water before evaporation of the solvents. The soybean protein was found to contain 1.5 g/100 g lipid material, assumed to be largely unsaturated, being derived from soybean oil.

### 2.4. Photocarcinogenesis induction

Groups of 15 mice were pre-fed the semi-purified diets for 4 weeks [8] before commencing SSUV irradiation. Mice were exposed on the dorsum to an incremental SSUV radiation regime for 10 weeks, being irradiated unrestrained with the wire cage tops removed on 5 days per week initially with 67% of the previously determined MED. This initial exposure time of 10 min was increased by 2 min every week until 20 min, which was then held constant until the end of the treatment period. The response of minimal erythema of the dorsal skin was maintained throughout the 10 weeks irradiation period. The cumulative doses were 111 kJ/m$^2$ UVB and 2106 kJ/m$^2$ UVA. Feeding of the prepared diets continued until day 232 from commencement of the UV irradiation, when the experiment was terminated. Tumour growth was monitored at intervals from first tumour appearance (day 84), counting tumours of 1 mm or greater diameter, and is expressed as the progressive tumour incidence (% of mice with tumours per group) or tumour multiplicity (average number of tumours per mouse per group). Significance of the differences in progressive tumour incidence was measured by the Mantel-Haenszel log rank test [23], and of tumour multiplicity by the Wilcoxon rank sum test.

Table 1

Fat content (% by weight of total diet) of the semi-purified diets

| Diet | Hydrogenated cottonseed oil | Sunflower oil |
|---|---|---|
| 1 | 20 | 0 |
| 2 | 15 | 5 |
| 3 | 10 | 10 |
| 4 | 5 | 15 |
| 5 | 0 | 20 |

### 2.5. Induction of contact hypersensitivity

This assay of T cell-mediated immunity has been optimised for the Skh hairless mouse [25,26] with sensitization on the unirradiated abdomen, as opposed to the irradiated dorsal skin, in order to measure the systemic response. Mice were sensitized with 3% w/v oxazolone (Sigma Chemical Co., St. Louis, MO) in ethanol, as previously described, either immediately following the final tumour count (day 232) for assay in chronically irradiated animals, i.e. 23 weeks after the final SSUV exposure; or 1 week following exposure to 1 MED of unfiltered UVB radiation on each of 3 consecutive days for assay of the acute reaction. One week later the mice were challenged by the application to each surface of the pinnae of 5 μl of the 3% oxazolone/ethanol. Ear thickness was measured with a spring micrometer (Mercer, St. Albans, UK) both before the challenge and at 18–24 h, recording the maximum ear thickness. The average net ear swelling was calculated as the difference between the pre- and post-challenge ear thicknesses. Significance between treatments was determined with Student's *t*-test.

For assay in chronically irradiated mice, those mice

(9–12 mice per group) remaining healthy in spite of their tumour loads, were tested. Age-matched unirradiated mice (8) which had been fed stock pellets provided controls for comparison. For assay in acutely irradiated mice, groups of 6 mice were exposed, or not exposed, to UVB radiation after 4 weeks pre-feeding, and the feeding was continued until the end of the assay.

## 3. Results

### 3.1. Composition of dietary fats

The fatty acid composition of the two dietary fats is shown in Table 2. In sunflower oil, the major fatty acid (64%) was C18:2 (linoleic acid), with a 26% component of C18:1 (oleic acid). However neither linoleic nor oleic acids was detected in the hydrogenated fat, in which the major fatty acid (69%) was *trans*-C18:1 (elaidic acid). The hydrogenated fat also contained significantly greater levels of C16:0 (21%) and C18:0 (9%).

The mice found the diets palatable, maintained normal body weight and showed no signs of essential fatty acid deficiency, even when fed 20% saturated fat. The deficiency was undoubtedly averted by the unscheduled provision of soybean oil from the protein source, which would have provided approximately 0.3% polyunsaturated fat, since the long-term feeding of hydrogenated oil previously in a similar semi-purified diet based on casein as the protein, in which contaminating milk fat would have been predominantly saturated, did result in essential fatty acid deficiency [29].

Table 2
Fatty acid composition (%) of the dietary fats

| Fatty acid | Sunflower oil | Hydrogenated fat |
|---|---|---|
| 14:0 | 0.54 | 1.19 |
| 16:0 | 6.74 | 20.77 |
| 18:0 | 3.24 | 8.79 |
| 18:1 (*cis*) | 25.67 | ND |
| 18:1 (*trans*) | ND | 69.25 |
| 18:2 | 63.62 | ND |
| 20:2 | 0.20 | ND |

ND, not detected.

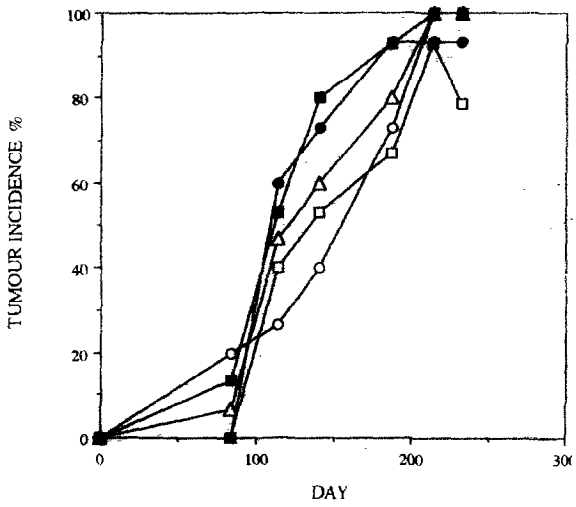


Fig. 1. tumour incidence in mice ($n = 15$) fed Diets 1–5. Open squares, Diet 1; filled circles, Diet 2; open circles, Diet 3; open triangles, Deit 4; filled squares, Diet 5.

### 3.2. Photocarcinogenesis

The average probability of survival of the experimental period was 0.88. Mice fed Diets 1 and 2 had the highest probability (0.93), mice fed Diet 5 had the lowest probability (0.80), while mice fed Diets 3 and 4 had an intermediate probability (0.87) of survival. Premature deaths in this study were all caused by euthanasia due to the presence of a tumour of greater than 1 cm diameter.

Tumours began to appear at day 84, shortly after the irradiation regime was completed (day 70) in mice fed Diets 3, 4 and 5, however first tumours were delayed in mice fed Diets 1 and 2 until day 113 (Fig. 1). A tumour incidence of 100% was attained in mice fed Diets 3, 4 and 5 by day 213, but the final tumour incidence at d232 in mice fed Diet 1 was 79%, and in mice fed Diet 2 was 93% (Fig. 1). The progressive tumour incidence was significantly ($P < 0.05$) less in mice fed Diet 1 compared with mice fed Diet 5, but there was no significant difference in progressive tumour incidence between the other dietary groups.

More significantly marked differences due to the diets were revealed by the progressive average tumour multiplicities (Fig. 2). The lowest final average multiplicity occurred in mice fed Diet 1 ($2.2 \pm 1.8$ tumours per mouse), and the highest in mice fed Diet 5

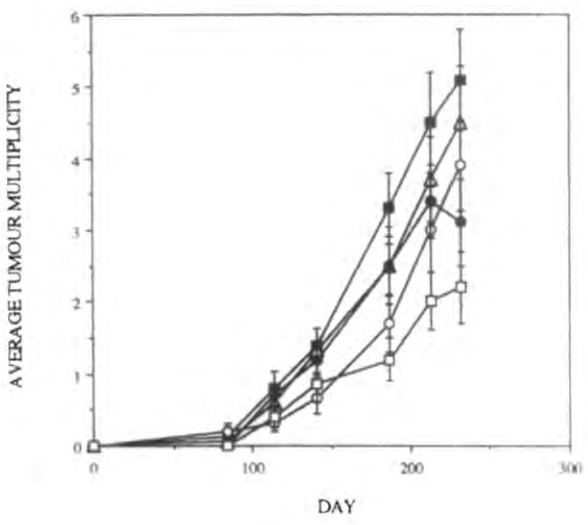


Fig. 2. Progressive average tumour multiplicity ± SE in mice ($n$ = 15) fed Diets 1–5. Open squares, Diet 1; filled circles, Diet 2; open circles, Diet 3; open triangles, Deit 4; filled squares, Diet 5.

(5.1 ± 2.5 tumours per mouse); intermediate diets resulted in intermediate multiplicities (Diet 2, 3.1 ± 2.1; Diet 3, 3.9 ± 2.3; Diet 4, 4.5 ± 2.9). The difference between the final tumour multiplicities in mice fed Diets 1 and 5 was highly significant ($P < 0.01$); differences between mice fed Diets 1 and 3, and Diets 1 and 4 were also significant ($P < 0.05$), but there was no significant difference between Diets 1 and 2.

The majority of the final tumours were papillomas, but a number of squamous cell carcinomas were confirmed; only a single squamous cell carcinoma developed in the mice fed Diet 1, but there were 4 or 5 carcinomas found in each of the other groups. The progressive tumour incidence for carcinoma formation is shown in Fig. 3, but statistical significance of differences could not be established between groups.

### 3.3. Contact hypersensitivity (chronic irradiation)

Otherwise healthy tumour-bearing mice were contact sensitised after the final tumour count at day 232 (Fig. 4). Mice fed Diets 1, 2 and 3 had similar CHS responses. These responses did not differ significantly from the response of non-irradiated but age-matched mice fed stock pellets. Therefore the responses of mice fed Diets 1, 2 and 3 can be regarded as normal, in spite of the mice having received chronic SSUV irradiation previously.

However the chronically irradiated mice fed Diets 4 or 5 displayed highly significant ($P < 0.001$) suppression of CHS, to 41% and 55% respectively of the response in the unirradiated control mice (no statistical difference). Thus Diets 1–3 appear to segregate as

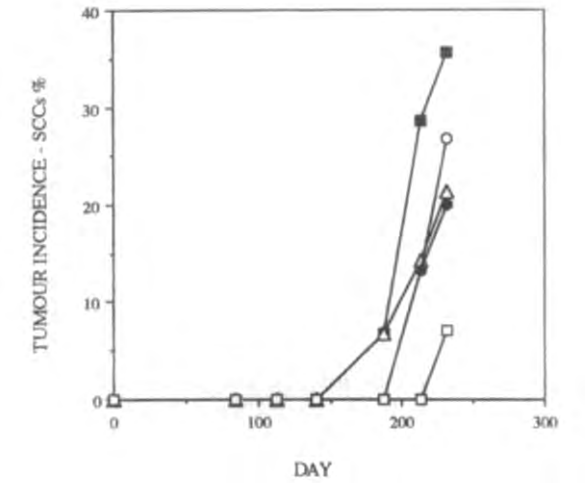


Fig. 3. Progressive tumour incidence of squamous cell carcinoma in mice fed Diets 1–5. Open squares, Diet 1; filled circles, Diet 2; open circles. Diet 3; open triangles, Deit 4; filled squares, Diet 5.

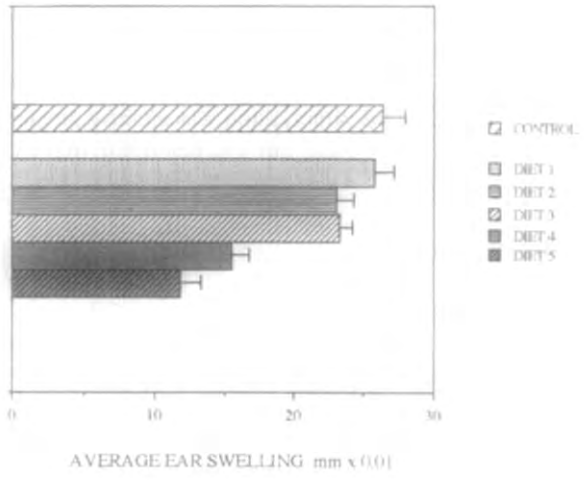


Fig. 4. Contact hypersensitivity responses, measured as average ear swelling ± SE, in mice ($n$ = 9–12) fed Diets 1–5, at 23 weeks after completion of chronic SSUV irradiation treatment. Control mice were fed stock pellets.

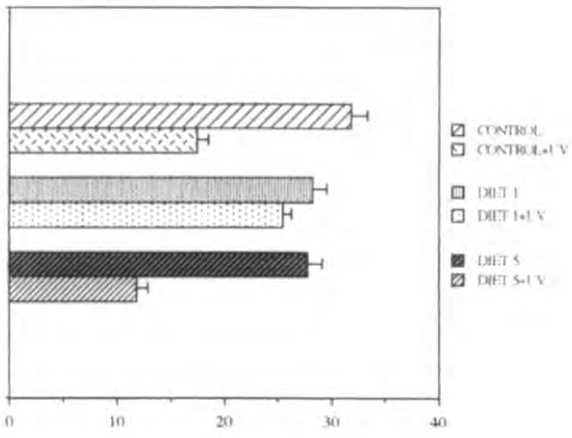


Fig. 5. Contact hypersensitivity responses, measured as average ear swelling ± SE, in mice ($n = 6$) fed Diet 1 or Diet 5, 1 week after acute exposure to 3 × MED of UVB radiation. Control mice were fed stock pellets.

having facilitated a normal CHS response following chronic irradiation, whereas Diets 4 and 5 appear to have rendered the mice susceptible to persistent immunosuppression.

### *3.4. Contact hypersensitivity (acute irradiation)*

CHS after acute irradiation was measured in mice fed Diet 1 or Diet 5, in comparison with mice fed stock pellets. Fig. 5 demonstrates that UVB-irradiation of control-fed mice resulted in 45% suppression of CHS. The immunosuppression was slightly greater (57%) in Diet 5-fed mice, but was negligible in mice fed Diet 1 (10%).

## 4. Discussion

This study demonstrates the sensitivity of photocarcinogenesis to the polyunsaturated dietary fat content of mixed fat diets fed to hairless mice, and is in accord with previous evidence for the enhancement of photocarcinogenesis by dietary polyunsaturated fat from single fat diets fed to hairless mice [2,3,5,29]. Both tumour incidence and tumour multiplicity were modulated significantly, the progressive tumour multiplicity reflecting a gradation of increasing severity as the polyunsaturated fat content of the diet was increased. Furthermore, probability of survival was reduced, and the progression of benign to malignant tumours was accelerated. The results are also consistent with the positive correlation with polyunsaturated fat of chemical carcinogenesis in organs other than the skin in various mouse strains and species [6,15,30].

However, in direct contrast, detailed studies of the promotion of chemically-initiated cutaneous carcinogenesis in the SENCAR mouse have consistently demonstrated tumorigenesis to be reduced as dietary polyunsaturated fat content was increased [19]. When mixed fat diets containing corn oil and coconut oil in graded proportions were fed in a protocol similar to the dietary regime we have used, the highest tumorigenesis resulted from diets with the highest saturated coconut oil content [18].

In the SENCAR mouse epidermis, but not extracutaneous tissue, the capacity to metabolise incorporated precursor fatty acids by either the cyclooxygenase or lipoxygenase pathways appears to differ from the epidermis of both hairless mouse and most conventional strains. Tumour promotion favours lipoxygenase products rather than cyclooxygenase products. A correlation was found between the promoter-induced $PGE_2$ level and the severity of tumorigenesis, but this was inversely correlated with the polyunsaturated fat intake and with the linoleate content incorporated into the epidermal phospholipids. This has revealed a competition in the epidermal incorporation of C18:2 with C20:4 into the phospholipids [20,21], thus providing a reduced substrate availability for eicosanoid synthesis. Conventional mice respond with predominantly prostanoid production, while the balance in the SENCAR is shifted in favour of lipoxygenase products.

Inhibitors of cyclooxygenase, such as indomethacin, likewise have suppressed tumorigenesis in the hairless [22,28] and conventional mice [32], but stimulated tumour promotion in the SENCAR mouse [12]. The SENCAR mouse studies have highlighted the pivotal role of the eicosanoid array in the control of skin tumour promotion, and one point of consensus between various mouse strains has been the correlation between the capacity for $PGE_2$ synthesis and the tumorigenic outcome. The inter-species and inter-strain differences in the epidermal linoleic acid metabolising enzyme patterns, the ability to elongate and desaturate epidermal C18:2 to C20:4, and the effec-

tive balance between cyclooxygenase and lipoxygenase activities during tumorigenesis which ultimately determines the tumour outcome in the skin, are worthy of further characterisation.

The saturated fat we fed contained a high proportion of *trans* C18:1, a product of the catalytic hydrogenation reaction. The potential of *trans* fats to affect tumorigenesis has been under scrutiny, since many processed foods including margarine may provide high levels of *trans* fatty acids in the human diet, but to date there is no evidence of such a relationship [14], nor of an influence of *trans* fatty acids on eicosanoid biosynthesis [33].

Because dietary fats have been shown to modulate photocarcinogenesis promotion rather than initiation, and because this phase of photocarcinogenesis is under immunological control, it has been interesting to observe the influence of the dietary fats on UVR-impaired CHS responses. Saturated fat-fed mice exposed in the short-term to an acute immunosuppressive dose of UVB radiation were spared compared with polyunsaturated fat-fed mice, in agreement with our recent observations in mice fed polyunsaturated sunflower oil in comparison with saturated butterfat [8]. We also observed that at a late time point in the photocarcinogenesis induction process, 23 weeks after the final UV exposure, when almost all mice had developed tumours, there remained significant differences in the ability of the mice to respond to a contact sensitiser, which reflected the severity of the tumorigenic outcome. When polyunsaturated fat intake had been increased, tumorigenesis had been exacerbated and a remarkable persistence of immunosuppression remained measurable. There was no difference apparent in the CHS responsiveness in mice fed 20% sunflower oil or saturated fat in the absence of UV irradiation, indicating that the persistent immunosuppression was likely to have been induced by the carcinogenic irradiation regime. Our findings thus correlate both the susceptibility to, and the persistence of, UVR-induced suppression of CHS with susceptibility to photocarcinogenesis, and add to the available evidence that dietary polyunsaturated fat enhances photocarcinogenesis by an immunological mechanism. It is notable in this context that when Black *et al* recently [4] compared 0.75% and 12% corn oil diets fed to hairless mice, there was a substantial decrease in the unirradiated delayed type hypersensitivity reaction in the high fat group; however the difference in the responses was no longer apparent by 24 wk, at which point significant differences in the carcinogenic expression would have been apparent. It appears therefore that if total dietary fat content is not held constant, the immunomodulating effects of polyunsaturated fats may differ; we have no ready explanation for the late divergence of immune function from carcinogenesis, in contrast to our observations.

Eicosanoid production provides a mechanism common to the regulation of both tumorigenesis and immunosuppression induced by UVR. When prostaglandin synthesis has been pharmacologically inhibited by indomethacin in vivo, both photoimmunosuppression [7,16,17] and photocarcinogenesis [22,28] have been shown to be reduced. The dependence of photoimmunosuppression in hairless mice on the dietary polyunsaturated fat has also been shown [8,10]. It is also of interest that in conventional Balb/c mice, the inhibition of the prostaglandin-dependent induction of a marker enzyme of tumour promotion, ornithine decarboxylase, by I-difluoromethylornithine, resulted in reduced photocarcinogenesis as well as an inability to reject a UV-tumour transplant [13], further supporting a common mechanism regulating both responses.

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