Retinoic acid protects Langerhans' cells from the effects of the tumour promotor 12-O-tetradecanoylphorbol 13-acetate

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SUMMARY

Retinoic acid prevents the decrease in epidermal Langerhans' cell (LC) density which occurs upon application of the tumour promotor 12-O-tetradecanoylphorbol 13-acetate (TPA) to murine skin. This occurred very rapidly, after only 1 week, and was still observed after 4 weeks of treatment. Retinoic acid alone increased the LC density, indicating that it could affect LC density independently of TPA. The induction of a contact sensitivity response which was inhibited by prior treatment with TPA due to the low LC density was also protected by retinoic acid. The anti-carcinogenic activity of retinoic acid is partially the result of its ability to inhibit tumour promotion. The loss of LC may be one of the important steps in tumour promotion as this would allow developing tumours to escape immune destruction. Our studies suggest that the ability of retinoic acid to suppress tumour promotion may be in part by protecting local antigen-presenting cells, thus allowing an immune response to be generated against tumours.

INTRODUCTION

Langerhans' cells (LC) are antigen-presenting cells which express high levels of class II major histocompatability complex (MHC) glycoproteins on their plasma membrane and are found in all stratified squamous epithelium, particularly the epidermis (Halliday & Muller, 1984). LC originate from a bone marrow precursor that migrates into the epidermis to form an even distribution of dendritic cells which take up antigen and then migrate via the dermal lymphatics to the local lymph node (Silberberg-Sinakin *et al.*, 1980). *In vitro* demonstration of the antigen-presenting capabilities of LC (Halliday & Muller, 1987a) indicate that in the lymph node it is likely that LC present the antigen to T lymphocytes.

We have found that the chemical carcinogen 7,12,dimethylbenz(a)anthracene (DMBA), which has both tumour-initiating and -promoting activity, depletes LC from murine epidermis (Muller, Halliday & Knight, 1985; Ruby, Halliday & Muller, 1989). Utilization of the tumour promotors 12-O-tetradecanoylphorbol 13-acetate (TPA) and teleocidin in the two-stage model of cutaneous carcinogenesis has demonstrated that LC are depleted during the promotor and not the initiator phase of

carcinogenesis (Halliday, MacCarrick & Muller, 1987). As LC are essential for initiating cutaneous immune responses, they may participate in anti-tumour immunity by presenting tumour-associated antigens to T lymphocytes. The reduction in LC during tumour promotion may allow suppressor cells to become activated in response to the developing tumour, enabling the neoplastically transformed cell to grow without initiating immunological defence mechanisms. This is supported by the observations that reduction in LC density disrupts the immunological functions of skin, allowing prolonged allograft survival of DMBA (Odling, Halliday & Muller, 1987) and TPA (Halliday *et al.*, 1988)-treated skin. In addition, application of contact sensitizers to DMBA- or TPA-treated skin activates specific long-lived suppressor cells rather than inducing hypersensitivity (Halliday & Muller, 1986, 1987b; Halliday *et al.*, 1988).

Retinoids have anti-carcinogenic activity (Elias & Williams, 1981; Graham, 1984), and in particular have been reported to inhibit the promotor phase of carcinogenesis (Verma, Conrad & Boutwell, 1982; Boutwell, 1983). The decrease in LC density may be one of the important events during tumour promotion, allowing neoplastic cells to escape immune destruction; here we show that retinoids protect LC from the effects of the tumour promotor TPA.

Abbreviations: ATPase, adenosinetriphosphatase; DAB, 3,3^l-diaminobenzidine; DMBA, 7,12 dimethylbenz(a)anthracene; DNFB, 2,4-dinitrofluorobenzene; LC, Langerhans' cells; MHC, major histocompatability complex; PBS, phosphate-buffered saline; solvent EDA, ethanol: dimethylsulphoxide: acetone (1:1:6, v/v); TPA, 12-O-tetradecanoylphorbol 13-acetate.

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MATERIALS AND METHODS

Animals
Young adult male BALB/c mice were obtained from the Central Animal House, University of Tasmania and used with approval from the Ethics Committee.

Treatment with TPA and retinoic acid

Mice were treated for 1 or 4 consecutive weeks by topical application to either the dorsal surface of each ear or the shaved dorsal trunk with 20 μl of reagent, commencing as indicated on Days -1 and/or 0, either (a) acetone once per week, Day 0; (b) 0·005% 12-O-tetradecanoylphorbol 13-acetate (TPA) in acetone (Sigma, St Louis, MO) once per week, Day 0 (Halliday *et al.*, 1987); (c) 0·5 mg/ml all trans retinoic acid type XX (Sigma) in solvent EDA (ethanol:dimethylsulphoxide:acetone, 1:1:6, v/v/v) twice per week, Days -1 and 0; (d) solvent EDA twice per week, Days -1 and 0; (e) solvent EDA twice per week, Days -1 and 0 and 0·005% TPA once per week immediately following solvent EDA on Day 0; or (f) 0·5 mg/ml retinoic acid in solvent EDA twice per week, Days -1 and 0 and 0·005% TPA once per week immediately following retinoic acid on Day 0. This concentration of retinoic acid (34 nmol) applied 24 hr and immediately prior to TPA has been shown to inhibit tumour promotion by TPA (Verma *et al.*, 1982).

One week following the final treatment mice were either killed by cervical dislocation and their treated ears removed for LC enumeration or they were sensitized through treated dorsal trunk skin.

Langerhans' cell and T-lymphocyte enumeration

Epidermal sheets were prepared as we have described previously (Halliday *et al.*, 1988) from mouse ear skin 1 week following the 1 or 4 weeks treatment with TPA and retinoic acid in the combinations described above. In brief, ear skin supported by cellophane tape applied to the stratum corneum was incubated for 2 hr at 37° in isotonic phosphate-buffered saline (PBS) containing 20 mM Na_4 ethylene diamine tetraacetic acid (Serva, Heidelberg, FRG), pH 7·3, in order to facilitate the epidermis being separated from the dermis in a single sheet.

Ia-positive LC in the epidermal sheets were visualized using light microscopy and identified by their dendritic morphology and staining by indirect immunoperoxidase with MKD6 cell supernatant containing anti-I-A^d monoclonal antibody followed by peroxidase-conjugated rabbit anti-mouse immunoglobulins (Dakopatts, Glostrup, Denmark) and 0·05% 3,3'-diaminobenzidine (DAB; Sigma) in PBS (pH 7·3) containing 1 drop/10 ml hydrogen peroxide. LC were similarly identified following staining for the cytoplasmic enzyme β-glucuronidase (Halliday *et al.*, 1988). The number of LC present in six microscopic fields was counted for each mouse, the size of the field being determined with a graticule. A total area of 4·1 mm^2 was counted per mouse, and the number of LC per mm^2 of epidermis calculated.

Sensitization and elicitation of contact sensitivity

Mice were sensitized with 2,4-dinitrofluorobenzene (DNFB; Sigma) by applying 25 μl of a 0·5% solution in acetone:olive oil (4:1, v/v) to their shaved dorsal trunk for 2 consecutive days (Halliday & Muller, 1986). The DNFB was applied to the area of skin that had received a final treatment with TPA or retinoic acid 1 week previously.

A contact sensitivity response was elicited 5 days following initial sensitization by applying 20 μl of 0·2% DNFB in acetone: olive oil (4:1, v/v) to the dorsal surface of the right ear. Thicknesses of the right challenged and left unchallenged ears were measured 24 and 48 hr later using an engineers' micrometer. Contact sensitivity was expressed as the percentage

increase in ear thickness following challenge by the following formula:

$$\frac{\text{thickness of right ear} - \text{thickness of left ear}}{\text{thickness of left ear}} \times \frac{100}{1}.$$

RESULTS

Effect of retinoic acid on TPA-induced LC depletion

One week following a single treatment with acetone the LC density was not significantly different from the number found in untreated epidermis, while TPA dissolved in acetone depleted LC by approximately one-third (Table 1). Neither retinoic acid dissolved in solvent EDA nor solvent EDA alone had any discernable effect on LC numbers compared to untreated epidermis, whereas they were significantly higher than the LC numbers observed in the TPA-treated group. However, retinoic acid prevented TPA from reducing the number of LC as the mice treated with both retinoic acid and TPA had a LC density that was not significantly different from the untreated group, and significantly higher than the TPA-treated mice. In contrast solvent EDA in which the retinoic acid had been dissolved did not prevent TPA from reducing the LC density as there was no significant difference from the group treated with TPA alone.

Similar results were obtained whether LC were identified by Ia or β-glucuronidase staining (Table 1); there were no significant differences in LC numbers identified by these two methods for any treatment group. The use of two staining procedures in association with the dendritic morphological characteristics confirmed that the cells being studied were LC.

When these treatments were continued for 4 weeks with the treated epidermis being examined for LC numbers 1 week following the final treatment, a different result was obtained for the group treated with retinoic acid alone, whereas all other treatments gave similar results to those obtained after 1 week of treatment (Tables 1 and 2). Again the mice treated with TPA had significantly fewer LC than the untreated group, whereas retinoic acid inhibited this effect of TPA since mice treated with both retinoic acid and TPA had a LC density that was higher than those who received TPA alone, and not significantly different from untreated mice. However, 4 weeks of retinoic acid treatment significantly increased the LC density compared to either the untreated or solvent EDA ($P < 0.05$)-treated mice. There was no difference in the results obtained when LC were identified by Ia or β-glucuronidase staining (Table 2). Neither TPA nor retinoic acid caused any observable alteration in LC morphology as determined by Ia or β-glucuronidase staining.

Effect of retinoic acid on TPA-induced suppression of contact sensitivity

To determine the functional effects of the protection of LC from TPA by retinoic acid, a contact sensitivity model was utilized (Table 3). Treatment with TPA in acetone significantly decreased the contact sensitivity response compared to mice treated with acetone. Neither solvent EDA nor retinoic acid in solvent EDA significantly altered the response compared to the acetone-treated group. Solvent EDA did not inhibit the TPA-induced decrease in contact sensitivity as this group was not significantly different to that treated with TPA alone and significantly lower than the mice treated with acetone. In

Table 1. Density of Ia- and β-glucuronidase-positive Langerhans' cells in BALB/c mouse ear epidermis 1 week following a single treatment with TPA and retinoic acid

Treatment of mice	Ia-positive LC			β-glucuronidase-positive LC		
	Mean/mm^2 (SD)	P*	P†	Mean/mm^2 (SD)	P*	P†
Untreated	823 (36·0)	—	<0·05	800 (28·3)	—	<0·05
Acetone	769 (50·3)	NS	<0·05	795 (42·4)	NS	<0·05
TPA	536 (51·9)	<0·05	—	551 (38·6)	<0·05	—
Solvent EDA‡	770 (29·9)	NS	<0·05	760 (36·6)	NS	<0·05
Retinoic acid	774 (17·2)	NS	<0·05	775 (50·0)	NS	<0·05
Solvent EDA‡ + TPA	526 (60·6)	<0·05	NS	514 (46·1)	<0·05	NS
Retinoic acid + TPA	768 (38·8)	NS	<0·05	781 (52·2)	NS	<0·05

* Significance of difference from untreated mice (ANOVA).

† Significance of difference from mice treated with TPA (ANOVA).

‡ Solvent in which the retinoic acid was dissolved, ethanol:dimethylsulphoxide:acetone (1:1:6 v/v).

No significant difference between Ia- and β-glucuronidase-positive LC for any of the treatment groups (paired Student's t-test).

NS, not significant; six mice in each group.

TPA treatment, 20 μl of 0·005% TPA once per week. Retinoic acid treatment, 20 μl of 0·5 mg/ml all trans retinoic acid 24 hr and immediately prior to TPA treatment.

F-stat for equal means (Ia), 49·68, df, 6·35; (β-glucuronidase), 48·60, df, 6·35.

Table 2. Density of Ia- and β-glucuronidase-positive Langerhans' cells in BALB/c mouse ear epidermis treated for 4 weeks with TPA and retinoic acid

Treatment of mice	Ia-positive LC			β-glucuronidase-positive LC		
	Mean/mm^2 (SD)	P*	P†	Mean/mm^2 (SD)	P*	P†
Untreated	823 (36·0)	—	<0·05	800 (28·3)	—	<0·05
Acetone	762 (54·0)	NS	<0·05	751 (42·5)	NS	<0·05
TPA	464 (25·4)	<0·05	—	451 (18·2)	<0·05	—
Solvent EDA‡	800 (50·5)	NS	<0·05	769 (24·5)	NS	<0·05
Retinoic acid	946 (51·6)	<0·05	<0·05	925 (50·1)	<0·05	<0·05
Solvent EDA‡ + TPA	481 (45·6)	<0·05	NS	463 (27·2)	<0·05	NS
Retinoic acid + TPA	766 (19.9)	NS	<0·05	777 (26.1)	NS	<0·05

* Significance of difference from untreated mice (ANOVA).

† Significance of difference from mice treated with TPA (ANOVA).

‡ Solvent in which the retinoic acid was dissolved, ethanol: dimethylsulphoxide:acetone (1:1:6, v/v).

No significant difference between Ia- and β-glucuronidase-positive LC for any of the treatment groups (paired Student's t-test).

NS, not significant; six mice in each group

TPA treatment, 20 μl of 0·005% TPA once per week. Retinoic acid treatment, 20 μl of 0·5 mg/ml all trans retinoic acid 24 hr and immediately prior to TPA treatment.

F-stat for equal means (Ia), 108·57, df, 6·35; (β-glucuronidase), 180·25 df, 6·35.

contrast retinoic acid in solvent EDA did inhibit the TPA-induced decrease in contact sensitivity as the response of these mice was significantly higher than that of the group treated with TPA alone, and not significantly different from the acetone or retinoic acid treated groups.

These results were obtained at both 24 and 48 hr following challenge, although the responses of each group had decreased by 48 hr. Hence treatment with retinoic acid curtailed the contact sensitivity response from being diminshed by treatment with TPA.

DISCUSSION

We have previously reported that the tumour promotors TPA and teleocidin, but not tumour initiators, deplete LC from murine epidermis (Halliday *et al.*, 1987), suggesting that one of the key steps in the promotor stage of carcinogenesis may be a reduction in LC, the local antigen-presenting cells. In the present study topical application of retinoic acid prevented TPA from reducing the LC density. In these experiments 34 nmol retinoic acid was applied twice per week, 24 hr before and

Table 3. Contact sensitivity response of BALB/c mice sensitized with DNFB through dorsal trunk skin treated for 4 weeks with TPA and retinoic acid

Treatment of mice	24 hr			48 hr		
	Mean percentage increase in ear thickness (SD)	P*	P†	Mean percentage increase in ear thickness (SD)	P*	P†
Acetone	37·9 (14·6)	—	<0·05	26·1 (8·0)	—	<0·05
TPA	11·1 (4·3)	<0·05	—	6·1 (9·4)	<0·05	—
Solvent EDA‡	35·8 (12·4)	NS	<0·05	27·1 (11·4)	NS	<0·05
Retinoic acid	41·3 (9·7)	NS	<0·05	30·0 (12·6)	NS	<0·05
Solvent EDA‡ + TPA	18·8 (3·1)	<0·05	NS	7·2 (3·5)	<0·05	NS
Retinoic acid + TPA	41·7 (10·1)	NS	<0·05	30·2 (15·4)	NS	<0·05

* Significance of difference from mice treated with acetone (ANOVA).
† Significance of difference from mice treated with TPA (ANOVA).
‡ Solvent in which the retinoic acid was dissolved, ethanol:dimethylsulphoxide:acetone (1:1:6 v/v).
NS, not significant; six mice in each group.
F-stat for equal means (24 hr), 10·15, df, 5·30; (48 hr), 6·67, df, 5·30.

immediately prior to TPA, which is a protocol previously shown to inhibit tumour promotion by TPA (Verma *et al.*, 1982). This effect of retinoic acid was rapid, being observed after 1 week of treatment. Extended treatments with retinoic acid alone for 4 weeks increased the LC density by 18%. Similar results were obtained using two markers, Ia and β-glucuronidase, for identifying LC.

Retinoic acid alone increased, whereas TPA decreased the LC density, suggesting that they effect independent biological mediators rather than having opposite effects on the same mechanism. LC migrate into a limited number of tissues where they form an even distribution of cells, but little is known on what mediators regulate their migration pattern. It is possible that TPA alters the production of one or more of these mediators in a way which either increases LC migration out of or decreases migration into the epidermis, while retinoic acid effects different signals so that increased numbers of LC migrate into the skin, although decreased numbers leaving is not excluded. These studies do not preclude the possibility that retinoic acid induces a maturation of LC precursors or induces LC division within the skin.

TPA-induced depletion of LC abrogates the induction of contact sensitivity through treated skin (Halliday *et al.*, 1988), while retinoic acid counteracted this effect of TPA, enabling a contact sensitivity response equal in magnitude to the controls to be induced, but did not increase the response when applied without TPA.

Retinoid treatment has previously been observed to have a stimulatory effect on LC (Tsambaas & Orfanos, 1981), increasing the number of LC in psoriatic skin lesions (Kanerva *et al.*, 1982; de Fleury *et al.*, 1986). When applied to mouse tail skin, parakeratotic epidermis, which does not contain LC, is converted to ortho-keratotic, and LC also appear at this site (Schweizer & Marks, 1977). Daily oral administration of the retinoid Ro 10-9359 to mice increases the number of Ia-positive LC during the first few days and then decreases their density over the next 2 weeks (Shiohara *et al.*, 1987). In addition Walsh, Seymour & Powell (1985) have shown that whereas human gingival tissue looses T6, HLA-DR and adenosine triphosphatase (ATPase)-

positive LC over a 72 hr culture period, the addition of retinol maintains HLA-DR and ATPase expression and enhances the density of T6-positive cells during the first 30 hr of culture. These studies are in agreement with our observations and suggest that while retinoic acid increases the number of epidermal LC it may also be protective of Lagerhans' cells in some circumstances.

Retinoids have also been shown to affect other antigen-presenting cells. Mice fed a diet supplemented with vitamin A acetate have increased numbers of both dendritic cells and macrophages in their lymph nodes (Katz *et al.*, 1987). Alveolar macrophages isolated from rats fed orally with high doses of retinoids are more efficient at opsonizing sheep red blood cells and killing tumour cell lines (Tachibana *et al.*, 1984). Culture of human monocytes with low doses of retinoic acid inhibits the increased expression of Fc receptors which normally occurs in the absence of retinoids and enhances the production of arginase (Rhodes & Oliver, 1980).

Whereas stimulatory effects of retinoid treatment on anti-tumour immunity were not examined in this study, there is substantial evidence that this may contribute to the anti-carcinogenic effects of retinoids. Intraperitoneal injection of 25–300 µg/mouse/day retinoic acid for 5 days increases the development of cell-mediated cytotoxicity upon challenge with allogeneic tumour cells (Lotan & Dennert, 1979). Treatment of human cancer patients with oral retinoids increases their delayed hypersensitivity reactions to recall antigens and augments the response of their lymphocytes to phytohaemagglutinin (Micksche *et al.*, 1977). Mice immunized with irradiated tumour cells have a prolonged survival when challenged with live tumour cells if they had been fed with a vitamin A acetate-rich diet; this was partially due to an increase in specific T-cell cytotoxicity (Malkovsky *et al.*, 1983).

Although there is considerable evidence that immunopotentiation can at least partially explain the anti-carcinogenic effects of retinoids, the mechanism by which they do this is unknown (Malkovsky & Medwar, 1984). Since retinoids increase the number or function of various antigen-presenting cell types, including LC, and as described here protect LC from tumour

promotors, it is possible that the immunopotentiating effects of retinoids may be due to their stimulatory effects on antigen-presenting cells, which would allow the more efficient induction of immune responses against tumours. Protection of local antigen-presenting cells during carcinogenesis may be an important mechanism by which retinoids exert their anti-carcinogenic effects.

We have found that one of the steps which occurs during the early stages of tumour promotion is a loss of epidermal LC, and topically applied retinoic acid increases the number of LC, overcoming the TPA-induced depletion. As this effect of retinoic acid enables these LC to induce an immune response against cutaneous antigens, whereas otherwise tolerance would result, protection of LC from promotors by retinoic acid may be one of the mechanisms by which retinoic acid inhibits tumour promotion, lowering tumour incidence. It is, however, unknown whether systemically or orally administered retinoids protect LC from the effects of TPA.

However, retinoic acid may not have this protective effect against all forms of carcinogenesis. Whereas TPA and DMBA deplete LC from the epidermis, we have observed that the carcinogens benzo(a)pyrene and catechol increase the epidermal LC density and alter their morphology (Ruby et al., 1989). These elevated numbers of morphologically altered LC have impaired function; it is not known whether retinoids also protect LC from these carcinogens.

ACKNOWLEDGMENTS

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