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PARALLEL ACTIVITIES OF FATTY ACID METHYL ESTERS AND ANALOGOUS PHORBOL DIESTERS TOWARD MOUSE LYMPHOCYTES

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Summary: Linear saturated fatty acid methyl esters were comitogenic with lectins for mouse lymphocytes, the degree of comitogenicity being strongly dependent on the length of the acyl group, and maximal for methyl tetradecanoate. Lesser effects were found for analogs with 10, 12 or 16 acyl carbon atoms, whereas those with fewer than 10 or more than 16 were inactive. Analogous structure-function relationships have been described for various membrane-active and tumor-promoting phorbol diesters, where there is a similar dependence on ester acyl group length for many activities. The fatty acid esters may therefore represent simple model compounds for studying mechanistic aspects of phorbol diester activity.

The diesters of the diterpenoid natural product phorbol have a wide variety of biological activities *in vitro* and *in vivo* (1). Among the former are mitogenic (2,3) and comitogenic (4,5) activities on various lymphoid and other cells, and effects on cell differentiation (2) and intercell communication (6,7). The most remarkable property of phorbol diesters *in vivo* is their ability to induce tumors in epidermis or certain other tissues pretreated with an otherwise subeffective dose of a carcinogen (1). The phorbol diesters of greatest *in vivo* potency are those esterified at the 12-and 13-hydroxyl groups with one each of long and a short chain fatty acyl group (8). Among this group of compounds the most active is TPA[2], this being true for the vast majority of effects mediated by this type of agent. The relative

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[2]Abbreviations: TPA, 12-O-tetradecanoylphorbol-13-acetate; PHA, phytohemagglutinin; FCS fetal bovine serum

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tumor-promoting activities of different phorbol diesters are determined not only by specific functions in the tetracyclic tigliadiene nucleus (8) but also by the acyl chain lengths of the 12-and 13-acyloxy substituents (8). Thus whereas 12-0-tetradecanoylphorbol-13-acetate and 12-0-acetylphorbol-13-tetradecanoate have comparable promoting activities, lower potencies are found for 12-0-alkanoylphorbol-13-acetates with alkanoyl groups longer or shorter than 14 carbon atoms, or for symmetrical diesters such as phorbol-12,13-didecanoate.

Although the above findings have been known for some time, little further attention has been paid to the role of the fatty acyl sidechains in the mechanism of action of phorbol diesters. In this respect, description of tumor-promoting activity for a saturated fatty acid methyl ester is limited to a single compound in one brief account (9). We therefore sought to examine whether simple fatty acid ester analogs of phorbol diesters were active on an *in vitro* cell response previously found to be modified by various phorbol diesters. The cell response chosen was the stimulation of mouse lymphocytes to proliferate by plant lectins, where phorbol diesters show synergistic activities with the lectins without displaying mitogenic activities *per se* (5). Different phorbol diesters also show the same order of activity toward this response as they do toward a large number of other *in vivo* and *in vitro*.

MATERIALS AND METHODS

Saturated fatty acid methyl esters (99% pure, Milton Roy Co., Delaware, Ohio) and 12-0-tetradecanoylphorbol-13-acetate (TPA, P. Borchert, Minneapolis, Minnesota) were made up as fresh solutions in acetone prior to dilution in Roswell Park Memorial Institute (RPMI) 1640 medium (GIBCO, Grand Island, New York) containing 10% fetal calf serum (FCS). Weanling (age 4-8 wks) female Balb/c mice (Jackson Memorial Labs, Bar Harbor, Maine) were sacrificed by cervical dislocation, and the spleens removed from a flank incision. After pressing through sterile stainless steel mesh, the lymphocytes were suspended in RPMI/10% FCS containing penicillin and streptomycin, washed and exposed for 2 minutes to 0.83% ammonium chloride solution to lyse erythrocytes. 5×10^5 cells per well were incubated in a microtitre plate in a total volume of 100 µl for 74 hr, in a humidified atmosphere of 95% oxygen/5% carbon dioxide. Phytohemagglutinin (PHA) and fatty acid

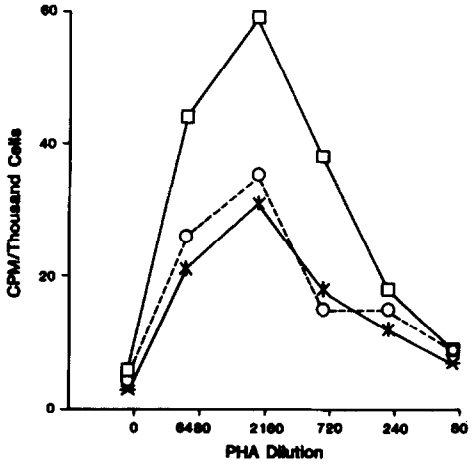

Figure 1. Uptake of tritiated thymidine into DNA of lymphocytes incubated with PHA alone (✱ — ✱), PHA + 120 μM methyl octanoate (0----0) or 120 μM tetradecanoate (□—□). Each point represents the average of six different experiments, and standard deviations were less than 10% of the mean.

esters or other agents were added where appropriate to experimental groups. After 48 hr incubation, 0.5 μCi tritiated thymidine were added to all cultures, and 24 hr after addition the cells were harvested onto glass fiber discs and washed using a semiautomated cell harvester. In order to measure incorporated radioactivity the discs were placed in 3 ml of Kew Solve (Kew Scientific, Columbus, Ohio) in a scintillation spectrometer. In the data shown, each value represents the mean of six replicate cultures, and the standard deviations were less than 10% of the mean. Except where indicated values from treatment groups were significantly different from those from corresponding controls (Student's t test, $p < 0.05$). Cell viability following ester treatments was determined by vital dye staining.

RESULTS AND DISCUSSION

Mouse spleen lymphocytes normally remain in a quiescent, nondividing state in culture, but in the presence of certain agents such as plant lectins, show a strong proliferative response. Uptake of tritiated thymidine can be used to estimate the degree of cell division, and an excellent correlation between uptake of radiolabel and cell number has been obtained in our laboratory previously. A characteristic maximum in radiolabel uptake was observed at a specific concentration (Fig. 1) of lectin, for example PHA. When incubated with fatty acid methyl esters with acyl groups from 6 to 18 carbon atoms in length, no proliferative stimulus was observed. If ester and lectin were present simultaneously, however, a

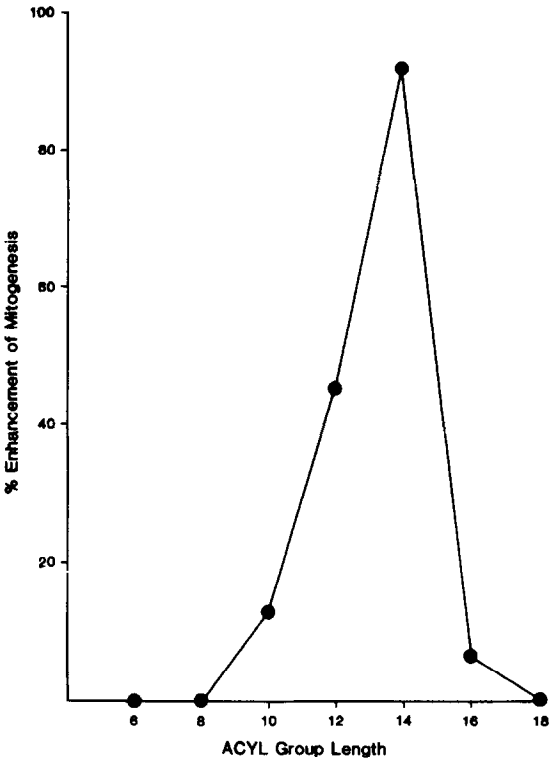

Figure 2. Dependence of comitogenicity of methyl esters on acyl group length in lymphocytes incubated with PHA. (Dilution 1:2160). Each point represents the average of six different experiments.

marked enhancement of the lectin-induced response was observed for certain esters (Fig. 1). Thus whereas methyl tetradecanoate induced a 100% increase in radiolabel uptake above lectin control, methyl octanoate had no significant effect. Both esters were present at a concentration of 120 μM, which was found to be the highest nontoxic concentration of any ester examined.

When a wider range of esters was examined, the dependence of activity on acyl chain length was confirmed in striking fashion (Fig. 2). At 120 μM concentration methyl tetradecanoate was found to have maximum activity, whereas methyl hexanoate, octanoate, and octadecanoate showed none. Other esters of intermediate length examined showed significant activity but less than that of the tetradecanoate. A similar structure-activity relationship was observed at ester concentrations of 60 μM, but the activities of

Table 1. Relative comitogenicities of methyl tetradecanoate and promoting agent analogs.

Comitogen	C_{50} (μM)[1]	Maximum Comitogenicity[2]
Tetradecanoylphorbol acetate	0.01	121 (1.6)
Tetradecane	16	300 (120)
Methyl Tetradecanoate	60	102 (120)

[1]Final concentration required to enhance by 50% mitogenesis induced by an optimal PHA concentration.

[2]The maximum value of $(P^T/PHA^T-1) \times 100$ at an optimal PHA concentration, where P^T is the tritiated thymidine uptake in cultures treated with compound P. Figure in parentheses refers to concentration (μM) of comitogen inducing the effect stated.

the hexadecanoate, and decanoate esters were not significant relative to controls (our unpublished results). At neither concentration did any of the esters displace the mitogenic response to lectin alone (e.g. Fig. 1).

In order to compare the activities of fatty acid methyl esters with those of other promoting agents reported active against murine lymphocytes, experiments were carried out comparing the potent phorbol diester TPA, and the linear alkane tetradecane to the fatty acid ester methyl tetradecanoate. As seen in Table 1, the activity of the phorbol diester was approximately three to four orders of magnitude greater than that of the other compounds, and the alkane was somewhat more active than the fatty acid ester. In all cases the agents displayed analogous activities, being comitogenic with the lectin, but not mitogenic alone.

A large number of biological and biochemical responses to phorbol diesters have been shown to be dependent on the chain length of the ester acyl functions (8). It has been proposed that most, if not all, of these responses are mediated by initial membrane binding at specific receptors (10). The finding that linear saturated fatty acid methyl esters and biologically active phorbol diesters of equivalent acyl chain length share a common activity against murine lymphocytes thus suggests that the long acyl functions of

the phorbol diesters play a significant role in their binding, and possibly in their mechanism of action. The reason why an acyl chain length of 14 carbon atoms results in optimal responses to phorbol diesters and other agents is intriguing, but presently unknown.

Up to the present we have shown the lectin-induced mitogenic response in mouse lymphocytes to be sensitive to several types of tumor-promoting agent, and to predict correctly their relative activities in vivo as promoters of epidermal carcinogenesis (5). In addition to demonstrating comitogenic activity, fatty acid esters show parallel behavior to phorbol diesters and alkanes in mouse lymphocytes in other respects. For instance the comitogenic response is maximal if lectin and ester are added simultaneously. If ester addition is delayed relative to lectin or vice versa reduced effects are observed (manuscript in preparation). In addition the three to four orders of magnitude difference in TPA and tetradecane activities and reduced activity of fatty acid ester relative to the corresponding alkane reflect differences observed in promotion of mouse epidermal carcinogenesis (9,11). Our findings therefore suggest that the mouse lymphocyte response may be valuable for investigating mechanisms of these agents in biological systems. Furthermore simple fatty acid esters may be useful model compounds for such investigations.

Currently a single report exists suggesting tumor-promoting activity for a saturated fatty acid ester, methyl dodecanoate (9). This finding, taken together with our results, suggest that this type of agent deserves further examination, especially since certain myristate esters are extensively used as emollients in cosmetic products (12).

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