Effects of retinoids and phorbol esters on the sensitivity of different cell lines to the polypeptide toxins modeccin, abrin, ricin and diphtheria toxin

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(Received 18 August 1980/Accepted 10 October 1980)

The effects of retinoic acid and 12-*O*-tetradecanoylphorbol 13-acetate on the sensitivities of a number of cell lines to the toxins modeccin, abrin, ricin and diphtheria toxin were studied. Retinoic acid and some other retinoids were found to protect a number of the cell lines against the toxins. HeLa cells that were protected bound much more retinoic acid than L-cells that were not protected. The tumour promoter 12-*O*-tetradecanoylphorbol 13-acetate was found to increase the sensitivity of cells to abrin, ricin and modeccin in the absence as well as in the presence of retinoic acid. Neither retinoic acid nor 12-*O*-tetradecanoylphorbol 13-acetate affected the extent of binding and pinocytotic uptake of toxins by the cells. Apparently retinoic acid and 12-*O*-tetradecanoylphorbol 13-acetate interfere with the entry of the toxins through the cell membrane.

Tumour promoters are substances that are not tumorigenic alone, but are able to induce tumours when given to mice previously treated with a carcinogen in a dose that does not alone induce tumours (Van Duuren *et al.*, 1978). 12-*O*-Tetradecanoylphorbol 13-acetate, which is present in croton oil, has been found to be a potent tumour promoter (Hecker, 1978; Van Duuren *et al.*, 1978). This hydrophobic compound binds to (Sivak & Van Duuren, 1971; Van Duuren *et al.*, 1978) and induces changes in the morphology and function of the cell membrane (Schimmel & Hallam, 1980; Wenner *et al.*, 1978; Wertz *et al.*, 1979).

Certain retinoids are known to counteract both the 12-*O*-tetradecanoylphorbol 13-acetate-induced tumour promotion and also some of the other effects of 12-*O*-tetradecanoylphorbol 13-acetate (Sporn & Newton, 1979; Wertz *et al.*, 1979; Lotan, 1980). Retinoids bind to plasma membranes as well as to a cytoplasmic protein (Sani, 1979) and are necessary for normal differentiation of epithelial cells (Sporn, 1978). Furthermore, they induce differentiation in several cell lines (Jetten & Jetten, 1979; Sporn & Newton, 1979).

Abrin, ricin, modeccin and diphtheria toxin are structurally related toxins that consist of two polypeptide chains. One of the chains binds to cell-surface receptors and the other chain is somehow able to penetrate the cell membrane and enter the cytoplasm, where it inhibits protein synthesis (Pappenheimer, 1977; Olsnes *et al.*, 1978). Little is known about the entry of the toxins into the

cytoplasm, but it is possible that they are taken up by mechanisms originally developed for physiologically important molecules like polypeptide hormones (Olsnes *et al.*, 1974). Since 12-*O*-tetradecanoylphorbol 13-acetate affects the action of epidermal growth factor and since retinoic acid increases the number of epidermal-growth-factor receptors (Jetten, 1980) and blocks the action of the polypeptide sarcoma growth factor (Todaro *et al.*, 1978), it was of interest to measure the effect of the tumour promoter 12-*O*-tetradecanoylphorbol 13-acetate and of retinoids on the sensitivity of cells to the toxic proteins. Furthermore, because of the selective toxicity of abrin and ricin on many tumour cells (Fodstad & Pihl, 1978), studies on the effect of tumour promoters and anti-promoters on the sensitivity of cultured cells to these toxins seemed warranted.

Materials and methods

Materials

Abrin, ricin and modeccin were prepared as described previously (Olsnes, 1978*a,b*). Diphtheria toxin was obtained from Connaught Laboratories, Willowdale, Ont., Canada. Nicked diphtheria toxin was produced from essentially un-nicked toxin by the method of Drazin *et al.* (1971). Other chemicals used and their sources are: 12-*O*-tetradecanoylphorbol 13-acetate (Consolidated Midland Corporation, Booster, NY, U.S.A.); 4 α -phorbol 12,13-

didecanoate, retinoic acid, retinol, retinal and retinol acetate (Sigma Chemical Co., St. Louis, MO, U.S.A). The other retinoids used and [^{14}C]retinoic acid were gifts from Hoffmann-La Roche.

Cells

Two HeLa cell lines were used in these experiments, one line that has been cultured in this laboratory for several years and HeLa S_3 cells, which were obtained from The American Tissue Culture Collection. Four clones of HeLa S_3 cells were isolated by us. The Hep 2 cell line was obtained from Dr. J. A. Espmark, Stockholm, Sweden. Also the 2T cell line was kindly provided by Dr. J. A. Espmark, who had obtained it from Dr. J. Pontén, Uppsala, Sweden. African-green-monkey kidney cells (Vero), sternal bone marrow cells (Det 6) and human amnion epithelium cells were obtained from the National Institute for Public Health, Oslo. HE and CCL 27 cells were obtained from Professor J. Jonsen, Oslo, Norway, and NRK and NRK ASV La 334 cells from Dr. T. Ege, Oslo, Norway. FME cells are a human melanoma line established in this laboratory (Tveit *et al.*, 1980).

Measurement of toxicity

The sensitivity of different cell lines to the toxins was measured by testing the ability of increasing toxin concentrations to inhibit cellular protein synthesis. The day before the experiment 10^5 cells per well were transferred to 'disposo' trays with 24 wells, 2 cm^2 each (Costar tissue culture cluster no. 3525; Costar 205, Broadway, Cambridge, MA, U.S.A.). The next day the cells adhered to the bottom of the wells. They were then washed with Hanks' solution and Eagle's minimum essential medium containing 21 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH 7.7, instead of bicarbonate and without leucine was added. Retinoids and 12-*O*-tetradecanoylphorbol 13-acetate were dissolved in dimethyl sulphoxide and added to the medium to give a dimethyl sulphoxide concentration of 0.1% and a concentration of retinoids and 12-*O*-tetradecanoylphorbol 13-acetate as indicated in the legends to the Figures. In experiments where retinoids and 12-*O*-tetradecanoylphorbol 13-acetate were used, the medium in the controls was adjusted to contain the same concentration of dimethyl sulphoxide (i.e. 0.1%). After incubating the cells at 37°C for 3.5 h with increasing amounts of toxins, the medium was removed and 0.5 ml of the same medium and 25 nCi of [^{14}C]leucine were added to each well. After 30 min more at 37°C , the medium was removed, the cells were dissolved in 0.1 M-KOH, trichloroacetic acid was added to a final concentration of 10% (w/v) and the acid-precipitable radioactivity was measured.

Binding studies

Modeccin, ricin and diphtheria toxin were labelled with ^{125}I by the lactoperoxidase method, essentially as described by Marchalonis (1969). To measure the total amount of ^{125}I -labelled toxin bound to cells, cells growing in wells of disposo trays (Costar) were incubated with ^{125}I -labelled toxin in serum-free medium for 60 min at 37°C . The cells were washed twice with Hanks' solution, dissolved in 0.1 M-KOH and the radioactivity associated with the cells was measured in an Inter-technique CG 30 automatic gamma spectrometer.

To measure the amount of toxin that could not be released from the cells with lactose (lactose-resistant toxin), cells were incubated with ^{125}I -labelled toxin for 60 min at 37°C , and then the medium was removed. The cells were then incubated with phosphate-buffered saline containing 0.1 M-lactose for 15 min to release surface-bound toxin. Finally, the cells were washed three times with this solution, dissolved in 0.1 M-KOH and the cell-associated radioactivity was measured.

Results and discussion

Effect of retinoic acid on the sensitivity of cells to modeccin, abrin, ricin and diphtheria toxin

To cells growing in wells of disposo trays increasing amounts of toxin were added in the absence and presence of retinoic acid and then, after 3.5 h, the ability of the cells to incorporate [^{14}C]leucine was measured. As shown in Fig. 1(a), in the presence of $10\text{ }\mu\text{M}$ -retinoic acid 100–1000 times more modeccin must be added to HeLa cells to obtain the same intoxication as in the absence of retinoic acid. The protection of cells against modeccin afforded by retinoic acid varied from one cell line to another. As shown in Fig. 1(b), the effect of retinoic acid on intoxication of Vero cells by modeccin was less than in HeLa cells, and retinoic acid had no protective effect in L-cells (Fig. 1c).

The data in Table 1 show that most of the cell lines tested are protected against modeccin, although to varying extents. Even different clones of HeLa cells responded differently. Other authors have found differences between different clones of B-16 melanoma cells in the extent to which retinoic acid inhibited cell growth (Lotan & Nicholson, 1979). Retinoic acid was also found to protect Vero cells against abrin, ricin and diphtheria toxin, although to a lesser extent than observed with modeccin. There was no increased protection if the cells had been incubated with retinoic acid for 4 h before addition of toxin, indicating a rapid effect of retinoic acid.

Binding of retinoic acid to cells

In attempts to elucidate the mechanism behind

Fig. 1. *Effect of retinoic acid on the sensitivity of HeLa cells (a), Vero cells (b) and L-cells (c) to modeccin*
To cells growing in 24 wells 'disposo' trays were added the indicated amounts of retinoic acid and increasing concentrations of modeccin. The cells were incubated for 3.5 h, then the medium was changed to medium containing [¹⁴C]leucine, the cells were incubated for 30 min more and then the incorporation of radioactivity into trichloroacetic acid-precipitable material was measured as described in the Materials and methods section. ×, No retinoic acid added; O, 10 μM-retinoic acid; Δ, 1 μM-retinoic acid.

Table 1. *Effect of retinoic acid on the sensitivity of cells to toxins*
Inhibition of protein synthesis by toxins was measured in the absence and presence of retinoic acid as described in Materials and methods section.

Tissue of origin	Cell	Decrease in sensitivity*			
		Toxin ... Modeccin	Abrin	Ricin	Diphtheria toxin
Human cervical carcinoma	HeLa	100–1000	1	1	
	HeLa S ₃	4			
	HeLa S ₃ , clone 1	13			
	HeLa S ₃ , clone 2	15			
	HeLa S ₃ , clone 3	4			
	HeLa S ₃ , clone 4	25			
African-green-monkey kidney	Vero	10–100	4–6	2–5	2
Heart	CCL27	7			
Human carcinoma laryngis	Hep 2	5			
Murine fibroblasts	L	1	1	1	
Human embryo	HE	1			
Human sternal bone marrow	Det 6	3			
Human melanoma	FME	10			
Human osteosarcoma	2T	10	1	1	1
Rat kidney	NRK	3			
Rat kidney (transformed)	NRK ASVL334	4			
Epithelial cells	Human amnion	4			

* Ratio between the toxin concentrations required to decrease protein synthesis to 50% of the control value in the presence and absence of 10 μM-retinoic acid.

the different extent of protection seen with the different cell lines, we compared the ability of cells to bind retinoic acid. These experiments were carried out at 0°C to decrease the pinocytotic uptake of the labelled compound. When [¹⁴C]retinoic acid was added to cells in serum-free medium,

there was a rapid binding of the labelled compound to the cells (Fig. 2a). Interestingly, L-cells that are not protected against modeccin by retinoic acid bound less [¹⁴C]retinoic acid than the HeLa cells (Fig. 2b). However, HeLa S₃ cells bound the same amount of retinoic acid as HeLa cells (results not

Fig. 2. Ability of HeLa cells and L-cells to bind retinoic acid

Cells growing in wells of 'disposo' trays were washed twice with Hanks' solution and then serum-free medium, and $[^{14}\text{C}]$ retinoic acid was added. In (a) $[^{14}\text{C}]$ retinoic acid was added to all wells to a concentration of $5\ \mu\text{M}$, whereas in (b) the amounts added are indicated on the abscissa. The cells were incubated at 0°C . After the indicated periods of time (a) or after 60 min (b) the cells were washed twice with Hanks' solution, dissolved in $0.1\ \text{M-KOH}$ and the radioactivity associated with the cells was measured as described in the Materials and methods section. Background values due to unspecific binding of $[^{14}\text{C}]$ retinoic acid to the plastic was measured in control wells without cells and subtracted from the experimental values. ●, HeLa cells; ×, L-cells.

Table 2. Ability of different retinoids to protect HeLa cells against modeccin

Inhibition of protein synthesis in HeLa cells by modeccin was measured as described in the Materials and methods section in the presence or absence of various retinoids.

	Chemical structure	R	Ability to protect against modeccin*
(1)		CO_2H CHO CH_2OH $\text{CH}_2\text{OOCCH}_3$	+ — — —
(2)		CO_2H	+
(3)		CO_2H COOC_2H_5	— —
(4)		COOC_2H_5	—
(5)		CO_2H	+

* The ability of a concentration of $10\ \mu\text{M}$ of each retinoid to protect HeLa cells under conditions as in Fig. 1(a) was measured. Those retinoids that had a protective effect were all equally efficient. +, Protection; —, no protection.

shown), although the protection of these cell lines differed. Clearly, the protective effect is not directly related to the amount of retinoic acid bound. When HeLa cells were incubated with [14 C]retinoic acid, then washed, removed from the monolayer by trypsin and sonicated, about 75% of the radioactivity was found in the pellet after short-time centrifugation at 12000 *g*. Most likely it was bound to the membranes. It should be noted that if the medium used in the binding assay contained 10% calf serum, there was no measurable binding of [14 C]retinoic acid to the cells, probably due to the fact that serum contains a protein that binds retinoic acid (Smith *et al.*, 1973; Lotan, 1980). In accordance with this, 10 μ M-retinoic acid did not protect HeLa cells against modeccin if 10% calf serum was present, indicating that retinoic acid exerts its effect directly on the cells.

Ability of different retinoids to protect HeLa cells against modeccin

The data in Table 2 demonstrate that only some retinoids protected HeLa cells against modeccin. With one exception (the carboxylic acid form of compound 3), these were the same ones as those previously shown to bind to the intracellular binding protein and to induce differentiation in teratoma cells (Jetten & Jetten, 1979). The retinoids that have a protective effect all have a terminal carboxylic acid group. Certain changes of the ring structure can occur without loss of protective effect. Those retinoids that are active in protecting cells against modeccin (indicated by + in Table 2) were equally efficient as retinoic acid.

Effect of phorbol esters on the sensitivity of cells to modeccin, abrin, ricin and diphtheria toxin

Since retinoic acid in many systems efficiently counteracts the tumour-promoting effect of phorbol esters like 12-*O*-tetradecanoylphorbol 13-acetate we decided also to study the effect of 12-*O*-tetradecanoylphorbol 13-acetate on cell intoxication. It was found that 12-*O*-tetradecanoylphorbol 13-acetate increased the sensitivity to modeccin of all cell lines tested. Thus, HeLa cells became approximately three times more sensitive in the presence of 1.6 μ M-12-*O*-tetradecanoylphorbol 13-acetate than in its absence (Fig. 3). 12-*O*-Tetradecanoylphorbol 13-acetate increased the sensitivity to modeccin of HeLa, FME, L, NRK and human amnion epithelial cells (results not shown). Furthermore, 12-*O*-tetradecanoylphorbol 13-acetate increased the sensitivity of HeLa, Vero, L- and human amnion epithelium cells to abrin and HeLa and L-cells to ricin.

The increase in the sensitizing effect was maximal at 1.6 μ M-12-*O*-tetradecanoylphorbol 13-acetate, although 0.16 μ M-12-*O*-tetradecanoylphorbol 13-

Fig. 3. Effect of 12-*O*-tetradecanoylphorbol 13-acetate and retinoic acid on the sensitivity of HeLa cells to modeccin

HeLa cells were incubated for 3.5 h with increasing concentrations of modeccin in the absence and presence of 12-*O*-tetradecanoylphorbol 13-acetate and retinoic acid. The medium was changed, [14 C]leucine was added and the incorporation of radioactivity was measured as in Fig. 1. Δ , 1.6 μ M-12-*O*-tetradecanoylphorbol 13-acetate present; $\circ$, 10 μ M-retinoic acid; $\bullet$, 10 μ M-retinoic acid and 1.6 μ M-12-*O*-tetradecanoylphorbol 13-acetate; $\times$, no additions.

acetate was almost as effective. Even 16 nM-12-*O*-tetradecanoylphorbol 13-acetate had a clear sensitizing effect, whereas no effect was seen with 1.6 nM-12-*O*-tetradecanoylphorbol 13-acetate. Pre-incubation of the cells with 12-*O*-tetradecanoylphorbol 13-acetate did not further increase the sensitivity to toxins. The phorbol ester 4 α -phorbol-12,13-didecanoate, which has no tumour-promoting activity, also had no effect on the sensitivity of cells to abrin, ricin and modeccin.

Cells that were incubated with modeccin in the presence of protective concentrations of retinoic acid became more sensitive to modeccin in the presence of 12-*O*-tetradecanoylphorbol 13-acetate (Fig. 3). Surprisingly, 12-*O*-tetradecanoylphorbol 13-acetate was found to protect rather than sensitize Vero cells to diphtheria toxin. Thus the cells became 10 times more resistant to diphtheria toxin in the presence of 12-*O*-tetradecanoylphorbol 13-acetate than in its absence (Fig. 4). Intact toxin and toxin where the A- and B-fragments were proteolytically cleaved from each other ('nicked toxin') gave the same result. As shown in Fig. 4, the slight protection

Fig. 4. Effect of 12-*O*-tetradecanoylphorbol 13-acetate and retinoic acid on the sensitivity of Vero cells to diphtheria toxin

Conditions were as described in the legend to Fig. 3.

●, 1.6 μ M-12-*O*-tetradecanoylphorbol 13-acetate; ○, 10 μ M-retinoic acid; Δ, 10 μ M-retinoic acid and 1.6 μ M-12-*O*-tetradecanoylphorbol 13-acetate; x, no additions.

afforded by retinoic acid was additive to the protection obtained with 12-*O*-tetradecanoylphorbol 13-acetate alone. 12-*O*-Tetradecanoylphorbol 13-acetate at a concentration of 0.16 μ M had no protective effect against diphtheria toxin. In accordance with the data with abrin, ricin and modeccin the non-promoter phorbol ester 4 α -phorbol 12,13-didecanoate had no effect on the sensitivity to diphtheria toxin.

Binding of toxin to cells in the presence of retinoic acid and 12-O-tetradecanoylphorbol 13-acetate

One possible mechanism of action of retinoic acid and 12-*O*-tetradecanoylphorbol 13-acetate is that they somehow change the binding of toxins to the cells. We therefore measured the ability of HeLa cells to bind 125 I-labelled modeccin in the absence and presence of retinoic acid (10 μ M) and 12-*O*-tetradecanoylphorbol 13-acetate (1.6 μ M). No significant differences were found (results not shown).

As previously shown, abrin, ricin and modeccin bound to cell-surface receptors can be released by incubating the cells in the presence of lactose (Olsnes *et al.*, 1974, 1978). If the cells are incubated with the toxins at 37°C, a fraction of the surface-bound toxin is transported to a state where it cannot be released with lactose (lactose-resistant toxin). This mainly represents toxin taken into the cells by

pinocytosis (Sandvig & Olsnes, 1979). Retinoic acid and 12-*O*-tetradecanoylphorbol 13-acetate did not change the rate with which modeccin was transferred to the lactose-resistant state. Also when the binding of 125 I-labelled ricin and diphtheria toxin to Vero cells was tested in the absence and presence of retinoic acid, no significant difference was observed and the amount of lactose-resistant ricin was the same under both conditions. Furthermore, 10 μ M-retinoic acid did not decrease the inhibitory effect of modeccin on protein synthesis in a cell-free system from rabbit reticulocytes (results not shown).

General discussion

The main findings in the present paper are that retinoic acid and certain related compounds protect cells against modeccin and to a lesser extent against abrin and ricin, whereas the tumour promoter 12-*O*-tetradecanoylphorbol 13-acetate increases the sensitivity of the cells to these toxins. Both types of compound protect cells against diphtheria toxin.

The reason for the effects here observed is not obvious. Retinoic acid is somehow involved in glycoprotein biosynthesis, possibly as a carrier of mannose through the cell membrane (Lotan, 1980). Changes in the glycoprotein composition in the plasma membrane could obviously result in altered sensitivity of the cells to the toxic lectins modeccin, abrin and ricin. This appears, however, unlikely for several reasons. Thus retinoic acid does not affect the gross binding of modeccin to HeLa cells or that of ricin to Vero cells. Furthermore, the short duration of the experiments here reported (4 h) makes it unlikely that a sufficiently great change in the surface-membrane glycoproteins could have taken place. Also, the protective effect of retinoic acid was not greater when retinoic acid was added 4 h before the addition of toxin than when retinoic acid and toxin were added simultaneously. These results and the fact that [14 C]retinoic acid binds rapidly to the cells, probably mainly to the membrane, indicate that the change in sensitivity is due to a rapid effect caused by the binding of [14 C]retinoic acid to the cells.

Although it is known that a binding protein for retinoic acid is present in the cytoplasm (Chytil & Ong, 1979; Jetten & Jetten, 1979), it is difficult to imagine how binding of retinoic acid to a cytoplasmic protein could affect the sensitivity of the cells to the toxin rapidly. It appears more likely that the binding of retinoic acid to the membrane (Sani, 1979) affects the transport of the toxins through the membrane.

It is interesting that 12-*O*-tetradecanoylphorbol 13-acetate, which in many other systems also has the opposite effect of retinoic acid (Lotan, 1980), sensitizes the cells to modeccin, abrin and ricin.

Abrin and ricin were found to have a cancerostatic effect in several model systems (Fodstad & Pihl, 1978; Olsnes & Pihl, 1980). It is not clear whether the effect observed here on the sensitivity of cells to the toxins is related to the ability of 12-*O*-tetradecanoylphorbol 13-acetate to act as a tumour promoter. The cells studied here were mostly malignant cells and 12-*O*-tetradecanoylphorbol 13-acetate was found to sensitize the human melanoma FME cell line to modeccin, in spite of the fact that 12-*O*-tetradecanoylphorbol 13-acetate has a differentiating effect on these cells (Tveit *et al.*, 1980). It is interesting, however, that the non-promoting phorbol ester 4 α -phorbol 12,13-didecanoate does not change the sensitivity of cells to toxins.

12-*O*-Tetradecanoylphorbol 13-acetate sensitizes leukaemic cells to macrophage- and granulocyte-inducing factor (Loten & Sachs, 1979). Furthermore, Balb/C 3T3 cells are sensitized by 12-*O*-tetradecanoylphorbol 13-acetate to epidermal growth factor and other growth factors (Frantz *et al.*, 1979) in spite of the fact that 12-*O*-tetradecanoylphorbol 13-acetate-treated cells have decreased affinity and number of cell-surface receptors for epidermal growth factor (Lee & Weinstein, 1978; Shoyab *et al.*, 1979). Clearly, the sensitizing effect of 12-*O*-tetradecanoylphorbol 13-acetate is on some process occurring after binding of epidermal growth factor, e.g. the internalization of the peptide. Possibly the mechanism behind the sensitization to toxins and growth factors is the same.

This work was supported by The Norwegian Cancer Society. The helpful discussion of Professor A. Pihl and the skilful technical assistance of Mrs. J. Jacobsen are gratefully acknowledged.

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