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DIFFERENTIAL SENSITIVITY OF MONOLAYER CELLS COMPARED WITH SUSPENSION CELL CULTURES TO TREATMENT WITH HIGH CONCENTRATIONS OF CROTON OIL FACTOR (TPA) AND THE SURFACTANTS TWEEN 80 AND TRITON X-100

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Abstract—The incorporation of ^{3}H-thymidine into HeLa and L-cells is inhibited by croton oil factor TPA and the surfactants, Tween 80 and Triton X-100. The relative inhibition is higher in cell cultures with low cell densities than in cultures with high cell densities, provided monolayer cultures are used. Comparative studies on the action of drugs on tissue culture cells in monolayers therefore should take into account the densities of the cultures used. This relation could not be observed in suspension cultures which in addition seem to be less sensitive than monolayer cultures.

HELA CELLS and L cells are widely used as *in vitro* test systems for biologically active substances. In this paper we report on the reactions of cell cultures to high concentrations of croton oil factor tetra-decanoyl-phorbol-acetate (TPA), Tween 80 and Triton X-100 as measured by the incorporation of ^{3}H-thymidine (^{3}H-Tdr). TPA has been purified and characterized[1,2] and tested extensively *in vivo*.[3] *In vitro* experiments using cell cultures have also been reported.[4–6] In the present study it was shown that the degree of the reaction of the cells studied to the addition of high concentrations of these compounds was very much dependent on the kind of culture system employed and the cell density if monolayer cultures were used.

MATERIAL AND METHODS

12-*O*-Tetra-decanoyl-phorbol-acetate (TPA) was prepared according to Bresch *et al.*[2] Tween 80 (Schuchardt), Triton X-100 (Fluka), Dimethylsulfoxide (DMSO; Merck) and ^{3}H-thymidine (^{3}H-Tdr; Amersham, CH_3-labelled, sp. act. 22·8 Ci/mM) were obtained from the quoted sources. 12-*O*-Tetra-decanoyl-phorbol-acetate-20-^{3}H was prepared as reported[7] (sp. act. 7·5 Ci/mM). L-cells were propagated as monolayers in a medium containing 70 per cent Hanks's balanced salt solution, 20 per cent calf serum and 10 per cent chicken embryo extract. HeLa cells were propagated as monolayers in a medium consisting of 90 per cent Gey's solution and 10 per cent calf serum. This medium was 0·25 per cent in lactalbumin (w/v).

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For monolayer experiments the cells grown in flasks were removed by trypsin (0·06 per cent trypsin in Dulbecco's solution without $CaCl_2$ and $MgCl_2$) and suspended in the case of L-cells in 90 per cent TCM 199 (Difco) and 10 per cent fetal calf serum (Flow), and in the case of HeLa cells in the above mentioned HeLa medium. One ml aliquots of the cell suspensions were distributed into roller tubes and rolled over night (12 rev/hr) to establish the monolayers. During this time the cell numbers did not increase significantly.

At the beginning of the experiments the medium was replaced by 1 ml TCM 199 (without or with 10 per cent calf serum) containing 0·5 per cent DMSO and one of the test compounds. Cultures containing 0·5 per cent DMSO only, served as controls.

For suspension experiments the cells were trypsinized as described and suspended in a medium containing 87·5 per cent Basal Eagles medium (Flow) supplemented with Earle's salts, 9·5 per cent calf serum and 3 per cent of a 4 per cent methylcellulose solution (final concn, 0·12 per cent; methocel MC 25 cp, Fluka). The cell suspensions were adjusted to 5×10^4 cells/ml, incubated in spinner flasks and stirred (60 rev/min) for 3 to 4 days at 37°. Cell multiplication was determined by repeated counting in a Neubauer chamber. At the beginning of the experiments the suspensions were adjusted to defined cell numbers, distributed to roller tubes (1 ml/tube) and rotated (120 rev/hr). Compounds were added in 5 μl DMSO (= 0·5 per cent DMSO). Cultures containing 0·5 per cent DMSO only served as controls.

Monolayer and suspension cultures were incubated with the test compounds for 6 hr. During the sixth hour ^{3}H-Tdr (2 μCi/culture) was added.

To stop the monolayer experiments 20 μl of a 10 per cent sodium dodecylsulfate solution (SDS) was pipetted into each culture. After whirling up and incubation at 37° for 30 min aliquots of the lysates were pipetted on to the filter paper disks as described earlier.[8]

Suspension experiments were stopped by pipetting 100 μl aliquots of the cell suspensions directly onto filter paper disks.

The disks were extracted following the Mans–Novelli procedure.[9] The radioactivity was measured in a Packard liquid scintillation counter with standard toluene scintillator.

The procedure for studying the binding of TPA-^{3}H to cells is described in the legend of Table 1.

TABLE 1. BINDING OF ^{3}H-TPA TO HELA MONOLAYER CELLS EXPRESSED IN PER CENT OF ADDED ACTIVITY

Cell no. ($\times 10^{-5}$ cm^{-1})	^{3}H-TPA concn (M)		
	10^{-5}	10^{-6}	10^{-7}
0·89	0·57	1·21	0·77
1·78	0·87	1·49	1·10
3·57	1·54	2·13	1·87

Different numbers of HeLa cells were plated in multiwell microtest-plates (TC quality, Linbro) and allowed to attach (2·5; 5·0 and 10×10^4 cells per well or per 28 mm^2). The medium (200 μl) was replaced by 200 μl TCM 199 containing the indicated concentrations of ^{3}H-TPA and 0·5% DMSO After a 60 min incubation at 37° in a CO_2 incubator the cells were washed twice with 200 μl TCM 199, lysed with 200 μl 1 per cent SDS and counted in a liquid scintillation counter using dioxane scintillator.

RESULTS

Cells cultured in monolayers were rolled over night as described in Methods and incubated with various concentrations of TPA, Triton X-100 or Tween 80 for 6 hr. In the sixth hour a thymidine pulse was given. Figure 1b shows that L-cell cultures with low cell densities were inhibited at lower concentrations of TPA than cultures with high cell densities. Thus given the same concentrations of TPA, cells in dense monolayer cultures are relatively less sensitive than monolayers with lower cell densities. At very low (10^{-7} M) and very high (10^{-4} M) concentrations the curves showing the relative inhibition merge.

Similar results were obtained with Triton X-100 and Tween 80 (Fig. 1c); here again cultures with low cell numbers were more sensitive than dense cultures.

The same holds also for HeLa cells (Fig. 1a). The results obtained with TPA are complicated, however, by the fact that even low concentrations of TPA inhibit the ^{3}H-Tdr incorporation into HeLa cells. This reaction is caused by a second biological effect exhibited by TPA on HeLa cells which is described more extensively elsewhere.[4,6,10] Although the curves are merging below the low level of about 50 per cent incorporation, the cell number dependency is clearly demonstrable at 10^{-5} M TPA.

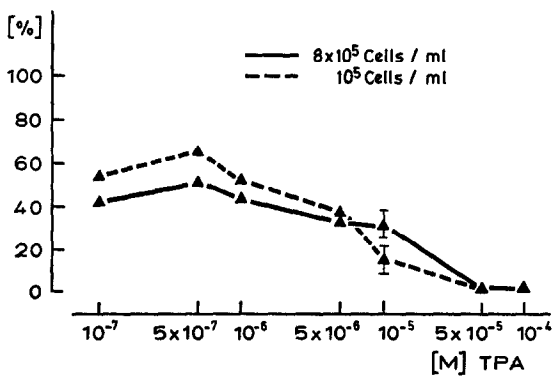

Fig. 1a

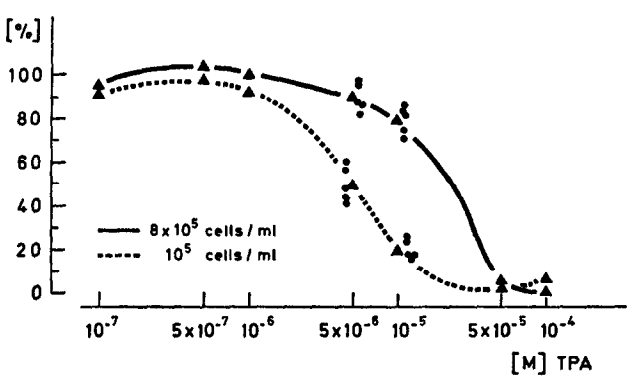

Fig. 1b

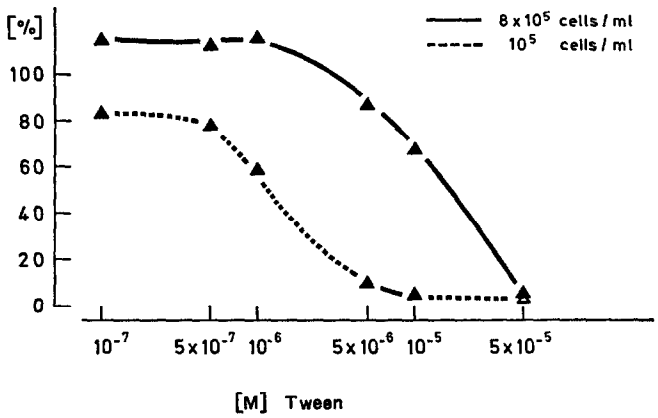

Fig. 1c

FIG. 1. Inhibition of ^{3}H-Tdr incorporation by different concentrations of phorbolester TPA (a, b) and Tween 80 (c) into HeLa cell (a) and L-cell (b, c) monolayer cultures expressed as per cent inhibition of the controls incubated with 0·5% DMSO only. In the case of (a) and (b) the maximal deviations or the values of the single cultures respectively have been plotted for the concentrations in question.

Figure 2 shows that with L-cells in a certain concentration range the same low levels of ^{3}H Tdr incorporation are observed either if the cell number is halved or the concentration of TPA is doubled. This result led to the idea that TPA might be largely removed by the cells from the medium which would be of course cell number dependent, thus decreasing the available active amount of TPA. This, however, is not the case as discussed later.

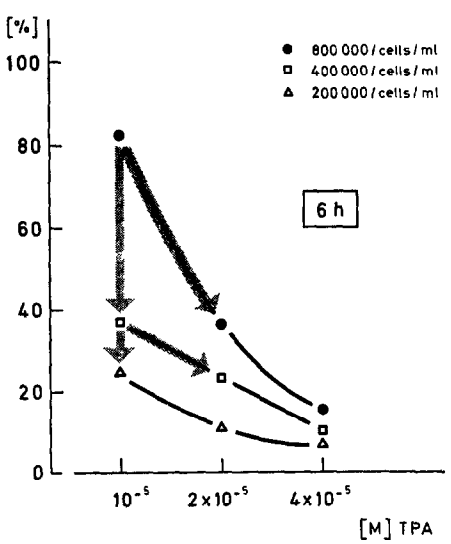

FIG. 2. Cell number and TPA-concentration dependent inhibition of ^{3}H-thymidine incorporation into L-cell *monolayers* expressed as per cent incorporation of the controls incubated with 0·5% DMSO only.

These results indicate that the incorporation of ^{3}H-Tdr into monolayer cells is influenced by the test compounds in a cell number dependent manner.

Cells in suspension, however, were inhibited quite independently of the cell density when incubated with TPA at various concentrations. Figure 3 shows such experiments for HeLa and L-cells. Tween 80 which was tested with L-cells acted exactly in the same way; its inhibitory action was again independent of cell numbers, provided suspension cultures were used.

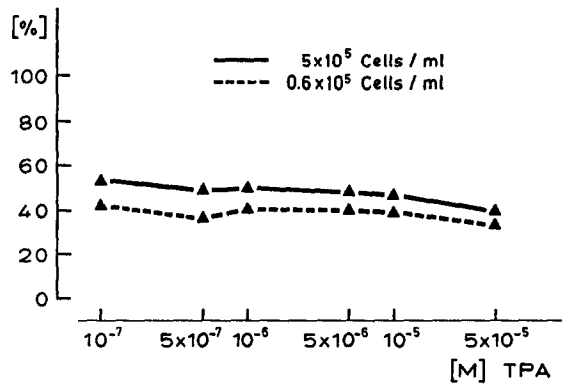

Fig. 3a

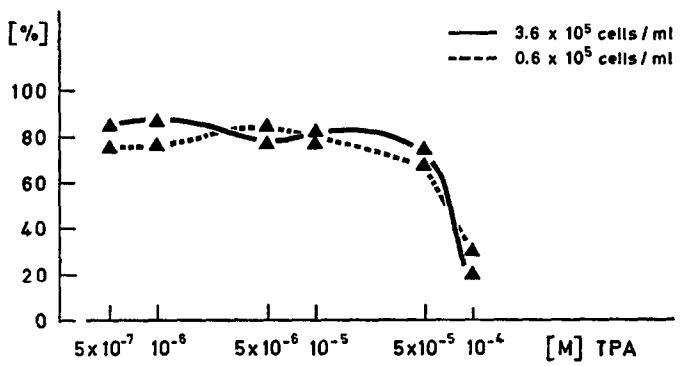

Fig. 3b

FIG. 3. Inhibition of ^{3}H-Tdr incorporation (by different concentrations of phorbol ester TPA) into HeLa (a) and L-cell (b) *suspension* cultures of different densities expressed as per cent incorporation of the controls incubated with 0·5% DMSO only.

Cell number dependent inhibition of Tdr incorporation thus is not a general phenomenon. This is further illustrated by experiments using hydroxyurea, which blocks DNA synthesis. In this case L-cells even in monolayers were affected completely independently of their actual cell density as shown in Fig. 4.

The fact that cells in suspension are influenced independently of their cell concentration argues against a high binding capacity of cells for TPA. This was shown directly by studying the binding of labelled TPA to HeLa cells (Table 1). The results demonstrate that only very little of the ^{3}H-TPA is bound to cells indicating that a

lack of TPA in the culture medium can not be the reason for the cell number dependent reaction of monolayer cells to TPA. The monolayer type of culture itself must be the basis for the phenomenon. Morphological studies support this view (Fig. 5a–c). A culture of HeLa cells was incubated for 6 hr with a high concentration of TPA (10^{-4} M), pictures were then taken of different parts of the culture showing different densities. Single cells or cells situated at the periphery of cell clusters were much more sensitive to the lytic action of TPA than cells surrounded completely by neighbours. Comparable results were obtained using Triton X-100 and Tween 80. A comparison of the monolayer and suspension experiments also gives evidence that suspension cells are in general less sensitive to the lytic action of the tested compounds. Whereas 5×10^{-5} M TPA for instance inhibits the Tdr incorporation into L-cell monolayers almost completely (Fig. 1b), the same concentration has only little effect if used in the suspension culture (Fig. 3b).

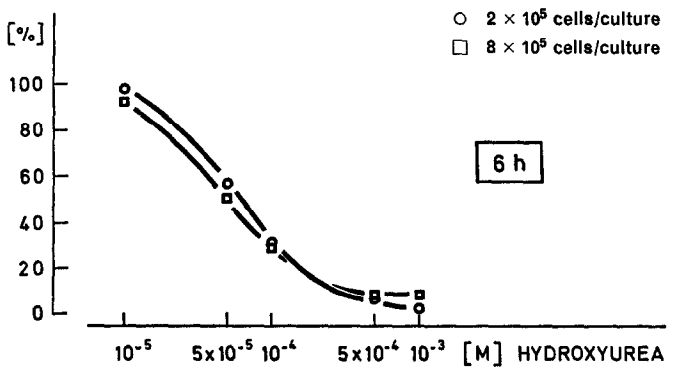

FIG. 4. Inhibition of ^{3}H-Tdr incorporation into L-cell monolayers by different concentrations of hydroxyurea expressed as per cent incorporation of the controls incubated with the medium only.

DISCUSSION

Why is a dense monolayer cell culture less sensitive to the lytic action of TPA, Triton X-100 and Tween 80 than monolayers with lower cell densities? That is, why is a cell less inhibited if it has neighbours? or more precisely, why are cells more easily lysed if they are grown in isolation? A stoichiometric relation between active compound and cells exposed might be the explanation.

The more cells present, the more substance is needed to reach a critical concentration within a cell which is necessary for lysis. If this is true, the same effect should be achieved: (a) by lowering cell numbers or (b) by increasing the concentration of the active compound.

It was shown that, given the right concentration range, such a relation really exists. Doubling the TPA concentrations has exactly the same effect as halving the numbers of the cells, suggesting a stoichiometric relation between TPA and the cells, which is critical for cell lysis.

If cells compete for TPA, one would expect that TPA would more or less completely disappear from the supernatant. However, experiments with ^{3}H-labelled TPA revealed that this is not the case.

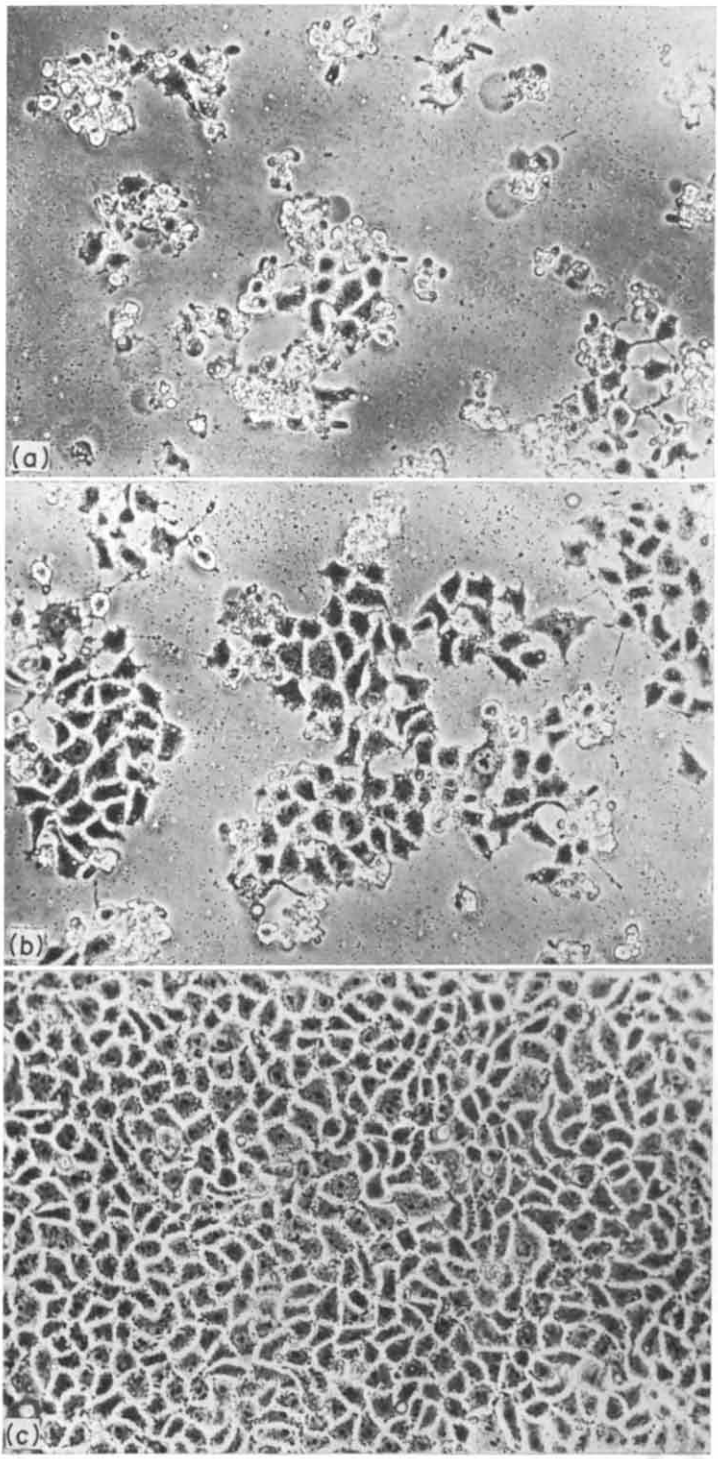

FIG. 5(a–c). HeLa cells from the same culture showing different densities (a–c) treated for 6 hr with 10^{-4} M TPA. (Magnification $\frac{1}{2} \times 180$).

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Suspension cultures are affected by TPA independently of their cell number which also makes a stoichiometric relation between TPA and the cells unlikely.

This leads to the conclusion, that the special conditions of monolayer culturing are responsible for the cell number dependent sensitivity of the cells against the croton oil factor TPA or the surfactants studied.

In contrast, hydroxyurea inhibited incorporation of thymidine into L-cells monolayers completely independently of the cell densities. This may suggest that surface active substances, applied in lytic concentrations, act in a cell number dependent fashion in monolayer cultures.

Morphological studies yielded detailed information about the cause. Cells situated at the periphery of cell clusters started to lyse first after the treatment. Surrounding cells possibly stabilize the single cell within a cluster.Thus the lower the total cell number, the more cells lie at the periphery or are isolated and the more cells are optimally exposed to the action of TPA, Tween 80 or Triton X-100. Very similar observations had been made using Walker cells in culture treated with lytic concentrations of antibodies. Here again isolated and "outside" cells were lysed first.*

In all these instances membranes had been attacked:

(a) TPA appears to be membrane bound.[5,11] Choline incorporation studies hinted at membranes in disorder after TPA application *in vivo* and *in vitro*.[6,10,12,13]
(b) Tween 80 and Triton X-100, established surfactants, may well lead to primary membrane damage and finally
(c) interactions between lytic antibodies and cells are supposed to occur at the outer cell membrane as well.

Hydroxyurea on the other hand, the effect of which is not modified by cell numbers, is known to inhibit an enzymatic step in DNA biosynthesis.[14]

In conclusion the effects of membrane attacking drugs on monolayer cultures are modified by cell numbers.

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