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Received December 12, 1966. P.S.E.B.M., 1967, v124.

Cytotoxicity of Dichlorodiphenylacetic Acid (DDA) Upon Cultured KB and HeLa Cells, and its Reversal by Mevalonic Acid. (31909)

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DDA [2,2-bis (p-chlorophenyl) acetic acid], constantly present in the tissues of most humans and other mammals, is the principal urinary metabolite of DDT [1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane](1). Structurally, it falls into the pattern of a large series of substituted arylacetic acids which have been shown to inhibit the incorporation of acetate and mevalonate into cholesterol(2). A typical example is diphenylylethylacetic acid, which has been extensively studied in this regard(3,4). DDA, like diphenylylethylacetic acid, has been shown to inhibit the acetylation of choline(5) and sulfanilamide(6) by interfering with the formation of acetyl-coenzyme A. The present studies are concerned with the cytotoxicity of DDA, and its reversal by mevalonic acid, in cultured KB and HeLa cells.

Materials and methods. Stock cultures of KB and HeLa cells were grown as monolayers in Roux and Brockway bottles in Mixture 199(7) as modified by Salk *et al*(8), supplemented by 5% calf serum. For rapid growth the medium was supplemented with 10% serum, and for maintenance the amount of calf serum was reduced to 3%. A cell suspension was obtained from a 5 day culture by trypsinizing (0.05-0.1% trypsin) the monolayer from the glass surface and dispersing the cells with gentle, repeated pipetting. Cell populations were determined by counting in Neubauer hemocytometer chambers.

Cytotoxic effects produced by the test compounds were graded according to the rating scale described by Toplin(9) in which the cytotoxic end-point (CE) is that concentration of test substance causing significant cellular degeneration, and the lethal end-point (L.E.), that concentration causing complete disintegration.

The DDA used in this work was prepared

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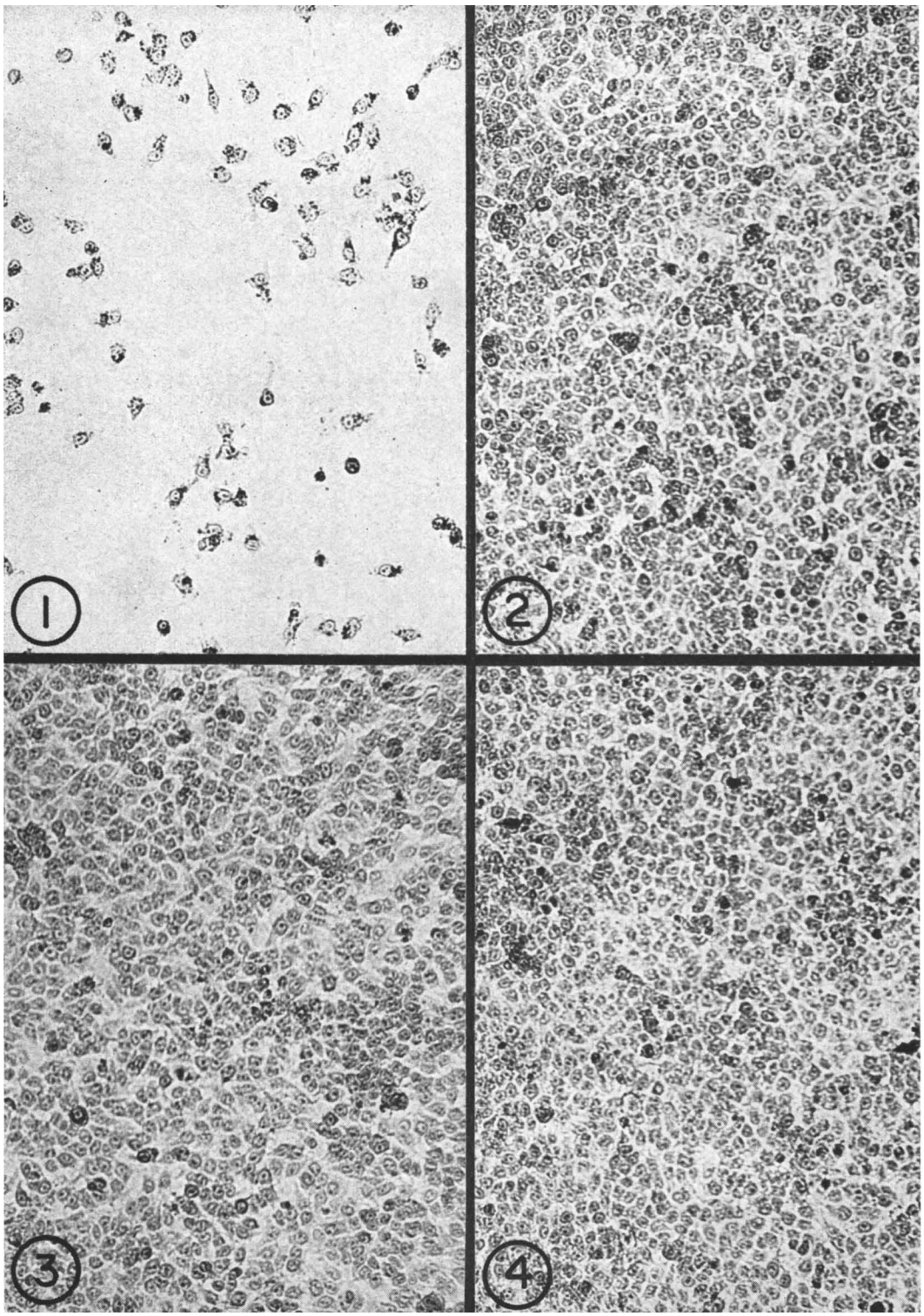
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Fig. 1.-4. Photomicrographs of KB cell monolayer showing cytotoxicity of DDA and reversal by mevalonic acid. (1) Effect of DDA (50 μg/ml); (2) KB control; (3 effect of DDA (50 μg/ml + mevalonic acid (25 μg/ml); (4) mevalonic acid (25 μg/ml) control. (X 175).

from DDT in this laboratory. Coenzyme Q_9 (ubiquinone-45) was a gift from Dr. W. E. Phillips, Food and Drug Research Laboratories. DL-Mevalonic Acid Lactone was purchased from Nutritional Biochemicals Corp. and used as such. Other materials employed were obtained from commercial sources.

Culture methods. Thirty ml disposable plastic tissue culture flasks (Falcon Plastics) and 18 mm pyrex test tubes were used as experimental vessels. Each vessel was inoculated with 5×10^5 KB or HeLa cells in a total volume of 5 ml of growth medium containing 5% calf serum. To induce monolayer formation the cultures were incubated for 24 hours at 36°C, following which the media was replaced by new media containing graded amounts of the test compounds. All tests were run in triplicate, and separate control vessels were set up for each run. All tests were run several times in both flasks and test tubes. The morphological changes in the monolayer of each culture flask as compared with the controls were recorded daily for 5 days. After the final microscopic examination for cytotoxic effect the cells were stained with 0.1% crystal violet solution in 0.1 M citric acid.

Results. DDA produced a significant cytotoxic effect at 10 μg/ml and lethal effect at 50 μg/ml upon both KB and HeLa cells. The ID_{50} in each case was 25 μg/ml. DDA at 50 μg/ml caused complete disruption of the established KB cell monolayer in 4 days (Fig. 1) as compared with the 5-day control (Fig. 2) in which no degeneration occurred until the 7th day. In the presence of 25 μg/ml of mevalonic acid no appreciable cytotoxic effect of DDA (50 μg/ml) was observed even after 5 days (Fig. 3). The same concentration of mevalonic acid gave approximately 50% protection against 100 μg/ml of DDA. Mevalonic acid itself at 25 μg/ml had no apparent effect on the KB cell monolayer (Fig. 4), its CE rating being 100 μg/ml. No reversal of the DDA effect was observed with either acetate (50 μg/ml) or cholesterol (10 μg/ml). The latter was additional to the cholesterol already present in the medium(7) and serum (13). Higher concentrations of cholesterol were toxic to the cells, which is in agreement with the effect of cholesterol on heart cells as noted by Pomerat and Leake(10).

Since mevalonic acid is an intermediate in the biosynthesis of the isoprenoid side chain of coenzyme Q (ubiquinone)(11), the possibility was considered that DDA toxicity might ultimately be due to suppression of coenzyme Q synthesis. However, no protective effect against DDA (50 μg/ml) was observed with a non-toxic concentration of coenzyme Q (50 μg/ml) in either KB or HeLa cells. It should be noted that most of the coenzyme Q was present as a suspension, and the extent of cellular absorption, if any, is not known.

Discussion. Mevalonic acid is a common intermediate in the biosynthesis of cholesterol and of the isoprenoid side chain of coenzyme Q(11). Thus, Shunk *et al*(12) observed that the specific activity of the coenzyme Q fraction of the liver, following the administration of 2-^{14}C-mevalonate to rats, was of the same order as that of the cholesterol fraction. In tissue culture, conversion of mevalonate to cholesterol has been demonstrated(13), but to our knowledge, not to coenzyme Q. However, the incorporation of radioactivity from mevalonic acid-2-^{14}C into coenzyme Q_9 has been shown to occur in rat liver slices(14) and homogenates(15,16). It is not surprising that addition of extra cholesterol to the growth medium failed to reverse the cytotoxic effect of DDA since mammalian cells in tissue culture take up considerable quantities of cholesterol from the serum present in the growth medium(17), and consequently are not dependent upon cellular synthetic processes to meet requirements of cholesterol. Thus it was considered highly unlikely that cholesterol synthesis was involved in the protective effect of mevalonic acid. However, the situation with regard to coenzyme Q is quite different, and there was a distinct possibility that DDA might be blocking coenzyme Q synthesis by inhibiting the formation of mevalonic acid. If the rate of biosynthesis of coenzyme Q is very slow, then one could

speculate that in a rapidly proliferating tissue there would be a low concentration of coenzyme Q. In this connection, it has been shown that regenerating liver and Novikoff hepatoma are low in reductase activity due to a lack of available coenzyme Q, and that marked enhancement of activity is produced by addition of coenzyme Q(18). The fact that, in our hands, addition of coenzyme Q to the culture medium failed to protect against DDA toxicity does not disprove coenzyme Q involvement, since the cells may not have absorbed the added coenzyme. It is worth noting that the inhibition of respiration which accompanies the growth inhibition of *Aspergillus niger* by 4-chloro-2-methylphenoxyacetic acid, structurally related to DDA, is reversed by addition of coenzyme Q(19).

The fact that mevalonic acid, but not acetate, reverses the cytotoxic effect of DDA suggests that DDA exerts its effect prior to the mevalonic acid stage on the sequence of reactions leading to sterol or coenzyme Q synthesis. Various studies(2,20) have shown that substituted arylacetic acids, including DDA(5,6), inhibit the reaction by which free acetate is activated to acetyl-coenzyme A. However, acetyl-coenzyme A can be generated at normal rates from other metabolic precursors, such as glucose and fatty acids, and inhibitors of acetate activation need not have any effect on mevalonic acid synthesis from these precursors. They could conceivably influence mevalonic acid formation by inhibiting any one of the conezyme A mediated reactions between acetyl-coenzyme A and mevalonic acid.

That DDA might be capable of influencing coenzyme Q levels in KB and HeLa cells, and in other rapidly proliferating tissues, is an intriguing possibility worthy of further investigation.

Summary. Dichlorodiphenylacetic acid (DDA) (50 μg/ml) completely disrupted the growth of cultured KB and HeLa cells. Mevalonic acid (25 μg/ml) provided essentially complete protection against the cytoxicity of 50 μg/ml of DDA. Neither acetate nor cholesterol afforded protection against the DDA effect.

We wish to thank Mr. Benjamin Korda for photomicrographs.

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Received December 15, 1966. P.S.E.B.M., 1967, v124.