# Diphenylhydantoin-Induced Inhibition of Nucleic Acid Synthesis in Cultured Human Lymphocytes (36722)

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(Introduced by C. M. Kunin)

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Diphenylhydantoin (Dilantin, DPH) therapy is associated with a variety of idiosyncratic reactions which include lymph node hyperplasias (1) and frank lymphomas (2). DPH has been demonstrated to stimulate proliferation of lymphocytes from a sensitized patient *in vitro* (3). In our study of 8 patients with major idiosyncratic reactions to DPH, however, stimulation of lymphocyte DNA synthesis was a rare and inconstant finding (4). The more significant and reproducible response of lymphocytes was inhibition of spontaneous DNA synthesis. This observation led to the present study in which we have studied dose–response curves of effect on DNA, RNA and protein synthesis, and the sequential changes of DNA synthesis over 24 hr exposure to DPH. The phytohemagglutinin-stimulated peripheral blood culture was used as a model of proliferating lymphocytes.

*Materials and Methods.* Heparinized normal blood was sedimented in 1% dextran (Abbott) and the supernatant plasma and leukocytes were mixed with culture medium 199 and phytohemagglutinin P (PHA) to make 20% plasma, 80% 199, 10 μg PHA/ml, 1000–2000 cells/mm$^3$. Pure lymphocyte cultures prepared by the fractionation technique of Rabinowitz (5) contained 400–500 lymphocytes/mm$^3$. Cultures were incubated at 37°.

DPH was dissolved in 50% ethanol, 40% propylene glycol–10% ethanol adjusted to pH 12, or water at pH 12, then diluted with deionized water to appropriate concentrations. The pH of cultures did not change when the drug was added. The final concentration of ethanol or propylene glycol (v/v) did not exceed 1% in any experiment. Ethanol concentrations of 0.2% or less were used for RNA synthesis experiments (6). Pairs of diluent controls were used in every experiment. Pure lymphocyte cultures contained ethanol-free DPH dissolved at pH 12 and diluted with deionized water. The changes observed were indistinguishable from those of mixed leukocyte cultures and the data were pooled for analysis.

DPH remained stable in culture medium at 37° as well as in various solvents at 4° as determined by gas chromatography (7).

The following isotopes were added to duplicate cultures at 1 μCi/ml; thymidine methyl-$^3$H, 0.12 mg/mCi; uridine-U-$^3$H, 0.0296 mg/mCi; leucine-4,5-$^3$H, 0.00226 mg/mCi. At the time of incubation with tritiated leucine tissue culture medium 199 was replaced by 199 without leucine to prevent loss of specific activity.

*Dose–response curves.* DPH was added to beginning cultures ($T_0$) in concentrations of 0.25 to 200 μg/ml. Radioactive precursors of DNA, RNA or protein were added at 72 hr. After incubation for 2 hr, the cells were killed. The incorporation of radioactivity into DNA, RNA and protein was measured and reported as percentage of diluent control.

*Time curve of inhibition.* DPH in the therapeutic range (10–25 μg/ml) was added to proliferating cell cultures at 72 hr. Tritiated thymidine was added at different time points subsequently and allowed to incubate for 2 hr before terminating the cultures. Paired control cultures containing diluent were studied at each time interval.

Radioactive extractions were prepared for liquid scintillation counting by centrifuging

the cultures at 200*g* for 20 min, washing the cell buttons twice at 0.15 *M* sodium chloride, and extracting as follows:

*DNA.* The cells were killed with ice-cold 10% trichloroacetic acid (TCA) and washed 3× with 5–10% TCA. The precipitate was dissolved in 1 ml Soluene (Packard), and added to 12 ml Bray's solution (8).

*RNA.* The cells were fixed with acetic acid methanol 1:3 and extracted by the perchloric acid (PCA) method of Feinendegen, Bond and Painter (9). Appropriate studies of the partition of radioisotopes into DNA and RNA were performed by this method.

Protein was extracted by the method of Yunis and co-workers (10).

The samples were counted in a Packard 3003 liquid scintillation counter and cpm converted to dpm by external standardization. Duplicate samples were used in all studies. Data were rejected from analysis if the duplicates differed from the average of the duplicates by more than 15%.

*Studies of cell viability.* Dye exclusion tests were performed by mixing 0.3 ml of 1% trypan blue or 0.4% erythrosin-B in phosphate-buffered 0.15 *M* sodium chloride with 1 ml of suspended cell culture and counting 100–200 cells after 3–15 min. Cell counts were performed on the Coulter counter Model F. DPH (10 or 100 μg/ml) was added to cultures with or without PHA. Cultures without PHA were studied daily by dye exclusion and leukocyte counting. Cultures containing PHA were examined by dye exclusion only.

Data were analyzed by Student's *t* test for paired samples.

*Results.* Dose response plots of thymidine incorporation into DNA showed variable effect at 0.25 to 2.5 μg DPH/ml. There was 50% inhibition at 10–100 μg DPH/ml culture (Table I). Leucine and uridine incorporation were less sensitive to DPH and no effect could be demonstrated in the therapeutic range (10–20 μg/ml).

Time of inhibition studies showed that thymidine incorporation was significantly depressed at 4 hr with further effect at 12 and 24 hr (Table II).

The possibility that DNA synthesis was not inhibited, but that thymidine was being diverted to other pathways was investigated. Sixty-five percent of the dpm in TCA precipitates was accounted for as DNA by PCA extraction. There was no difference in extraction efficiency between control and DPH incubated cultures, and no discernible change in results over 24 hr incubation with DPH.

TABLE I. Dose–Response Determinations: Thymidine, Uridine and Leucine Incorporation into DNA, RNA and Protein in Cultures Exposed to DPH for 72 hr (% of control).[a]

| | DNA | | | RNA | | | Protein | | |
|---|---|---|---|---|---|---|---|---|---|
| DPH (μg/ml culture) | Mean | SE[b] | *N*[c] | Mean | SE | *N* | Mean | SE | *N* |
| 0.25 | 102 | 13 | 6 | 77 | 11 | 4 | 83 | 9 | 4 |
| 1.0 | 81 | 11 | 6 | 95 | 24 | 5 | 91 | 4 | 4 |
| 2.5 | 83 | 11 | 6 | 82 | 2 | 3 | 94 | 7 | 3 |
| 5.0 | 86 | 5 | 6 | 89 | 20 | 4 | 87 | 11 | 3 |
| 10 | 50[d] | 4 | 5 | 70 | 8 | 4 | 95 | 8 | 3 |
| 20 | 48[d] | 4 | 6 | 68 | 13 | 6 | 100 | 4 | 4 |
| 100 | 53[f] | 6 | 3 | 55 | 15 | 5 | 55 | 32 | 3 |
| 200 | 20[f] | 3 | 3 | 9[f] | 1 | 3 | 32[e] | 8 | 3 |

[a] DPH was added to beginning phytohemagglutinin-stimulated lymphocyte cultures. Radioisotopes were added at 72 hr; after 2 hr incubation, the cells were extracted for liquid scintillation counting. Incorporated radioactivity is reported as percentage of diluent control.

[b] Standard error of the mean.

[c] $N$ = number of experiments.

[d] Differs from control, $p = < 0.025$; differs from 5 μg, $p = < 0.05$.

[e] Differs from control, $p = < 0.01$; [f] $p = < 0.005$.

TABLE II. Changes in Incorporation of Tritiated Thymidine into DNA During Prolonged Exposure to DPH (% of control).[a]

| | DNA | | |
|---|---|---|---|
| Exposure to drug (hr) | Mean | SE[b] | $N$[c] |
| 2 | 85 | 8.0 | 8 |
| 4 | 84[d] | 2.4 | 5 |
| 6 | 77 | 4.1 | 5 |
| 8 | 82 | 7.7 | 6 |
| 10 | 84 | 6.2 | 3 |
| 12 | 67[d] | 8.5 | 3 |
| 24 | 73[d] | 9.0 | 5 |

[a] DPH 10–25 μg/ml was added to established lymphocyte cultures at 72 hr (hour 0), and tritiated thymidine was added for 2 hr prior to the times indicated. Percentage of control is ratio of dpm in Dilantin-treated cultures to diluent-containing cultures removed at the same time.

[b] Standard error of the mean.

[c] $N$ = number of experiments.

[d] $p = < 0.05$.

DPH caused a loss of 15% of cells on the third day of incubation at 100 μg (Table III).

*Discussion.* We have observed dose and time dependent inhibition of incorporation of thymidine into proliferating lymphocytes at concentrations of drug found in patients receiving DPH for treatment of epilepsy. Uridine and leucine incorporation were inhibited at higher concentrations. Partitioning of thymidine radioactivity showed that 65% of the incorporated thymidine was in DNA in control cultures as well as drug treated cultures. This suggested that inhibition of thymidine incorporation was not due to shunting of the radioisotope to other pathways.

In order to show that the effect of the drug was selective and not due to nonspecific toxicity, we studied its effect on cell numbers and vital dye incorporation. These studies indicated that cell death was not a sufficient explanation for drug effect. Selectivity of the drug effect was further shown by the lesser degree of inhibition of leucine and uridine incorporation compared to thymidine.

Previous studies support our observations. Trowell (11) showed that DPH at a concentration of 10–20 μg/ml caused pyknosis of rat lymph node lymphocytes. McIntyre and Ebaugh (12) showed data suggesting that DPH inhibited DNA synthesis in their origi-

TABLE III. Cell Number and Vital Dye Staining During Prolonged Exposure to DPH (% of Control).[a]

| | Unstimulated | | | | | | Trypan blue PHA | | |
|---|---|---|---|---|---|---|---|---|---|
| | Trypan blue[b] | | | Cell counts | | | | | |
| Day of exposure | Mean | SE[c] | $N$ | Mean | SE | $N$ | Mean | SE | $N$ |
| 1 | 101 | 0.7 | 4 | 109 | 6.0 | 3 | 100 | 1.2 | 4 |
| 2 | 91 | 0.9 | 4 | 74 | 8.1 | 3 | 97 | 1.3 | 4 |
| 3 | 99 | 2.0 | 4 | 81 | 13 | 3 | 98 | 1.0 | 4 |
| 7 | 102 | 1.6 | 4 | 83 | 10 | 3 | | | |
| | | | | DPH 10 μg/ml | | | | | |
| Days | | | | | | | | | |
| 0 | 100 | 0.3 | 5 | 99 | 1.8 | 5 | 100 | — | — |
| 1 | 97 | 0.6 | 5 | 98 | 2.3 | 5 | 111 | 9.2 | 4 |
| 2 | 96 | 1.9 | 5 | 91 | 2.1 | 5 | 95 | 1.7 | 4 |
| 3 | 85 | 3.2 | 3 | 84 | 2.7 | 3 | 95 | 4.6 | 4 |
| | | | | DPH 100 μg/ml | | | | | |

[a] DPH 10 or 100 μg/ml was added to beginning cultures. Leukocyte counts and trypan blue dye studies were performed on unstimulated cultures, trypan blue studies on phytohemagglutin-stimulated cultures on days indicated.

[b] Trypan blue negative.

[c] Standard error of the mean.

nal description of PHA-stimulated peripheral blood lymphocytes. Tisman, Herbert and Brenner (13) have presented results similar to ours in abstract (13).

If DPH causes a metabolic disorder in proliferating lymphocytes, we would expect that lymphocyte numbers should be reduced in patients receiving the drug. Our studies of patients receiving DPH for treatment of epilepsy (4) showed that there was a significant negative correlation between DPH blood levels and absolute lymphocyte count. Other blood cells did not show similar changes.

*Summary.* Diphenylhydantoin inhibited DNA synthesis of phytohemagglutinin-stimulated lymphocytes. The inhibition was dose and time dependent, and was significant in the therapeutic range (10–20 μg/ml). At higher concentrations RNA and protein synthesis were also inhibited.

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