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The Effect of Diphenylhydantoin and Cortisol on the Cell Cycle[1] (42724)

A. A. MACKINNEY, JR.,*†,[2] L. KNOBELOCH,* AND D. NORBACK‡

Hematology Laboratory, William S. Middleton Memorial Veterans Hospital, 2500 Overlook Terrace, Madison, Wisconsin 53705, and Departments of †Medicine and ‡Pathology, University of Wisconsin, 600 Highland Avenue, Madison, Wisconsin 53792

Abstract. Normal human lymphocytes cultured in the presence of phytohemagglutinin were blocked in G_0G_1 when diphenylhydantoin (DPH) or cortisol 3.6×10^{-4} *M* was added at the beginning of culture. The suppression of culture growth was analyzed by flow cytometry and confirmed by [^{3}H]thymidine incorporation and mitotic rate analysis. The correlation of these measurements with flow cytometry was good for DNA synthesis and excellent for mitosis. There was an additive effect on the G_0G_1 retention of cells when both drugs were present in the culture. These data may partially explain the suppression of cell-mediated immunity which occurs in DPH-treated patients.

Diphenylhydantoin (DPH) is a valuable anticonvulsant with an immunosuppressive potential which has been extensively studied *in vitro* and *in vivo*. It suppresses delayed hypersensitivity (1) and decreases immunoglobulin concentration (2) and lymphocyte numbers in circulation (3). It is associated with an increased risk of lymphoma (4–7). DPH suppresses DNA synthesis of phytohemagglutinin (PHA)-stimulated lymphocytes *in vitro* (8, 9).

Recent work has demonstrated biologic similarities between DPH and glucocorticoids. Both drugs cause cleft palate in mice. Studies of cleft palate formation suggest that an inactive glucocorticoid may block the DPH effect (10). We have shown that DPH lyses rat thymocytes *in vitro* and inhibits cortisol-induced lysis (11). Since it is now known that glucocorticoids also block malignant lymphocytes in G_0G_1 (12), we set out to determine if DPH has a similar effect on cultures of human lymphocytes. We used flow cytometry, thymidine incorporation, and metaphase arrest to compare DPH and glucocorticoid activities.

Materials and Methods. Six samples of human peripheral blood from normal volunteers were anticoagulated with heparin, sedimented in 1% dextran (Abbott Laboratories, North Chicago, IL), and fractionated on Ficoll–Hypaque gradients. The mononuclear cells (1×10^6/ml) were incubated with a mixture of 80% RPMI 1640 containing 25 m*M* Hepes buffer and supplemented with L-glutamine, 20% autologous plasma, and 1% PHA. Cultures were incubated at 37°C for 3 days. DPH in bicarbonate buffer, pH 11, or cortisol in phosphate-buffered saline, 3.6×10^{-4} *M*, was added at the initiation of cultures using appropriate diluent controls. Colchicine, 10 μg/ml, was added for the final 18 hr. [^{3}H]Thymidine (NEN: 20 Ci/mmole; 2 μCi/ml) was added for the final 2 hr to separate pairs of cultures.

Cells for flow cytometry were washed three times in 70% ethanol and refrigerated until ready for use. Cells were then pelletted and 2 ml of mithramycin 100 μg/ml in 0.9% sodium chloride was added for 20 min. Cells were filtered through a 20-μm mesh and counted in an Ortho Cytofluorograf (System 50H with 2140 computer) using a 5-W watercooled argon-ion laser (window 250–300 mW; WL 457 n*M*; 70-μm orifice). Seven thousand cells were counted and doublets were excluded by setting the appropriate gate on the area versus peak green fluorescence plot. Cells in regions G_0G_1, S, and G_2M were quantitated by setting regions on the green fluorescence histogram, based on the width of the G_0G_1 and G_2 peaks at half-height.

Colchicine-treated cells were incubated

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[2] To whom all correspondence and reprint requests should be addressed at Hematology Laboratory, William S. Middleton Memorial VA Hospital, 2500 Overlook Terrace, Madison, WI 53705.

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with hypotonic KCl, fixed in acetic acid:methanol (1:3), and blown on glass slides. One thousand cells were counted to determine the mitotic index. [^{3}H]-Thymidine-treated cultures were washed with phosphate-buffered saline, precipitated on glass filters with 5% trichloroacetic acid (TCA), and counted by liquid scintillation spectroscopy.

Data were analyzed by t test for paired samples.

Results. The effect of cortisol or DPH (3.6×10^{-4} M) on cultured human lymphocytes was similar (Table I); both drugs suppressed [^{3}H]thymidine incorporation and mitosis, increased the fraction of cells in G_1, and decreased the fraction of cells in S and G_2M. The only significant difference between the two drugs was the greater suppression of thymidine incorporation by cortisol ($P < 0.05$). When the drugs were present together for the duration of culture, the effect on thymidine incorporation and G_0G_1 was enhanced.

Flow cytometry estimates of S phase were well correlated with thymidine uptake in paired cultures ($r = 0.77$) (Fig. 1). There was even better correlation between cell cytometry estimates of G_2M and the mitotic index using colchicine ($r = 0.95$) (Fig. 2). This correlation included both control and test samples.

As a positive control, we studied the effects of colchicine on flow cytometry estimates of the cell cycle. Colchicine (10 μg/ml for terminal 18 hr of culture) decreased cells in G_0G_1 and S, and tripled the fraction in G_2M (Table I).

Discussion. Our study demonstrated that PHA-stimulated human lymphocytes, like neoplastic cells, are arrested in G_0G_1 by cortisol, and that DPH has the same effect. This is the second cortisol-like action of DPH which we have described (thymocyte lysis was the first (11)) and the fourth analogous action reported (10, 13). We have previously noted (11) that although DPH and cortisol lyse rat thymocytes, they have no similar effect on PHA-stimulated normal human peripheral blood lymphocytes at drug concentrations up to 3.6×10^{-4} M. Consequently, the cell cycle data in this report cannot be interpreted as due to cell lysis.

TABLE I. EFFECTS OF PHENYTOIN, CORTISOL, AND COLCHICINE ON CELL CYCLE

	[^{3}H]Thymidine $\times 10^3$DPM		Mitosis/1000		G_1 (%)		S (%)		G_2M (%)	
	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
Control	222.5	118	245	108	55.7	5.5	28.7	6.1	15.7	1.5
Phenytoin[a]	131.3	92	119	46	73.3	12.0	18.0	9.0	8.7	3.4
Cortisol[a]	39.7	27	88	67	81.8	8.8	12.4	5.5	5.6	3.6
Phenytoin + cortisol[a]	9.3	12.5	17	7.5	88.2	7.9	7.8	6.6	4.0	1.5
Colchicine[b]					25.8	7.7	19.7	2.8	52.7	4.7
	t	P	t	P	t	P	t	P	t	P
Phenytoin vs control	4.07	<0.01	9.06	<0.001	6.08	<0.005	6.92	<0.001	4.87	<0.005
Cortisol vs control	4.08	<0.01	7.65	<0.001	12.5	<0.001	8.9	<0.001	5.0	<0.005
Phenytoin vs cortisol	3.01	<0.05	1.99	NS	2.3	NS	1.95	NS	2.2	NS
Phenytoin + cortisol vs cortisol	4.81	<0.005	2.52	NS	2.60	<0.05	2.37	NS	1.89	NS

Note. $n = 6$.
[a] 3.6×10^{-4} $M \times 72$ hr.
[b] Colchicine 10 μg/ml $\times$ 18 hr.

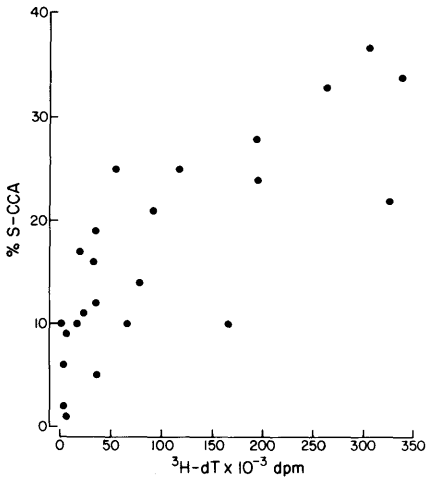

FIG. 1. Correlation of fraction of cells in S phase of cell cycle by flow cytometry (mithramycin label) with [^{3}H]-thymidine incorporation by scintillation spectroscopy. r = 0.77.

Our study extends previous work (3) which demonstrated that DPH decreases DNA synthesis in stimulated human lymphocytes. The current study shows that there is less DNA synthesis because fewer cells move from G_0G_1 to S phase. The action of DPH on DNA synthesis appears complex. We had previously found that DPH inhibits not only uptake of thymidine, but also uptake of uridine, orotic acid, leucine, deoxyglucose, phosphate, and acetate. DPH also partially blocked sodium–potassium-dependent ATPase, suggesting a membrane effect (14). Transport studies, however, did not conclusively demonstrate competitive inhibition of thymidine by DPH. However, the kinetics of 60-sec soluble and insoluble uptake of thymidine appeared to be different, favoring inhibition of a separate membrane transport mechanism (unpublished).

We had previously found that DPH arrests lymphocytes in mitosis (15). In the present study, G_2M and mitoses/1000 cells were not increased in the presence of DPH because the drug was added at the beginning of culture. Under this experimental condition, the suppressive effect on the movement of cells through the cycle overrode the metaphase-arresting property. DPH, like colchicine, must be added near the end of the culture to assay its mitosis-arresting properties.

Colchicine was the classic drug used in previous cell cycle studies, and we confirmed that in this culture system, colchicine, added at the end of the culture period, increased cells in G_2M and diminished the pool of cells in G_1 and S phase (as expected for a metaphase-arresting drug), in agreement with the work of Harmon and co-workers (12).

It has been known for years that cortisol can, under certain conditions, depress lymphocyte proliferation (16, 17). In 1977, Smith and co-workers reported that PHA stimulates lymphocyte glucocorticoid receptor production and cell division, but that glucocorticoid suppression of growth is not dependent on the number of receptors (18). Harmon and co-workers found that dexamethasone arrests a lymphoid cell line in G_0G_1 (12). In the same year, Gillis *et al.* discovered that dexamethasone also blocks production of T-cell growth factor (TCGF) (19). However, dexamethasone was ineffective if the culture had been previously exposed to TCGF (20, 21). Subsequently, Bowen and Fauci (22) reported that hydrocortisone blocks B lymphocytes in G_0 based on inhibition of early activation markers. It is attractive to speculate that glucocorticoids and DPH block cells in G_0 by inhibiting the release of TCGF.

In our study, simultaneous addition of DPH and cortisol augmented the block in

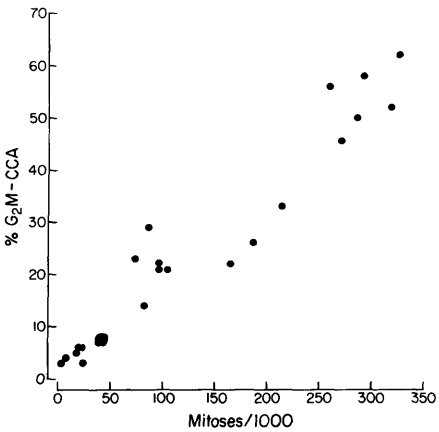

FIG. 2. Correlation of fraction of cells in G_2M phase of cell cycle by flow cytometry with mitoses estimated by manual counting of 1000 cells after 18 hr exposure to colchicine. r = 0.95.

[^{3}H]thymidine uptake and the retention of cells in G_0G_1. We previously reported that DPH augments the suppression of thymidine incorporation by cortisol in rat thymocytes. Our present study amplifies this observation: DNA synthesis is inhibited by arresting cells in G_0G_1, with both drugs acting separately or in concert. Since both compounds are present in DPH-treated patients, this additive G_0G_1 block may partially explain the immune impairment described in these individuals.

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1. Sorrell TC, Forbes IJ. Depression of immune competence by phenytoin and carbamazepine. Clin Exp Immunol **20**:273–285, 1975.
2. Grob PJ, Herold GE. Immunological abnormalities and hydantoins. Brit Med J **1**:561, 1972.
3. MacKinney AA Jr, Booker HE. Diphenylhydantoin effects on human lymphocytes in vitro and in vivo. Arch Int Med **129**:988–992, 1972.
4. Saltzstein SL, Ackerman LV. Lymphadenopathy induced by anticonvulsant drugs and mimicking clinically and pathologically malignant lymphomas. Cancer **12**:164–182, 1959.
5. Hyman GA, Sommers SC. Development of Hodgkin's disease and lymphoma during anticonvulsant therapy. Blood **28**:416–427, 1966.
6. Anthony JJ. Malignant lymphoma associated with hydantoin drugs. Arch Neurol **22**:450–454, 1970.
7. Li FP, Willard DR, Goodman R, Vawter G. Malignant lymphoma after diphenylhydantoin (Dilantin) therapy. Cancer **36**:1359–1362, 1975.
8. MacKinney AA Jr, Vyas R. Diphenylhydantoin-induced inhibition of nucleic acid synthesis in cultured human lymphocytes. Proc Soc Exp Biol Med **141**:89–92, 1972.
9. Bluming A, Homer S, Khiroya R. Selective diphenylhydantoin-induced suppression of lymphocyte reactivity in vitro. J Lab Clin Med **88**:417–422, 1976.
10. Goldman AS, Baker MK, Piddington R, Herold R. Inhibition of programmed cell death in mouse embryo plate *in vitro* by cortisol and phenytoin: Receptor involvement and requirement of protein synthesis. Proc Soc Exp Biol Med **174**:239–243, 1983.
11. MacKinney AA Jr, Knobeloch L. Diphenylhydantoin inhibits cortisol-induced lysis of thymocytes. Proc Soc Exp Biol Med **182**:474–477, 1986.
12. Harmon JM, Norman MR, Fowlkes BJ, Thompson EB. Dexamethasone induces irreversible G_1 arrest and death of a human lymphoid cell line. J Cell Physiol **98**:267–278, 1979.
13. Katsumata M, Gupta C, Baker MK, Sussdorf CE, Goldman AS. Diphenylhydantoin: An alternative ligand of a glucocorticoid receptor affecting prostaglandin generation in A/J mice. Science **218**:1313–1315, 1982.
14. MacKinney AA Jr, Vyas R. The assay of diphenylhydantoin effects on growing human lymphocytes. J Pharmacol Exp Ther **186**:37–43, 1973.
15. MacKinney AA Jr, Vyas R, Powers K. Morphologic effect of hydantoin drugs on mitosis and microtubules of cultured human lymphocytes. J Pharmacol Exp Ther **204**:195–202, 1978.
16. Ilfeld DN, Krakauer RS, Blaese RM. Suppression of the human autologous mixed lymphocyte reaction by physiologic concentrations of hydrocortisone. J Immunol **119**:428–434, 1977.
17. Heilman DH. Failure of hydrocortisone to inhibit blastogenesis by pokeweed mitogen in human leukocyte culture. Clin Exp Immunol **11**:393–403, 1972.
18. Smith KA, Crabtree GR, Kennedy SJ, Munck AU. Glucocorticoid receptors and glucocorticoid sensitivity of mitogen stimulated and unstimulated human lymphocytes. Nature (London) **267**:523–526, 1977.
19. Gillis S, Crabtree GR, Smith KA. Glucocorticoid-induced inhibition of T-cell growth factor production. J Immunol **123**:1624–1631, 1979.
20. Sloman JC, Bell PA. Cell cycle-specific effects of glucocorticoids in phytohaemagglutinin-stimulated lymphocytes. Clin Exp Immunol **39**:503–509, 1980.
21. Smith KA. T-cell growth factor and glucocorticoids: Opposing regulatory hormones in neoplastic T-cell growth. Immunobiology **161**:157–173, 1982.
22. Bowen DL, Fauci AS. Selective suppressive effects of glucocorticoids on the early events in the human B cell activation process. J Immunol **133**:1885–1890, 1984.

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