Effects of Nitrogen Mustard, Methotrexate and Hydrocortisone on Cell Replication in Rat Ileum and Spleen[1] (36476)

JOSEPH HOFFMAN AND JOSEPH POST

New York University Research Service, Goldwater Memorial Hospital, Welfare Island, New York 10017

The use of metabolic inhibitors as cancer chemotherapeutic agents and as immunosuppressants has been accompanied by cytotoxic effects upon replicating normal host cells of the hematopoietic and gastrointestinal systems (1–3). Previous studies in this laboratory with 5-iodo-2′-deoxyuridine (4) have demonstrated the perturbation of the cell kinetics of rat spleen and ileal crypt cells associated with reduced DNA synthesis. These changes have occurred in the absence of either cytoarchitectural organ changes, weight loss, diarrhea, anemia or leukopenia. The present experiments deal with the effects of sublethal doses of nitrogen mustard (HN_2), methotrexate (MTX) and hydrocortisone (HC) upon the cell replication kinetics of ileal crypt cells and spleen lymphocytes in the 3-week-old male rat. The clinical states, the respective organ histology and the hemograms were also followed.

Methods. Three-week-old male Wistar rats were caged individually and fed Purina checkers *ad libitum*. Methotrexate, HN_2 and HC were injected ip once daily for 5 days in the following doses: MTX, 0.5 mg/kg body weight; HN_2, 0.4 mg/kg body weight; and HC, 100 mg/kg body weight. Four hours after the last dose, tritiated thymidine (^{3}HTdR), 1.0 μCi/g body weight, was administered and the animals were sacrificed at frequent intervals thereafter. Autoradiographs of spleen and ileum were made using NTB-3 emulsion and storing the slides in the cold for 30 days. They were stained with Delafield's hematoxylin. Mitotic labeling, interphase labeling and mitotic index were determined. The animals were weighed daily and the percentages of body weight change were recorded at the end of the experiment. At the time of sacrifice complete blood counts were done using a Coulter counter, Model S. The generation time (T_c) was determined from the mitotic labeling curve by measuring the interval between the initiation of labeling in successive cycles. The DNA synthesis time (DNAS) was estimated as the interval between the ascending and descending limbs of the mitotic labeling curves at the 50% intercepts. Appropriate untreated age controls were also studied.

Results. All of the animals remain free of diarrhea and hemorrhagic phenomena. The controls increase their body weights by almost 25%. Those treated with HC have weight gains of about 22% but those which received HN_2 and MTX show only slight increments (Table I). Following HN_2 there is a sharp decrease in the white blood count (WBC) from a control value of 10,400 to 3400 cells/mm^3. Methotrexate produces a significant fall in the red blood count (RBC), hemoglobin (Hb) and hematocrit (HCT). Hydrocortisone does not alter the blood significantly. Histologically, the ileum is normal after HN_2 and HC; MTX produces flattening of the villi without necrosis. With HC there is vacuolization of the hepatocytes and loss of stainable RNA (5). After each of the drugs there is a reduction in the density of

[1] This work was supported by Grants No. 5 R01 CA 03917-11 from the United States Public Health Service, by the United States Atomic Energy Commission under contract AT(30-1)-2778 and by the National Aeronautics and Space Administration NGR 33-016-102. Presented in part at the 51st Annu. Session of the Amer. College of Physicians in Philadelphia, PA, Apr. 12–17, 1970.

TABLE I. Summary of Weight Changes, Blood Counts and Histology.

	No. of animals	Wt (% change)	Blood			HCT ± SD[a] (%)	Histology		
			WBC ± SD[a]	RBC ± SD[a]	Hb. ± SD[a] (g)		Spleen[b]	Ileum[c]	Liver[d]
Control	10	+ 24.6	10444 ± 2290	$7.12 \times 10^6 \pm 7.7 \times 10^5$	15.1 ± 1.4	43.4 ± 3.9	0	0	0
Nitrogen mustard	10	+ 2.6	3400 ± 1100	$7.03 \times 10^6 \pm 7.98 \times 10^5$	15.4 ± 1.4	42.2 ± 4.0	+	0	0
Methotrexate	10	+ 7.6	6250 ± 1800	$4.85 \times 10^6 \pm 5.28 \times 10^5$	11.1 ± 1.1	31.0 ± 3.5	+	+	0
Hydrocortisone	10	+ 21.5	7350 ± 1470	$6.31 \times 10^6 \pm 4.87 \times 10^5$	13.3 ± 1.1	38.9 ± 3.1	+	0	+

[a] SD = standard deviation; + = present; 0 = absent.
[b] Depletion of lymphocytes and sinusoidal congestion.
[c] Flattening of villi.
[d] Vacuolization of hepatocytes.

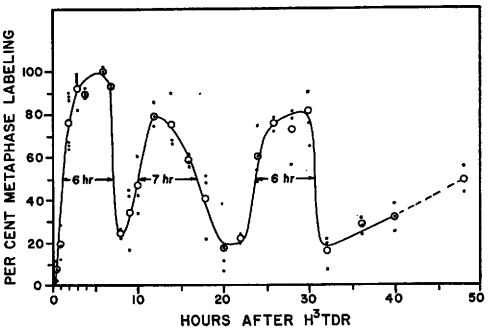

FIG. 1. Ileum, control. ^{3}HTdR, 1 μCi/g body weight. (•) individual rat. (○) mean for the group. These symbols are used in all the figures. [Reproduced from Post and Hoffman (17) with the permission of Academic Press].

spleen lymphocytes and a concomitant sinusoidal congestion with erythrocytes.

The T_c for ileum in the normal control rat is about 9 hr and the DNAS is about 6 hr (Fig. 1) (17). Three complete cycles are visualized and there is 100% metaphase labeling in the first cycle. Nitrogen mustard causes disruption of the cyclic pattern of ileal cell replication so that the T_c and DNAS cannot be determined (Fig. 2). Metaphase labeling never reaches 100%. The shaded areas in this figure and in the following ones represent the patterns of the untreated controls. Following MTX the changes in the mitotic labeling curve are similar to those after HN_2 (Fig. 3). Hydrocortisone produces a lengthening of the T_c in ileal crypt cells to about 11 hr but DNAS is about 7 hr (Fig.

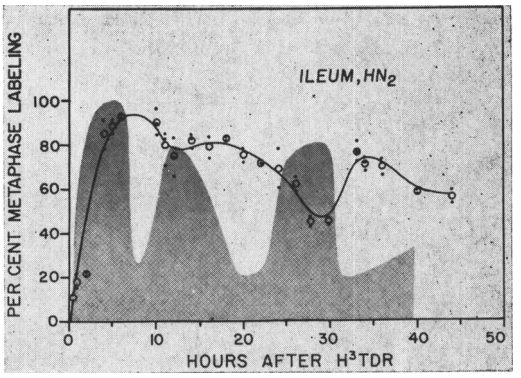

FIG. 2. Ileum, after HN_2. The shaded background is the control.

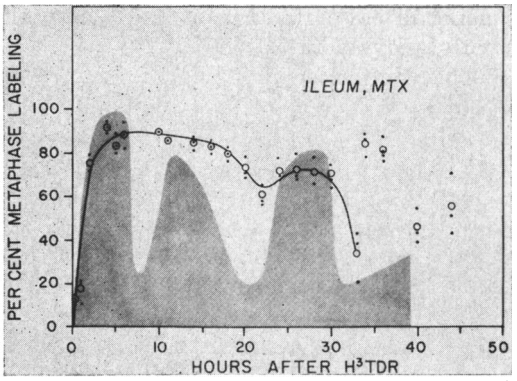

FIG. 3. Ileum, after MTX. The control rat is the shaded portion.

4). After the first cycle the curve loses its regular wave-like character.

The spleen lymphocytes of the 3-week-old control animal have a generation time of about 12 hr and the DNAS is about 8 hr (Fig. 5) (18). Following HN_2 the control pattern of the mitotic labeling curve is altered so that the T_c and the DNAS are not measurable (Fig. 6). Methotrexate and HC produce similar changes in the mitotic labeling curves (Figs. 7, 8).

The interphase labeling rates (LI) and the mitotic indices (MI) of spleen and ileal cells were measured 2 hr after the injection of ^{3}HTdR. The spleen lymphocytes after HN_2 and after MTX exhibit marked reductions in the labeling index from a control value of 51.5% to 17.4 and 19.5%, respectively. Hydrocortisone has little effect on this parameter. The MI after HN_2 falls to 0.8% from a control value of 2.5%, while after MTX the MI is 1.2%. Hydrocortisone produces a drop in the MI to 1.6%.

Nitrogen mustard has no effect on the LI of the ileum but causes a fall in the MI from 8.4 to 2.0%. Methotrexate, on the other hand, effects a rise in the LI of ileal cells from 55.7 to 67.9% with an accompanying fall in the MI to 3.8%. After HC the LI remains at the control levels while the MI drops to 5.3% (Table II).

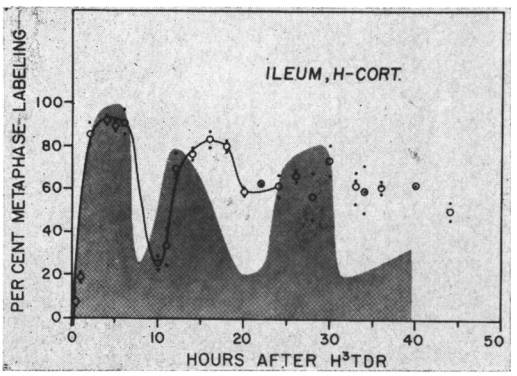

FIG. 4. Ileum, after HC. The shaded background is Fig. 1.

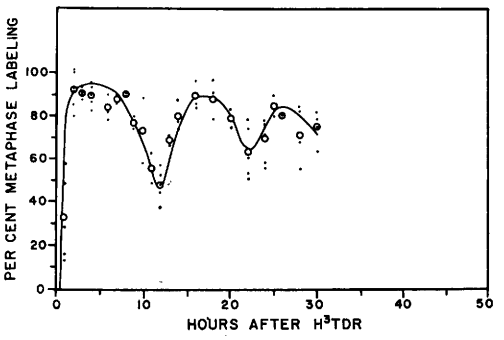

FIG. 5. Spleen, control. ^{3}HTdR, 1 μCi/g body weight. (Reproduced from Post and Hoffman (18) with the permission of New Engl. J. Med.)

Discussion. The data show profound alterations in the cell cycle kinetics of ileal and spleen cells after sublethal amounts of HN_2, MTX and HC. However, the animals remain in good physical appearance although their weight gains are minimal after HN_2 and MTX. They do not exhibit signs of diarrhea or of hemorrhage, as evidence of damage to either the gastrointestinal or the hematopoietic systems. Each drug affects the hemogram differently, yet each one causes remarkably

TABLE II. Labeling and Mitotic Indices (%).

	Ileum		Spleen	
	Interphase labeling	Mitotic index	Interphase labeling	Mitotic index
Control	55.7	8.4	51.5	2.5
HN_2	58.3	2.0	17.4	0.8
MTX	67.9	3.8	19.5	1.2
HC	53.7	5.3	46.2	1.6

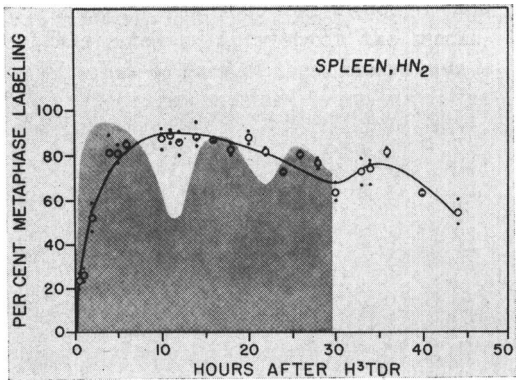

FIG. 6. Spleen, after HN_2. The shaded portion is the control rat.

similar changes in the replication cycle of the spleen lymphocyte. Nitrogen mustard produces a moderate leukopenia while MTX causes anemia. Hydrocortisone has little effect on the blood count. All three drugs appear to have a greater effect on the spleen, inducing loss of lymphocytes and sinusoidal congestion along with the changes in cell kinetics. On the other hand, except for the flattening of the villi observed after MTX, the changes in the ileal cell replication occur in the absence of significant histological abnormalities.

Of interest is the fact that although each agent has acted as a cytotoxin the mechanism of action of each of these compounds is different. Nitrogen mustard is an alkylating agent that accomplishes its cytotoxic action by producing crosslinking of the DNA strands and thus interrupting replication of the DNA (1, 6). Hampton (7) has shown that in the mouse ileum HN_2 (2.5 mg/kg), produces arrest of cell replication in DNA synthesis (S) or in the postsynthetic state (G_2) and that it also causes ribosomal depletion and cell loss. Ehrlich ascites tumor cells treated with HN_2 *in vivo* are also blocked in G_2 (8). Wheeler and co-workers (9) using cultured Hep No. 2 cells indicated that in low but toxic doses HN_2 does not interfere with the progression of cells through G_1 and into S, but does retard their advancement to mitosis, thus acting as a block in S and/or in G_2. The data in the present experiments indicate that DNA synthesis in the spleen is reduced and there is a decrease in mitosis. In the ileum however, the unchanged LI with a diminished MI suggests that there is a block in G_2, confirming the conclusions of other investigators (7–9).

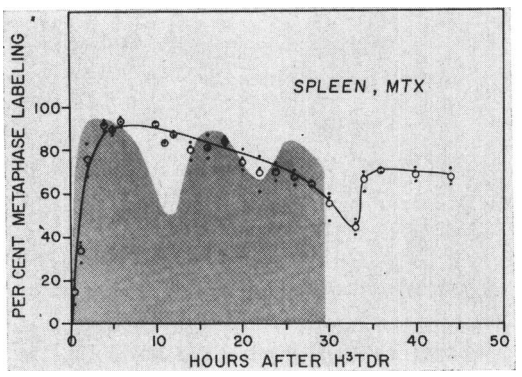

FIG. 7. Spleen, after MTX. The background is Fig. 5.

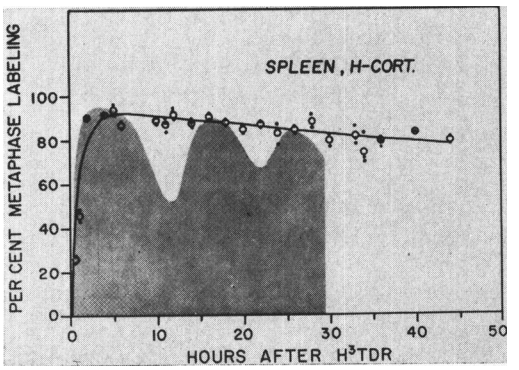

FIG. 8. Spleen, after HC. The control is the shaded portion.

Methotrexate blocks DNA synthesis by inhibiting the catalytic action of dihydrofolate reductase in the conversion of deoxyuridylic acid to thymidylic acid. Therefore, the maximum lethal effects of MTX are manifested among replicating cells (10, 11). The effects of MTX on the coexistent ileal and spleen cells of the rat are similar to those of HN_2. DNA synthesis and mitosis are sharply reduced in the spleen. In the ileum increased LI and the falling MI point to a block in G_2.

Glucosteroids have been reported to alter the cell cycle. During regeneration, and during growth HC depresses DNA synthesis in the rat liver (5, 12, 13). Hoffman *et al.* (5) also showed that mitosis and polyploidization after CCl_4 injury are reduced. Inhibition of mitosis in the hematopoietic system has also been reported for cortisone and prednisolone (14, 15). Kollmorgen and Griffin (16) demonstrated that 6 hr after addition of hydrocortisone the doubling time of HeLa Chessen cells changes from 18 to 35 hr. They showed that the block is in the postmitotic state (G_1). When the cells are returned to the control medium doubling time reverts to 18 hr. In the present experimental design there are alterations of the mitotic labeling curves of the ileal crypt cell and the spleen lymphocyte, more marked in the latter. These changes are associated with a small reduction in the MI, but they do not permit precise localization of a cycle block.

Conclusion. Cell replication is perturbed in the rat ileal crypt cells and spleen lymphocytes following 5 days of HN_2, MTX or HC. At the dosage levels employed HN_2 produces leukopenia, MTX causes anemia and HC does not affect the blood count. Histologically there is a loss of spleen lymphocytes following the administration of each of the drugs. Methotrexate also causes flattening of the intestinal villi without evidence of necrosis.

The authors thank Tatiana Miheyev, Dr. Bruce Pachter and Dr. Robert Sklarew for their technical assistance.

1. Scott, R. B., Brit. Med. J. 4, 259 (1970).
2. Henderson, E. S., Semin. Hematol. 6, 271 (1969).
3. Frei, E., III, Fed. Proc., Fed. Amer. Soc. Exp. Biol. 26, 918 (1967).
4. Post, J., and Hoffman, J., Cancer Res. 29, 1859 (1969).
5. Hoffman, J., Himes, M. B., Lapan, S., and Post, J., AMA Arch. Pathol. 60, 10 (1955).
6. Wheeler, G. P., Cancer Res. 22, 651 (1962).
7. Hampton, J. C., Radiat. Res. 30, 576 (1967).
8. Layde, J. P., and Baserga, R., Brit. J. Cancer 18, 150 (1964).
9. Wheeler, G. P., Bowdon, B. J., Adamson, D. J., and Vail, M. H., Cancer Res. 30, 100 (1970).
10. Borsa, J., and Whitmore, G. F., Cancer Res. 29, 737 (1969).
11. Bruce, W. R., Meeker, B. E., and Valeriote, F. A., J. Nat. Cancer Inst. 37, 233 (1966).
12. Rizzo, A. J., Heilpern, P., and Webb, T. E., Cancer Res. 31, 876 (1971).
13. Henderson, I. C., Fischel, R. E., and Loeb, J. N., Endocrinology 88, 1471 (1971).
14. Cardinali, G., Cardinali, G., De Caro, B. M., Handler, A. H., and Aboul-Enein, M., Cancer Res. 24, 969 (1964).
15. Nowell, P. C., Cancer Res. 21, 1518 (1961).
16. Kollmorgen, G. M., and Griffin, M. J., Cell Tissue Kinet. 2, 111 (1969).
17. Post, J., and Hoffman, J., Radiat. Res. 34, 570 (1968).
18. Post, J., and Hoffman, J., Exp. Cell Res. 60, 103 (1970).

Received Jan. 10, 1972. P.S.E.B.M., 1972, Vol. 140.