Effect of Orally Administered 3′,5′-Dichloroamethopterin on Formate-C^{14} Incorporation in Leukemic Mice*

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SUMMARY

The influence of the route of administration on the inhibition by 3′,5′-dichloroamethopterin (DCM) and amethopterin (MTX) of formate-C^{14} incorporation *in vivo* into acid-soluble adenine of mouse spleens infiltrated with leukemia L1210 was investigated. At equal dose levels, oral treatment was less effective than subcutaneous administration in inhibiting formate incorporation. Maximum inhibition occurred within ½ hour after subcutaneous, and about 2 hours after oral, administration of DCM. The extensive inhibition produced by parenterally administered DCM was not obtained with oral administration, even at much higher dose levels.

The greatly enhanced antileukemic effectiveness in mice (8) of 3′,5′-dichloroamethopterin (DCM)[1] as compared with that of its parent compound, amethopterin (MTX),[1] has encouraged clinical investigations of the drug (7). However, whereas parenteral administration has been employed routinely in the animal experiments (8), most of the patients received the drug *per os* (7). This has been in line with the generally accepted practice of employing the oral route of administration in antifolic therapy of leukemia. In the case of MTX, oral therapy is generally considered (3) to be about as efficacious in inducing and maintaining remissions as the parenteral route. With DCM, however, there are indications[2] that oral administration in the patient may not procure as satisfactory an antileukemic action as might have been expected from the previous studies in which mice received the drug subcutaneously (8). Accordingly, a study was undertaken in this laboratory to determine the comparative effectiveness of DCM by oral and parenteral administration in the treatment of mice bearing systemic leukemia L1210. The results of this study show that oral administration was much less effective in increasing survival time and reducing tumor size than was subcutaneous injection (19).

The question arose whether DCM, when administered orally, was fully effective at the site of neoplastic infiltration. Our previous studies have indicated that inhibition of formate-C^{14} incorporation *in vivo* into the acid-soluble adenine of infiltrated spleens of leukemic mice can serve as a quantitative measure of the effect of DCM and MTX (16). The present paper deals with the application of this method to the comparison of the effects of DCM and of MTX when administered by the oral and by the subcutaneous route.

MATERIALS AND METHODS

Hybrid male C×DBA mice [(BALB/cAn × DBA/2J)F_1], 11–14 weeks old and weighing 25–30 gm., were given inoculations subcutaneously, in the right inguinal region, of leukemia L1210 (about 2 million cells), as described previously (16). Experiments were conducted on day 7 or day 8 after inoculation. At that time the local tumors had reached diameters of 9–12 mm. The weights of the infiltrated spleens are listed with the different experiments (average weight of a normal spleen, 0.1 gm.) and are indicative of the degree of systemic infiltration.[3] Survival time of untreated

* Presented in part before the American Association for Cancer Research, Inc., April, 1960.

† National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

[1] The following abbreviations have been used: DCM = dichloromethotrexate (3′,5′-dichloroamethopterin); MTX = methotrexate (amethopterin).

[2] E. J. Freireich, personal communication.

Received for publication April 20, 1960.

[3] Comparison of the data from the different experiments suggests that, at equal dose levels of DCM, inhibition of formate incorporation was more pronounced with increased

mice was 9 to 11 days. The animals received Purina Laboratory Chow pellets and water ad libitum.

DCM[1, 4] and MTX[1, 4] were dissolved in 2 per cent sodium bicarbonate on the day of the experiment and administered at 0.01 ml/gm body weight. Controls received 2 per cent sodium bicarbonate solution. Subcutaneous injections were given in the axillary region, while oral administration was performed by gavage as described elsewhere (19). Sodium formate-C^{14} (1 $\mu c/\mu$mole) was dissolved in saline and administered intraperitoneally at 0.2 μc/gm body weight. The animals were killed by cervical dislocation 20 minutes after injection of the formate. Acid-soluble adenine was isolated from the pooled spleens of three mice used in each experimental group and its specific activity (counts/min/μmole) determined according to the procedure described previously (16).

TABLE 1

INHIBITION OF FORMATE-C^{14} INCORPORATION INTO THE ACID-SOLUBLE ADENINE OF LEUKEMIC MOUSE SPLEENS* BY SUBCUTANEOUSLY (S.C.) AND ORALLY ADMINISTERED 3′,5′ DICHLOROAMETHOPTERIN (DCM)

Interval (hours)†	Per cent inhibition‡ according to dose and route					
	40 mg/kg		10 mg/kg		2.5 mg/kg	
	S.C.	Oral	S.C.	Oral	S.C.	Oral
0.25	—	10	—	21	—	0
0.5	96	11	95	1	87	0
1	95	58	92	8	87	8
2	85	23	56	17	31	0
3	65	7	71	0	28	0
4	66	12	43	25	18	0
8	47	31	20	13	—	0
16	15	15	36	0	—	—

* Average spleen size, 0.31 ± 0.06 gm. (standard deviation).

† The mice were given injections of formate C^{14} at the stated time intervals after administration of DCM and killed another 20 minutes later.

‡ $100 \times \left(1 - \frac{\text{counts/min/}\mu\text{mole of treated mice}}{\text{counts/min/}\mu\text{mole of controls}}\right)$. Zero signifies no inhibition, and a dash no experimental point.

RESULTS

It was shown previously (16) that DCM inhibited formate-C^{14} incorporation into acid-soluble adenine of leukemic mouse spleens to the same extent when administered subcutaneously or intraperitoneally. This was true even when the drug was injected only 5 minutes before the labeled precursor. Maximum inhibition was observed within 20 minutes after parenteral administration of DCM. As the time interval was extended, inhibition was found to diminish. This indicated that the effect of DCM was transitory (16).

Table 1 indicates that, with oral treatment, the effect of DCM on the spleen was less rapid in that maximum inhibition occurred only after 1 or 2 hours. In addition, over a wide range of intervals after administration of DCM, the drug was much less effective when given orally.[3]

These findings are corroborated by dose-response experiments carried out at several time intervals between the administration of DCM and of formate. The results, which are illustrated in Chart 1, show that the maximum inhibition levels produced by parenterally administered DCM could not be reached with oral administration, even when the dose was increased considerably. The dose-response curves for MTX (Chart 2) resembled the ones obtained with DCM. The previous finding (16) that the effect of MTX was more persistent than that of DCM was confirmed for both subcutaneous and oral administration. This is apparent from Table 2, which indicates the doses required for 50 per cent inhibition[3] at the different time intervals, as derived from the curves shown in Charts 1 and 2. In agreement with previous findings (16), increasing amounts of parenterally administered DCM were needed with increasing time intervals between administration of drug and of formate, in order to effect the same inhibition. With the oral route, however, the amount of DCM required to produce 50 per cent inhibition was lower at the 2-hour than at the 1-hour interval, and increased sharply as the interval was extended beyond 2 hours. This would appear to indicate that the peak concentration of DCM in the spleen was reached about 2 hours after oral administration. It is of interest to compare the oral to the parenteral doses that produced the same level of inhibition (Table 2). The ratio of these doses was found to decrease with increasing time after administration, and appeared to approach a limit value of about 2, both with DCM and MTX. Thus, even after absorption was expected to be virtually complete (1, 6), twice as much drug was required orally as parenterally to produce the same inhibitory effect.

average spleen weights. This is consistent with the previously reported observation (16) that formate incorporation is inhibited to a proportionately greater extent in leukemia-infiltrated than in normal spleens.

[4] Obtained from the Lederle Division, American Cyanamid Company, Pearl River, New York.

DISCUSSION

Studies on the physiological disposition of orally and parenterally administered folic acid antagonists have been the subject of several reports in the literature (11). Two principal methods have been employed for determining blood, tissue, and urine levels of MTX. Burchenal's microbiological assay (1) and Freeman's fluorometric procedure (6) have yielded results that were qualitatively similar but showed quantitative differences. Thus, Burchenal reported that 40–57 per cent of orally ingested MTX in man was excreted in the urine within 24 hours and none after that time (1), whereas Freeman reported complete recovery in the urine during the first 24-hour period (6). Residual levels of MTX are, however, retained in tissues such as liver, kidney, and, to a lesser extent, spleen long after excretion has been completed and plasma levels have fallen to zero (2, 5). It has been emphasized (2) that MTX persists in these tissues long after its effects have disappeared.

Although inhibition of formate incorporation into the acid-soluble adenine of leukemic spleens is not a direct index of drug concentration, it is a measure of a metabolic effect of the drug, at the site of leukemic infiltration. The inhibition of folic reductase (21) by MTX is thought to be responsible for the interference with the biosynthesis of purine nucleotides and thymidylic acid (10, 11). The hypothesis that the antileukemic effect of MTX and its congeners is related to inhibition of the formation of these essential constituents of DNA (20) is strengthened by the finding that less or no inhibition occurs in antifolic-resistant sublines of leukemia (4, 9, 12, 15, 17, 18).

Burchenal (1) reported that peak serum levels of MTX in the fasting adult were reached in less than an hour after oral administration, and that food intake delayed absorption. Freeman's (6) data indicate maximal plasma levels after 1 or 2 hours. Rall (14) reported that peak plasma levels in man of DCM, measured by Freeman's procedure, were reached 2–4 hours after oral ingestion. The present results suggest that DCM

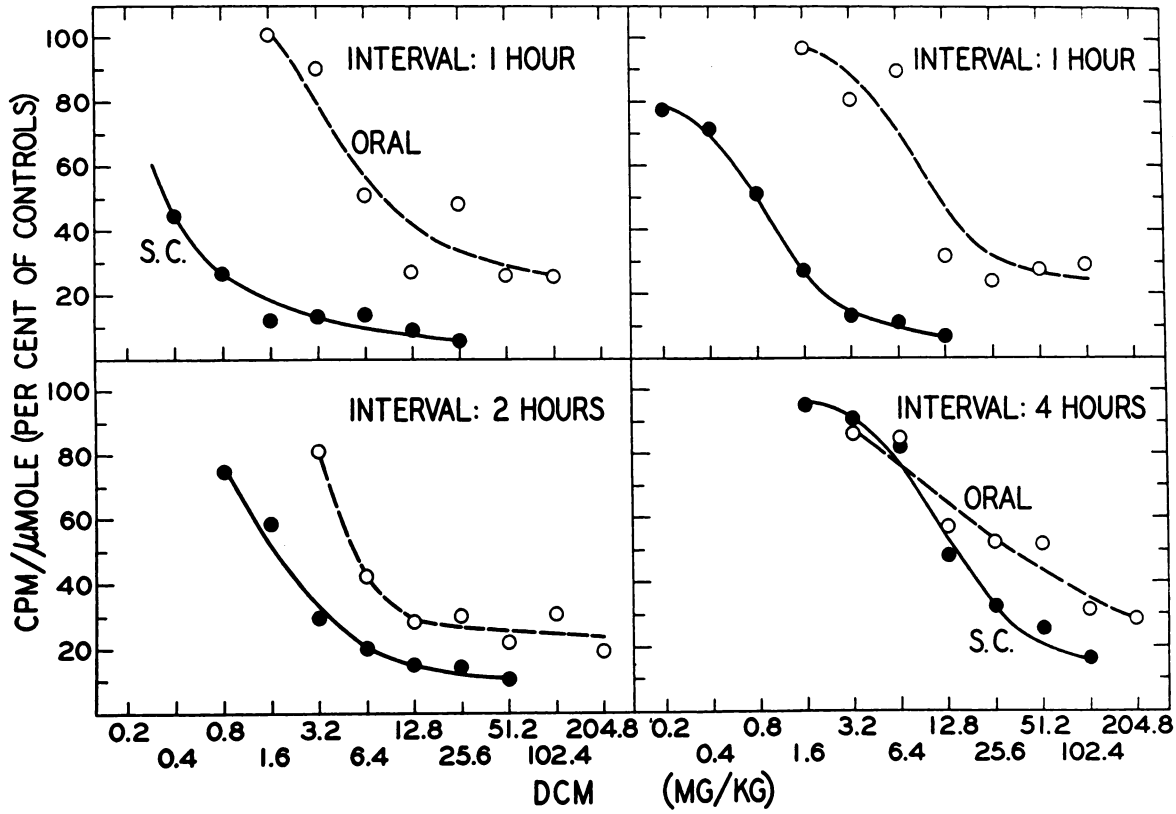

CHART 1.—Dose-response for the inhibition of formate incorporation into the acid-soluble adenine of leukemic spleens by orally and subcutaneously (S.C.) administered 3′,5′-dichloroamethopterin (DCM).

The mice were given injections of sodium formate-C^{14} at the stated time intervals after administration of the drug and killed another 20 minutes later. Results are expressed as:

$$100 \times \frac{\text{counts/min/}\mu\text{mole (treated)}}{\text{counts/min/}\mu\text{mole (controls)}}$$

reaches its maximum effect in mouse spleen about 2 hours after oral administration.

It has been shown that MTX appears in the urine very shortly after ingestion and that significant amounts are excreted before peak plasma levels are reached (1, 6). In view of the more gradual concentration build-up after oral administration, one might expect that a given dose would produce a lower peak plasma concentration orally than parenterally, even if all the drug were eventually absorbed. Burchenal (1) has measured the serum concentration of MTX in a patient after oral and intramuscular administration and found no significant difference. The present results, which show that a larger dose of MTX is required orally than parenterally in leukemic mice to produce the same inhibition in the spleen, suggest that a smaller fraction of a given dose reaches the tissue by the oral route.

In the case of DCM, the contrast between the effects of oral and parenteral administration is much greater than with MTX, both with respect to antileukemic action (19) and to formate incorporation. The more rapid disappearance of the effect of DCM (16)[5] could account for this difference, since an increased rate of disappearance relative to the rate of absorption would lead to decreased peak plasma and tissue levels. The ratios of the oral to the parenteral doses of DCM that produce equal inhibition of formate incorporation at the various time intervals (Table 2) are of the same order of magnitude as the inverse ratios of the plasma concentrations in man (14). It should, however, be pointed out that this agreement may be fortuitous, since Rall (14) has emphasized that

TABLE 2

DOSES OF 3',5'-DICHLOROAMETHOPTERIN (DCM) AND AMETHOPTERIN (MTX) REQUIRED FOR 50 PER CENT INHIBITION OF FORMATE-C^{14} INCORPORATION INTO ACID-SOLUBLE ADENINE OF LEUKEMIC SPLEENS

Drug	Interval* (hours)	Dose (mg/kg)†		Dose ratio oral/s.c.	Av. spleen wt. (gm. ± s.d.)
		Oral	S.C.‡		
DCM	1§	8.4	0.35	24	0.56 ± 0.10
"	1#	11.9	0.82	14	0.43 ± 0.11
"	2	5.3	1.7	3.1	0.56 ± 0.10
"	4	34	15	2.3	0.43 ± 0.11
MTX	1	1.0	0.16	6.3	0.68 ± 0.08
"	6	1.15	0.48	2.4	0.44 ± 0.10

* Sodium formate-C^{14} was injected at the stated time intervals after administration of the drug.

† Derived from the curves shown in Charts 1 and 2.

‡ Subcutaneous.

§ Upper left panel of Chart 1.

Upper right panel of Chart 1.

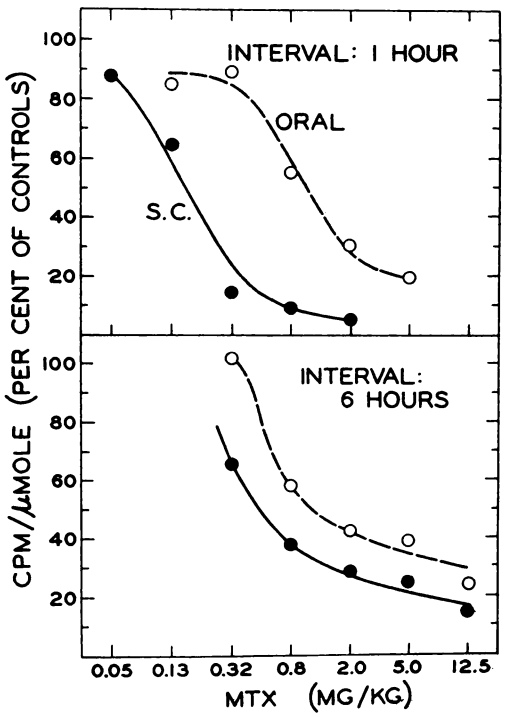

CHART 2.—Dose-response for the inhibition of formate incorporation into the acid-soluble adenine of leukemic spleens by orally and subcutaneously (S.C.) administered amethopterin (MTX). For details, see Chart 1.

[5] It has been reported recently (13) that DCM is both excreted as such and metabolized, while MTX is apparently excreted unchanged.

the plasma half-life of DCM varied greatly from species to species.

Whereas the present findings can account for the greatly reduced antileukemic efficacy of orally administered DCM, they do not explain the fact (19) that host toxicity on daily treatment is not decreased to the same extent. Further studies may be needed to clarify this point.

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Cancer Res 1960;20:1457-1461.