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Dihydrofolate reductase activity of leukemia L1210 during development of methotrexate resistance

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DEVELOPMENT of resistance to folate analogs in murine leukemia has most often been found to be associated with an increased dihydrofolate reductase (EC 1.5.1.3) activity of the leukemic cells. The selection process leading to these events has been discussed in detail.[1–4] Consecutive passage through treated mice seemed required to achieve resistance to a given drug regimen,[1,4,5] in contrast to human leukemia, which becomes refractory in the patient during therapy.[6] It became, therefore, of interest to determine whether dihydrofolate reductase levels were in fact already increased during the initial transfer generation of systemic leukemia L1210 under treatment with methotrexate (MTX).

CDF_1 hybrid male mice were inoculated s.c. with spleen suspensions of leukemia L1210, as described previously.[1] MTX (0·75 mg/kg s.c.) was injected daily, and tumor reinoculations were carried out as in the establishment of Friedkin's subline FR-1.[1] Acetone powders were prepared from the solid tumors growing at the site of inoculation and from the spleens infiltrated with leukemic cells, stored at −20°, and extracted with buffer, pH 7·4, for determination of dihydrofolate reductase activities.[1,7] In comparing the present results with Friedkin's data,[1] it should be noted that carrying out the rate determinations at 28°[7] instead of 32° and using a $\Delta\epsilon$ value of 12,300[8] instead of 11,300[1] in the spectrophotometric assay have resulted in somewhat lower specific activities.

Dihydrofolate reductase activities in the local tumors and spleens of leukemic mice treated daily with MTX from day 5 after tumor inoculation were measured 48 hr after the last injection of the drug (Table 1).

The decrease during the first 18 days after inoculation, as compared to the specific activities in untreated animals, was attributable to partial binding of the enzyme to the drug.[9,10] When treatment was continued to day 20 after inoculation or longer, dihydrofolate reductase activity in the local tumors was increased markedly. Resistance, as reflected by increased enzyme activity, thus became evident only after at least 15 days of treatment and shortly before the expected death of the treated animals. The activity in the tumors of animals treated until day 20 was not increased further when the interval between the last injection of MTX and tumor removal was extended to 72 hr (Table 1), and it was identical with that observed in animals of the next untreated transfer generation (Table 2). Therefore, bound MTX produced no significant inhibition of enzyme activity 48 hr after injection once the cells had become partly resistant, possibly because of synthesis of new enzyme during that period or because of the emergence of cells with decreased permeability to MTX.

The increased dihydrofolate reductase activity in the local tumors of mice treated from day 5 to 20 was not observed in the spleens (Table 1). In agreement with previous findings,[11] these spleens became much larger in leukemic mice treated with MTX than in untreated control animals. The much lower enzyme activity per unit of protein in the spleens, as compared to the tumors, on days 22 and

TABLE 1. DIHYDROFOLATE REDUCTASE IN LEUKEMIC MICE TREATED WITH METHOTREXATE*

Days after inoculation		Specific activity		Average spleen wt. (g)
Treatment	Sacrifice	Tumor	Spleen	
Untreated	7	75	60	0·63†
5–8	10	40	40	0·84
5–12	14	20	30	1·21
5–16	18	35	30	1·28
5–20	22	250‡	50	1·73
5–22	24	240	85	1·61

* Mice (three per group) were inoculated s.c. with spleen suspensions of sytemic leukemia L1210 and injected daily with 0·75 mg/kg methotrexate s.c. Dihydrofolate reductase activities (mμmoles/hr/mg protein) were determined at pH 7·4 and 28° in extracts of acetone-dried powders, which were prepared immediately after the animals were killed.

† Spleens of untreated nonleukemic mice weighed 0·10–0·15 g.

‡ The specific activities in tumors removed on days 21 and 23 from mice treated until day 20 were 110 and 260 respectively.

TABLE 2. DEVELOPMENT OF RESISTANCE TO METHOTREXATE IN LEUKEMIA L1210*

Transfer generation	Day of treatment iniation	Day of tumor reinoculation into next generation	Median survival time (days)		Dihydrofolate reductase activity	
			Treated	Untreated	Tumor	Spleen
1	5	23	24·5	8	75	60
2	3	8	10	8	240†	170‡
3	0	7	8	8	460	420
4					450	390

* Mice carrying systemic leukemia L1210 were injected daily with 0·75 mg/kg of methotrexate s.c. Treatment was started on the day indicated after s.c. inoculation of a spleen suspension and continued until the day prior to reinoculation in the donor mice used for tumor transfer, and until death in the mice used for survival studies. Dihydrofolate reductase activity (mμmoles/hr/mg protein) was determined at pH 7·4 in extracts of acetone-dried powders from another group of mice, receiving no treatment, which were killed on day 7 after inoculation.

† This value was 460 in mice treated with methotrexate on days 3–7 and killed on day 9.

‡ This value was 380 in mice treated on days 3–7 and killed on day 9.

24 might suggest that the increase in spleen size above the level of the untreated control mice represents hyperplasia of normal spleen tissue rather than infiltration with leukemic cells. This greatly increased spleen weight in animals treated with MTX was not observed in the second transfer generation§ when the tumor had already become partly resistant and the treated mice died shortly after the untreated controls. The ratio of activities of spleens and local tumors in generation 2 (Table 2) would suggest 70–80 per cent infiltration of the former with leukemic cells.

Dihydrofolate reductase activity in the local tumors of the second transfer generation treated with MTX rose to six times the level found in the original leukemia L1210 (Table 2). No further increase occurred after an additional treated generation, in which the drug produced no increase in survival time over that of untreated controls. Therefore, maximal enzyme activity and complete resistance to

§ It should be noted that this generation was obtained by inoculation with suspensions of the first-generation spleens.

the drug dose used was achieved by serial passage and treatment with a constant dose of MTX for only two generations. By comparison, dihydrofolate reductase activity in the FR-1 subline rose to about ten times the original level.[1]

It may be noted that fewer treatments were administered in each transfer generation with the ascitic sublines of L1210, in which more passages were required to achieve resistance to MTX.[3-5,7] Thus establishment of resistance of a cell line with a given mutation frequency to a given dose may be a function of the total duration of exposure. At the end of a course of treatment of systemic leukemia L1210 for at least 15 days, the tumor exhibits an increase in dihydrofolate reductase levels, which in turn may contribute to the failure of therapy. The results of treatment of murine leukemia with MTX thus parallel the therapeutic situation in man and suggest use of this technique for the study of drug resistance in the clinical situation.

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Persistent reduction of serum bilirubin levels after treatment of Gunn rats with some acidic compounds

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THE EXCRETION of bilirubin is largely dependent on its conjugation with glucuronic acid to form the water soluble glucuronide which is then excreted into the bile. Gunn rats exhibit a type of hereditary jaundice due to their inability to conjugate bilirubin with glucuronic acid. This jaundice is characterized by fairly constant serum levels of unconjugated bilirubin of 5–15 mg per cent which persist throughout the life-time of the animal. A single dose of a variety of acidic compounds (e.g. sulphisoxazole,[1] salicylate[2]) have been shown to reduce the level of serum bilirubin in Gunn rats presumably by competitive binding on albumin which results in displacement of bilirubin into other tissues,