Effect of Folic Acid on *in vitro* Uptake of Labeled Thymidine by Leukemic Cells.* (26953)

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Long term cultivation of hemic cells *in vitro* is difficult, although de Bruyn (1) reported success with cells from lymphosarcoma and myelosarcoma of mice and Osgood and Brooke (2) established continuous cell lines from human leukemias using gradient culture. Of the virus-induced leukemias, only cells from the fowl leukosis complex have been cultivated. Beaudreau *et al.* (3) have successfully grown myeloblastosis, and Lagerlof (4), erythroleukemia. Although it is possible to propagate at least 2 mouse leukemia viruses in mouse embryo cells, Friend's (5) and Moloney's (6), the cells themselves do not proliferate continuously.

Fischer's observations that the ascitic form of the mouse leukemia L5178 could be grown *in vitro* only in the presence of high concentrations of folic acid stimulated interest in determining the relationship of this compound to leukemias (7,8). L5178 cells are deficient in the enzyme which converts dihydrofolic acid to tetrahydrofolic acid (7). In addition, in human leukemia the folic acid content in white cells is higher than in the normal cells (9,10).

Because of the implication that folic acid is intimately involved in the leukemic process, it was decided to study the synthesis of deoxyribonucleic acid (DNA) in different virus-induced and non-virus-induced leukemias and lymphosarcomas growing in culture in presence of increased amounts of folic acid. This was done by adding labeled thymidine (H^3) to cultures and determining the number of labeled cells using radioautographic techniques (11). Mitotic indices were determined on the same specimens.

Materials and methods. Cultures. To obtain a monolayer of cells, the explant was placed on a coverslip in a very thin plasma clot. Preparations were made from spleens of mice infected with Friend's leukemia§ (12), Gross's leukemia (Passage A)§ (13), Moloney's leukemia§ (14), and L1210‡. A mouse lymphosarcoma isolated from random-bred stock mice and kept in serial passage was also used. Normal splenic cultures served as controls. The cultures were divided into 2 groups, one grown in medium A consisting of Eagle's basic medium (15) with 15% inactivated horse serum and 1 mg/liter of folic acid (15,16); the other grown in medium B, identical with medium A except that it contained 10 mg/liter of folic acid.

Radioautographs. After 10-12 days culture the explants had grown out as monolayers and the plasma clots were completely digested. Tritiated thymidine (specific activity 3 curie/mM) was added to the medium to give a final concentration of 0.1 μc/ml. After 3-48 hours, cultures were sacrificed and the number of labeled cells determined. The cultures were washed in distilled water and were fixed in methyl alcohol-acetic acid (3:1). They were washed again in distilled water and allowed to dry. The stripping film technique described by Fitzgerald *et al.* (17) was used; the film was Kodak AR-10. After developing, slides were stained with Giemsa at pH 7. It was found that after 24 hours half of the cells

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‡ L1210 was induced by carcinogenic agents by Dr. L. W. Law and was obtained from Dr. Joseph Burchenal of this Institute.

TABLE I. Incorporation of Tritiated Thymidine by Various Tissues and % Mitosis.

Tissue	Media*	% labeled cells	% mitosis
Friend's leukemia spleen	A	25 ± 4	.9 ± .1
	B	49 ± 4	1.4 ± .1
Gross's leukemia spleen	A	26 ± 1	.7 ± .1
	B	43 ± 1	1.5 ± .1
Moloney's leukemia spleen	A	25 ± 2	.7 ± .1
	B	42 ± 1	1.3 ± .2
L1210 leukemia spleen	A	28 ± 2	.7 ± .2
	B	29 ± 3	.9 ± .1
Lymphosarcoma	A	31 ± 3	1.1 ± .3
	B	25 ± 2	1.0 ± .3
Normal spleen	A	42 ± 1	1.5 ± .1
	B	44 ± 2	1.4 ± .1

* Eagle's media containing 1 mg/l (A) or 10 mg/l (B) folic acid.

were labeled, so this time was chosen for the experiments.

Cell counts. One thousand cells per culture were counted using the oil immersion objective, and the number of cells containing grains in the nucleus was determined. The number of grains in the nucleus varied considerably from a few to many, but this was disregarded, since the same variability was noted in all groups. The results are reported as the per cent of labeled nuclei in the total number of nuclei counted. The number of mitoses per 100 cells was also recorded.

Results. Growth in tissue culture. The number and types of cells which grow out from splenic cultures, whether malignant or normal, are of many different types whose exact origin is unknown. In this study they were classified mainly as 2 types, a fibroblast-like cell and a round basophilic cell. In the L1210, Gross's and Moloney's leukemia, and lymphosacroma, the latter type predominated; in cultures of the Friend leukemic spleen most cells were also basophilic but larger than the others.

Incorporation of tritiated thymidine and per cent mitosis. The results are shown in Table I. The uptake of tritiated thymidine was more pronounced in normal spleen than in any of the leukemic spleens or in the spleen from the mouse having lymphosarcoma. These results agreed with those of others (18,19). In the presence of 10-fold folic acid (medium B), the virus leukemic spleens took up significantly more labeled thymidine than did the L1210 or the lymphosarcoma. This appeared to be a reflection of increased growth, since in most instances the mitotic index almost doubled.

The Friend leukemia agent was cultivated in Scherer's medium (20) which contains purines and pyrimidines available for nucleic acid synthesis. In this medium, 29% of the cells were labeled. When ten times the amount of folic acid was added, however, the number of labeled cells was 40%. Thus, the stimulatory effect was apparent.

To determine if uptake of thymidine was related to DNA synthesis and mitosis, a correlation analysis was done by the method of Snedecor (21). Fig. 1 shows the adjusted values and the real values. The low values represent the L1210 and lymphosarcoma in both media and the 3 virus leukemias in medium A. The higher values represent the normal spleen (both media)

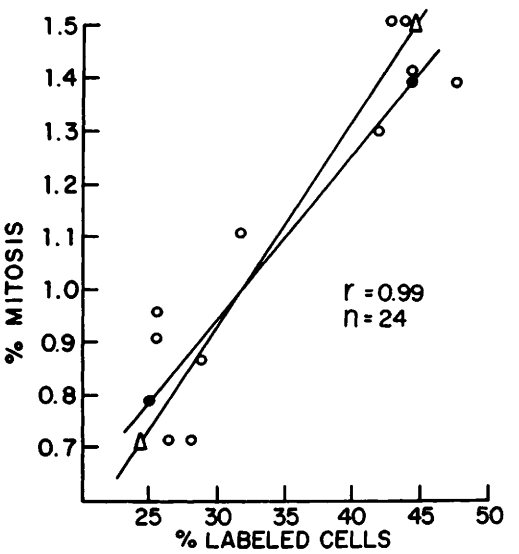

FIG. 1. Correlation analysis showing the relationship of uptake of tritiated thymidine to DNA synthesis and growth: adjusted values of per cent mitosis (●); adjusted values of per cent labeled cells (△); real values (○).

TABLE II. Incorporation of Tritiated Thymidine into Normal Splenic Cells Infected with Friend's Leukemia Virus.

Type of tissue	Media*	% labeled cells
Normal spleen infected with virus	A	17 ± 3
	B	31 ± 5
Normal spleen, control	A	40 ± 2
	B	44 ± 4

* Eagle's media containing 1 mg/l (A) or 10 mg/l (B) folic acid.

and the 3 virus leukemic spleens in the media with the increased amounts of folic acid. The correlation coefficient shows that uptake of tritiated thymidine is related to DNA synthesis and growth as measured by the mitotic index.

To see if there were some relation between the folic acid effect and the presence of virus, another type of experiment was set up. Normal spleen was grown out in tissue culture and, after 8 days, infected with the Friend leukemia virus. Radioactive thymidine was added 3 days later. It can be seen (Table II) that the normal spleen infected with virus had a much lower per cent of labeled cells than did the control uninfected spleen grown in the same medium. However, when the infected spleen was grown in medium with excess folic acid, the per cent of labeled cells almost doubled, although the number of labeled cells was not as great as in the normal control cultures.

Discussion. The tissue culture experiments reported here agree with the findings of Gavosto *et al.* (19) and Craddock (18) who showed that in short-term experiments the uptake of tritiated thymidine is less in human acute leukemic cells than in normal white cells. It was also demonstrated that certain leukemic spleens need a supplement of folic acid in order to get values equal to that of normal spleen.

Folic acid serves as a coenzyme in utilization of one carbon compounds (22) which serve as the building blocks in biosynthesis of purines, pyrimidines, and the amino acids, serine and methionine. It is required for growth of cells in tissue culture at a level of 1 mg/liter, as shown by Eagle (16). This amount proved to be adequate for the growth of normal mouse spleen, but the 3 virus-infected spleens studied required 10 times that amount. This was true even in the presence of purines and pyrimidines in the medium. The fact that these are virus-induced leukemias and that similar results were obtained with normal spleens infected with virus suggests that perhaps this is a phenomenon associated with viral infection. However, it has been reported (7,8,23) that two types of mouse leukemias (L5178 and P815), supposedly not of viral origin, have increased requirements for folic acid; the need for folic acid may be a matter of individual cellular requirements rather than of the etiologic agent. The difference shown may be more complex than it appears, since attempts to grow the Friend leukemia virus in continuous passage with increased amounts of folic acid have not enhanced the production of virus.

Summary. The uptake of tritiated thymidine by leukemic and normal spleens has been studied. The values obtained for the normal spleen were always higher than those for the leukemic spleen. When virus-induced leukemic spleens were cultured in high concentrations of folic acid their uptake equalled that of normal spleen. This was not true of a non-viral leukemia or of a lymphosarcoma. When normal splenic cultures were infected with the Friend leukemia virus, the number of labeled cells decreased; but when high concentrations of folic acid were added, the number increased.

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Efficiency of Plaquing Certain Poliovirus on Tissue Cultures at Higher Temperatures as a Function of L-Cystine Concentration.* (26954)

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In 1956 3 wild-type polioviruses were shown to require exogenously supplied cystine for efficient plaque production on monkey kidney tissue cultures at 36° (1). Subsequently, it was shown that their efficiency of plaque production on cultures not supplied with cystine is dependent on the temperature during plaque development: lowering the temperature below 37° increased this efficiency (2).

That the temperature of the host cell has a marked influence on growth of poliovirus has recently been well recognized [see reviews by Lwoff and Lwoff (3) and by Sabin (4)]. However, the relation of cystine to this influence of temperature has not been adequately explored; the results of such investigation are described here.

Materials and methods. Polioviruses. The wild-type virus, the 30°-adapted (ca^{30}) mutant (5), and the 23°-adapted (ca^{23}) mutant (6) of the Akron strain of antigenic type 1 poliovirus were used. *Growth of monkey kidney tissue cultures (MKTC's).* Kidney cells from cynomolgus monkeys (*Macaca irus*) or rhesus monkeys (*M. mulatta*) were dispersed with trypsin (7), suspended in lactalbumin growth medium (8), delivered to Petri plates, and then kept at 37°-38° in an atmosphere of 3% CO_2 and 97% air for 5-9 days, after which time suitable sheets of cells had developed. *Inoculations and plaque development.* The sheets were washed twice, each time with 4 ml phosphate-buffered saline (PBS) (9), then were inoculated with the appropriate virus concentrations, 0.3 ml inoculum per plate. Inoculated sheets were put at 37° for 30 minutes. Then a sheet received 6 ml altered Eagle's maintenance medium (AE_m) (2), adjusted with HCl and/or NaOH usually to pH 6.9-7.1, containing 0.9% washed (9) agar and 0, 0.025, 0.050, 0.20, or 1.0 mM

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