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Effect of 1-β-D-Ribofuranosyl-1,2,4-Triazole-3-Carboxamide (Ribavirin) on Friend Leukemia Virus Infections in Mice (38647)

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The triazole nucleoside 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (ribavirin,[1] ICN 1229) has notable broad spectrum antiviral activity *in vitro* (1–6) and *in vivo* (1, 4, 7–10). *In vivo*, the drug has been particularly effective when administered parenterally against influenza and parainfluenza viruses (1, 4, 8), and when applied topically to herpes and vaccinia virus-induced eye infections in rabbits (7). Inhibition of adenocarcinoma 755, L1210 leukemia, and Ehrlich ascites carcinoma (Khwaja *et al.*, Abstr. Amer. Assoc. Cancer Res. **13**, 19, 1972), and of spontaneous leukemia in AKR/5 mice (Randawa *et al.*, Abstr. 207, 14th ICAAC, 1974), by ribavirin in mice has also been reported.

In view of these antiviral and antitumor properties, we were interested in determining if ribavirin treatment could also influence neoplastic diseases induced by introduction of oncogenic viruses. The Friend leukemia virus (FLV) infection in laboratory mice was selected for our initial experiments, which are described in this report.

Materials and Methods. Drug preparation. The ribavirin used in these studies was synthesized at this Institute according to the method described by Witkowski *et al.* (2). The chemical was dissolved in sterile physiological saline for the present studies.

Mice. Random bred male Swiss Webster mice weighing 14–16 g. were obtained from Hilltop Lab Animals, Chatsworth, CA.

Virus. FLV was prepared as a cell-free centrifuged spleen homogenate from the 166th passage in Swiss mice. This strain, obtained originally from Dr. H. Christine Reilly, Sloan-Kettering Institute for Cancer Research, Rye, NY, and then utilized in extensive studies at Southern Research Institute, Birmingham, AL, produces a biphasic response (11, 12) characterized by malignant proliferation of reticular cells with involvement of the spleen and liver, and extensive polycythemia. Data of similar experiments in which this virus was used have previously been reported (13–15).

Testing procedures. The anti-FLV activity of ribavirin was evaluated using three parameters: Splenomegaly inhibition, reduction of infectious virus titer in the spleen and reduction of virus titer in the plasma of infected, treated mice as compared with virus control mice which were treated with saline only.

In all experiments, mice were inoculated intraperitoneally (ip) with 0.2 ml of approximately 10^4 of the 50% infectious dose (ID_{50}) of FLV. This quantity of virus was sufficient to induce an average spleen weight of approximately 1300 mg (normal weight approximately 160 mg) in 21 days. Groups of 10 or 15 mice were used in all virus-infected groups receiving a particular drug treatment. Twenty or 25 mice were utilized as virus controls. The animals were killed on day 21 and the spleens removed, weighed, and then frozen at −70°. Plasma was pooled from each experiment group and also frozen at −70°. Dilutions (10^{-1}, 10^{-2}, and 10^{-3}) of supernates from centrifuged spleen homogenates and plasma from virus control mice were prepared in MEM containing 1% sorbital, 100 units of penicillin, 100 μg of streptomycin/ml, and 50 μg of gentamicin/ml. Each dilution was inoculated ip in 0.2 ml vol into 20 mice. Supernates of spleen homogenates and plasma from the test mice were diluted 10^{-1} and also inoculated

[1] Ribavirin is the name approved by the U.S. Adopted Names Council for this compound, previously identified as Virazole.

into groups of 20 recipient mice. All indicator mice were killed 21 days later, spleens were removed and weighed, and the mean spleen weights were plotted against the dilution of the inoculum. A straight line plot on a semilog graph of the data from the virus control animals' spleens and plasma was used to determine the virus concentration. Spleens of indicator mice inoculated with spleen homogenates or plasma from drug-treated animals were compared to the standard virus control plot, and the reduction in virus titer was then calculated. This procedure was adapted from the method of Chirigos *et al.* (16).

Since host weight loss may affect the degree of splenomegaly induced by FLV (14), animal weights were determined in each experiment. Toxicity control mice which were inoculated with virus diluent only and treated with ribavirin were run in parallel with infected, treated animals in all studies, and weight loss, failure to gain weight, reduction of normal spleen weight and mortality was observed in different groups.

Results. Multiple treatment therapy. Table I summarizes the data obtained using ribavirin administered twice daily for 14 days beginning 2 hr previrus inoculation. Significant, dose dependent inhibition of increases in spleen weight was observed. Virus titer reductions were demonstrable in both the pooled spleens and in the plasma of ribavirin treated animals, however, these reductions were somewhat variable and apparently not dose-dependent. Relatively high dose levels of ribavirin administered 15 min pre- and then 3, 6, and 9 days postvirus inoculation were also quite inhibitory to splenomegaly and virus development in spleens and plasma (Table II). In both experiments less host weight gain was seen in the treated groups receiving the high dosage levels which were moderately lethal in toxicity, but toxicity control spleen weights were not significantly reduced using any of the dosage levels. In the mice receiving lower dose levels of ribavirin, the animals gained weight in essentially a normal manner.

Single treatment therapy. When 400 mg/kg ribavirin was administered in a single injection 15 min postvirus inoculation, marked inhibition (79% $P < 0.01$) of splenomegaly, accompanied by reductions in spleen and plasma virus (2.0 and 1.4 $\log_{10}$, respectively, $P < 0.05$) was exhibited. All treated animals gained weight normally, spleen weight loss was not seen in the toxicity controls, and all toxicity control mice survived. Single treatment effects were studied further, with treatment times designed to affect the viral infection at different phases of development: 15 min prior to virus inoculation, 4 hr postvirus inoculation, shortly after probable transformation of spleen cells by virus (17), 8 hr postvirus inoculation, during the "eclipse" phase when no virus is usually detectable in spleen and plasma (13), and 24 hr postvirus inoculation, near the time virus can initially be demonstrated in the

TABLE I. EFFECT OF MULTIPLE TREATMENTS WITH RIBAVIRIN ON FRIEND LEUKEMIA VIRUS INFECTIONS IN MICE.[a]

Drug dosage (mg/kg/day)	Toxicity control Surv/Total	Infected, treated Surv/Total	Spleen wt (mg)	Splenomegaly inhibition (%)	Plasma virus titer[b] (Log_{10})	Spleen virus titer[b] (Log_{10})
75.0	4/5	10/15	0.49	63[d]	2.1	2.4
37.5	5/5	15/15	0.71	46[c]	2.0	1.3[c]
18.8	5/5	15/15	0.74	44[c]	2.1	1.5[c]
9.4	5/5	14/15	0.83	37	1.5[c]	1.4[c]
0	—	20/20	1.32		2.6	2.9

[a] Intraperitoneal treatment began 2 hr previrus inoculation and continued twice daily for 14 days.

[b] Virus titer in plasma and spleen determined by 21-day splenomegaly in indicator Swiss mice inoculated with cell-free preparations of the material.

[c] $P < 0.05$ (t test).

[d] $P < 0.01$ (t test).

TABLE II. EFFECT OF TREATMENT WITH RIBAVIRIN EVERY THREE DAYS ON FRIEND LEUKEMIA VIRUS INFECTIONS IN MICE[a]

Drug dosage (mg/kg/day)	Toxicity control surv/total	Infected, treated surv/total	Splenomegaly inhibition[b] (%)	Plasma virus titer reduction[c] (Log_{10})	Spleen virus titer reduction[d] (Log_{10})
400	2/5	3/10	78[e]	1.0[e]	1.2[e]
200	5/5	10/10	61[d]	1.1[d]	1.0[d]
100	5/5	10/10	41[d]	0.4	0.6
50	5/5	10/10	41[d]	0.5	0.5

[a] Intraperitoneal treatments were administered 15 min pre- and 3, 6, and 9 days postvirus inoculation.
[b] Average virus control spleen weight = 1.35 g (20 mice).
[c] Virus titer in plasma and spleen determined by 21-day splenomegaly in indicator Swiss mice inoculated with cell-free preparations of the material.
[d] $P < 0.05$ (t test).
[e] Insufficient samples for statistical evaluation.

spleen and plasma (13). Treatments 15 min pre- and 4 hr postvirus inoculation appeared approximately equal in efficacy; treatments 8 hr and 24 hr post were moderately less effective than the earlier therapy, particularly at the lower drug dosage, although definite inhibition of splenomegaly was still demonstrable. Virus titer reductions were not determined in this experiment. These latter single treatment results are summarized in Table III. The treated animals gained weight normally during the study, and the spleens of toxicity control mice remained normal in size. All toxicity control mice again survived the duration of the study.

TABLE III. EFFECT OF SINGLE TREATMENT WITH RIBAVIRIN AT VARYING TIMES RELATIVE TO VIRUS INOCULATION ON FRIEND LEUKEMIA VIRUS INFECTIONS IN MICE.

Time of treatment[a]	Drug dosage (mg/kg)	Infected, treated surv/total	Splenomegaly inhibition[b] (%)
15 min pre	400	10/10	43[c]
	200	10/10	38[c]
	100	10/10	23
4 hr post	400	10/10	49[c]
	200	10/10	44[c]
	100	10/10	30
8 hr post	400	9/10	37[c]
	200	10/10	19
	100	10/10	13
24 hr post	400	10/10	40[c]
	200	10/10	32
	100	10/10	0

[a] Relative to virus inoculation.
[b] Average virus control spleen weight = 1.35 g (20 mice).
[c] $P < 0.05$ (t test).

Discussion. These experiments indicate that ribavirin had a moderate but significant effect on FLV infections in mice, whether the drug was administered in multiple or in single injections. The effects of the therapy were seen by all parameters used in the study.

Ribavirin's efficacy seemed to be related to the time of treatment relative to infection. Apparently FLV is in a stationary, or "eclipse" phase, in which no infectious virus can be detected until at least 24 hr after virus inoculation (13). Oncogenic RNA viruses are thought to carry information for both virus multiplication and for expression of oncogenicity (18). Therefore, during the FLV eclipse phase, events leading to neoplastic cell transformation may have been initiated. Recent studies by Rossi *et al.* (17) using DBA/2 mice indicate hemopoietic cells in the spleen are transformed into neoplastic cells 3 hr after infection. Since transformation occurs in this short time, it presumably does not require production of complete virus, but may need the synthesis of viral nucleic acid and/or virus-specified protein. It would seem apparent, then, that drugs having efficacy against FLV infections may express that activity through antiviral or antineoplastic actions, or as a combination of both actions.

Ribavirin, as the 5′-phosphate, inhibits

at least three enzymes involved in *de novo* purine biosynthesis (9, 19, J. F. Henderson, University of Alberta Cancer Research Unit, Edmonton, Canada, *personal communication*) which result in a decreased quantity of guanylate nucleotides available for nucleic acid synthesis. This enzyme inhibition probably could prevent synthesis of viral nucleic acid, and could also interfere with the DNA synthesis of the rapidly dividing newly transformed cells.

In the present studies, ribavirin treatment initiated at about the time of infection may have prevented the synthesis of virus RNA, or interfered with enzymes necessary for cell transformation. Treatment 4 or more hr after infection could interfere with completion of virus replication and with division of the transformed cells. Treatment with ribavirin as late as 24 hr would seem to reflect antineoplastic cell properties of the drug. The low dose multiple treatments, also quite effective in this study, probably prevented, or markedly slowed the virus replication and eventual transformation of new cells during the treatment period.

Since both virus and drug were injected ip, the possibility of a direct action of the drug on the virus must be considered. Although specific virucidal studies using ribavirin with FLV have not been accomplished, it may be pertinent to note that the drug has had no direct action on other viruses, including type 1 herpes simplex, vaccinia, type 2 adeno, type 3 parainfluenza and type 13 rhino viruses (1, 5). The efficacy noted when ribavirin was administered up to 24 hr postvirus inoculation would also support our premise that the drug is acting by an other than virus inactivating effect, especially in view of the report by Rossi *et al.* (17) showing transformation of spleen cells in mice three hr after ip injection of FLV.

Chirigos and March (20) have shown that spleen cells are of major importance as a site of FLV replication, and they attribute the anti-FLV activity of many drugs to a specific action on these cells. The liver can also become an erythropoietic organ in mice infected with FLV, however, as shown particularly in experiments with splenectomized animals (21). Blood producing cells are also involved in this early disease, which results in marked lymphocytosis (21). Data from our laboratory (J. Miller, *unpublished observations*) indicate that in mice treated by oral gavage with tritium-labeled ribavirin, 44% and 6% of the administered dose can be found in the liver and spleen, respectively, within 30–60 min, with a half-life in each organ of approximately 12 hr. Approximately 25% of this recovered drug is in the form of the 5′-phosphate, a quantity considered adequate to completely inhibit the inosine monophosphate dehydrogenase in the cells. Temporary inhibition of this enzyme, which is important in the synthesis of nucleic acid, has a minimal effect on host cells, since cells have a variety of alternate repair pathways for maintaining the integrity of their host DNA. These metabolism and biochemical data suggest ribavirin to be suitable for use in treatment of infections such as that induced by FLV.

Summary. Ribavirin, when administered intraperitoneally to mice infected with Friend leukemia virus, significantly inhibited development of the disease as determined by inhibition of virus-induced splenomegaly and viable virus titers in the spleen and plasma of the infected animals. The drug was effective whether administered ip in multiple daily treatments, treatments every three days, or in a single injection. Greatest efficacy was seen when therapy began early in the infection, presumably while the virus was in its eclipse phase.

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1. Sidwell, R. W., Huffman, J. H., Khare, G. P., Allen, L. B., Witkowski, J. T., and Robins, R. K., Science **177**, 705 (1972).
2. Witkowski, J. T., Robins, R. K., Sidwell, R. W., and Simon, L. N., J. Med. Chem. **15**, 1150 (1972).
3. Suganuma, T., and Ishida, N., Chemotherapy (Jap.) **20**, 795 (1972).
4. Suganuma, T., and Ishida, N., Tohoku J. Exp. Med. **110**, 405 (1973).
5. Huffman, J. H., Sidwell, R. W., Khare, G. P., Witkowski, J. T., Allen, L. B., and Robins,

R. K., Antimicrob. Ag. Chemother. **3,** 235, (1973).
6. Togo, Y., Antimicrob. Ag. Chemother. **4,** 641 (1973).
7. Sidwell, R. W., Allen, L. B., Khare, G. P., Huffman, J. H., Witkowski, J. T., Simon, L. N., and Robins, R. K., Antimicrob. Ag. Chemother. **3,** 242 (1973).
8. Khare, G. P., Sidwell, R. W., Witkowski, J. T., Simon, L. N., and Robins, R. K., Antimicrob. Ag. Chemother. **3,** 517 (1973).
9. Simon, L. N., Sidwell, R. W., Khare, G. P., Streeter, D. G., Miller, J. P., Witkowski, J. T., Huffman, J. H., and Robins, R. K., *in* "Virus Research" (C. F. Fox and W. S. Robinson, eds.), p. 415. Academic Press, New York (1973).
10. Sidwell, R. W., Simon, L. N., Witkowski, J. T., and Robins, R. K., *in* "Proceedings of the Eighth International Congress of Chemotherapy" *in press* (1975).
11. Mirand, E. A., Prentice, T. C., Hoffman, J. G., and Grace, J. T., Jr., Proc. Soc. Exp. Biol. Med. **106,** 423 (1961).
12. Mirand, E. A., Hoffman, J. G., Grace, J. T. Jr., and Trudel, P. J., Proc. Soc. Exp. Biol. Med. **107,** 824 (1961).
13. Sidwell, R. W., Dixon, G. J., Sellers, S. M., Chirigos, M. A., and Schabel, F. M., Jr., Cancer Chemother. Rep. **48,** 31 (1965).
14. Sidwell, R. W., Dixon, G. J., Sellers, S. M., Maxwell, C. F., and Schabel, F. M., Jr., Proc. Soc. Exp. Biol. Med. **119,** 1141 (1965).
15. Sidwell, R. W., Dixon, G. J., Sellers, S. M., and Schabel, F. M., Jr., Cancer Chemother. Rep. **50,** 299 (1966).
16. Chirigos, M. A., Luber, E., March, R., and Pettigrew, H., Cancer Chemother. Rep. **45,** 29 (1965).
17. Rossi, G. B., Cudkowicz, G., and Friend, C., J. Nat. Cancer Inst. **50,** 249 (1973).
18. Martin, G. S., Nature (London) **227,** 1021 (1970).
19. Streeter, D. G., Witkowski, J. T., Khare, G. P., Sidwell, R. W., Bauer, R. J., Robins, R. K., and Simon, L. N., Proc. Nat. Acad. Sci. U.S.A. **70,** 1174 (1973).
20. Chirigos, M. A., and March, R. W., *in* "Antimicrobial Agents and Chemotherapy—1966" (G. L. Hobby, ed.), p. 489. American Society Microbiology, Ann Arbor, Michigan (1967).
21. Metcalf, D., Furth, J., and Buffett, R. F., Cancer Res. **19,** 52 (1959).

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