Reduction of Antileukemic and Immunosuppressive Activities of 5-Azacytidine in Mice by Concurrent Treatment with Uridine[1] (34660)

S. VADLAMUDI, M. PADARATHSINGH, E. BONMASSAR, AND A. GOLDIN

Microbiological Associates, Inc., and National Cancer Institute, Bethesda, Maryland 20014

A cytidine analog, 5-azacytidine (NSC-102816, 5-AzaCyd), was shown to be active against *E. coli* bacteria in culture and against lymphomas in AKR mice (1, 2). Treatment of mice with the drug suppressed DNA and RNA syntheses of large lymphocytes and mature myeloid cells (3). Also, incorporation of the drug into RNA of bone marrow cells was observed in mice treated with the drug (4). The drug was reported (5, 6) to possess antileukemic activity against L1210 in mice. Also, reversal of the antileukemic and toxic activities of 5-AzaCyd by simultaneous treatments with cytidine was demonstrated (5, 7). Immunosuppression by pyrimidine nucleoside analogs and the inhibition of immunosuppressive effect of cytosine arabinoside by concurrent treatment with deoxycytidine has been reported (8).

The following experiments were conducted to study (a) the effect of administration of 5-AzaCyd and uridine (NSC20256) on the antileukemic activity and toxicity of the drug in leukemic (L1210) and nonleukemic mice; (b) the effect of treatment with 5-AzaCyd and uridine on the bone marrow cells and colony-forming ability of these cells in nonleukemic mice; and (c) the effect of concomitant administration of 5-AzaCyd and uridine on hemagglutinin synthesis and hemolytic plaque-forming ability of the spleen cells of nonleukemic mice.

Materials and Methods. Ten to 12-week-old CDF_1 (BALB/c female $\times$ DBA/2 male) F_1 male mice, weighing 22–25 g, were employed in the studies on survival time, toxicity, and suppression of hemagglutinins; and BDF_1 (C57BL/6 female $\times$ DBA/2 male)F_1 male mice were employed for the assay of hemolytic plaque-forming cells (PFC). Six to 8-week-old BALB/c male mice were used as recipients in the spleen colony assay. CDF_1 male mice were inoculated subcutaneously (sc) into the right hind leg with 10^6/0.25 ml of leukemia (L1210) ascites cells. The leukemia (L1210) ascites line is maintained in this laboratory by weekly intraperitoneal (ip) passage in DBA/2 mice.

5-AzaCyd was suspended in steroid suspending vehicle and the pH was adjusted to 4.5. Uridine (Calbiochem) was dissolved in saline, pH 4.5. The drugs were injected intraperitoneally (ip) at a total volume of 0.01 ml/g of body weight. In combination treatment administration of 5-AzaCyd was followed within 5 min by uridine.

Six nonleukemic CDF_1 mice from each group were sacrificed 24 hr after treatment with the drugs, the femurs were removed, a 3-ml bone marrow cell suspension was prepared, and the cell concentrations of these suspensions were determined by employing a Coulter counter Model F. Bone marrow cell suspensions were adjusted to 10^5 cells/0.2 ml and injected intravenously (iv) into 6 BALB/c mice, which had been X-irradiated previously with 400 R (200 kV, 15 mA, 0.25 mm Cu and 0.55 mm Al filtration; target distance 54 cm, dose rate 122 R/min). Eight days after inoculation of the cells, recipient mice were sacrificed, the number of spleen colonies in 6 mice were counted, and the colony-forming units (CFU) per milliliter of cell suspension were estimated as described previously (9). The number of surviving

[1] Part of this study was presented by the authors at the Expermental Hematology Bone Marrow Conference held in Atlantic City, April 14, 1969. Supported in part by Contract PH43-68-1283 from Chemotherapy, National Cancer Institute, NIH.

cells and CFU as a fraction of untreated control values were estimated.

In order to study the influence of uridine on the immunosuppressive effect of 5-AzaCyd, nonleukemic mice were injected with 0.25 ml ip of 30% sheep erythrocytes (SRBC) 24 hr before or after treatment with the drugs, and were bled at designated intervals. Sera from 6 mice from each group were pooled and tested for hemagglutination (HA) titer by the micro-HA method described by Stavitsky (10).

The assay for direct PFC test was carried out according to the method of Jerne *et al.* (11), with some modifications suggested by Shearer *et al.* (12) in preparing spleen cell suspensions.

BDF_1 mice were immunized with 4×10^8 SRBC and treated 24 hr later with 5-AzaCyd alone, or in combination with uridine. Four mice were sacrificed on days 2, 6, 8, and 11, and 8 mice on day 4 for the PFC test.

Results. The influence of treatment with uridine on the effect of 5-AzaCyd in leukemic or nonleukemic mice is summarized in Table I. Treatment with 5-AzaCyd (20–90 mg/kg) alone increased the median survival time (MST) of leukemic mice by 57–79% over the untreated controls (7 days). Doses of 150 and 250 mg/kg of 5-AzaCyd were lethal to leukemic or nonleukemic mice. Combination treatment with uridine (600 or 360 mg/kg) reduced the antileukemic activity of 5-AzaCyd (doses 20–54 mg/kg) given

TABLE I. Influence of Concurrent Treatment with Uridine on the Effect of 5-AzaCyd in Leukemic and Nonleukemic Mice.[a]

	Leukemic mice[b] (L1210)						Nonleukemic mice	
	5-AzaCyd		5-AzaCyd + uridine[b] (600 mg/kg)		5-AzaCyd + uridine[b] (360 mg/kg)		5-AzaCyd alone	5-AzaCyd + uridine[b] (600 mg/kg)
Treatment dose (mg/kg)	MST (days)	ILS[c] (%)	MST (days)	ILS[c] (%)	MST (days)	ILS[c] (%)	No. of mice surviving Day 70	
Untreated controls (L1210)	7 (6–8)	0					8	
5-AzaCyd (mg/kg)								
20	11 (9–13)	57	8.5 (8–10)	21	9 (8–11)	29	8	8
33	11.5 (9–13)	64	8 (8–11)	14	10 (8–11)	43	8	8
54	12.5 (6–14)	79	10 (8–13)	43	11 (10–13)	57	8	8
90	12 (4–14)	71	11 (9–11)	57	12 (7–24)	71	8	8
150	5.5 (4–13)		11 (7–11)	57	12.5 (11–22)	79	4 (2–4)	8
250	2 (1–2)		7.5 (3–12)	7	7 (3–13)	0	0 (1–3)	7 (2)
Vehicle control	7.5 (7–8)	7					8	
Uridine			8 (7–8)	14	8 (7–9)	14		8

The values in parentheses show the range of days of death of animals.

[a] 10–12-week-old CDF_1 male mice 8/group—leukemic mice inoculated subcutaneously with 10^6/0.25 ml of leukemic (L1210) ascites cells.

[b] Concurrent treatment intraperitoneally: 5-AzaCyd followed by uridine.

[c] ILS = increase in life-span over untreated controls.

alone. The MST of leukemic mice was almost similar when the drug was given at 90 mg/kg alone or in combination with uridine. At the higher doses (150 and 250 mg/kg) the toxicity of the drug was reduced by combination treatment with uridine. The increase in survival time of mice treated with 150 mg/kg of 5-AzaCyd plus 360 mg/kg of uridine was 79%, which was similar to the increased lifespan (ILS) obtained with 54 mg/kg of 5-AzaCyd alone. In nonleukemic mice, this concurrent treatment with uridine also reversed the toxicity of 5-AzaCyd (150 or 250 mg/kg) and is reflected in the number of 70-day survivors observed in the groups treated with these doses (15/16, compared to those in the groups treated with 5-AzaCyd alone, 4/16).

The results of the influence of combination treatment with uridine on the effect of 5-AzaCyd on bone marrow cells and CFU of mice sacrificed 1 day after drug administration are summarized in Fig. 1. The surviving bone marrow cells in mice on day 2 after treatment with 5-AzaCyd alone (20–250 mg/kg) varied from 30 to 6% of the untreated control values, while the ability of these cells to form CFU was only 2% of the untreated control values. Treatment with 600 mg/kg of uridine reduced the toxic effect of the drug on bone marrow cells at all dose levels of 5-AzaCyd. The surviving cells varied from 65 to 18% of the untreated control values. Although similar elevation of CFU was observed in the groups treated with uridine plus the lower dose levels of 5-AzaCyd (20–90 mg/kg), the colony-forming ability of bone marrow cells of mice treated with 150 or 250 mg/kg remained suppressed.

The results of the effect of treatment with 5-AzaCyd alone, or 5-AzaCyd plus uridine, on hemagglutinin synthesis in mice are summarized in Fig. 2A and B. A single treatment with 5-AzaCyd (doses 20–250 mg/kg) reduced the hemagglutinin synthesis in nonleukemic mice given before or after SRBC. The HA titer of the sera from the groups treated after SRBC (Fig. 2A) varied from 1:128 to 1:16, and the titer in the untreated controls was 1:256. The lowest titer, 1:16, was observed in the sera of mice treated with 150 mg/kg of 5-AzaCyd. Similar results were observed when mice were injected with the drug 1 day before antigenic stimulation (Fig. 2B). Treatment with cyclophosphamide (250 mg/kg) before or after SRBC was more effective than 5-AzaCyd in suppressing immune response in mice. Uridine (600 mg/kg) alone had no effect on hemagglutinin synthesis as reflected in the HA titers of sera. However, in mice treated with a combination of 600 mg/kg of uridine and 20–250 mg/kg of

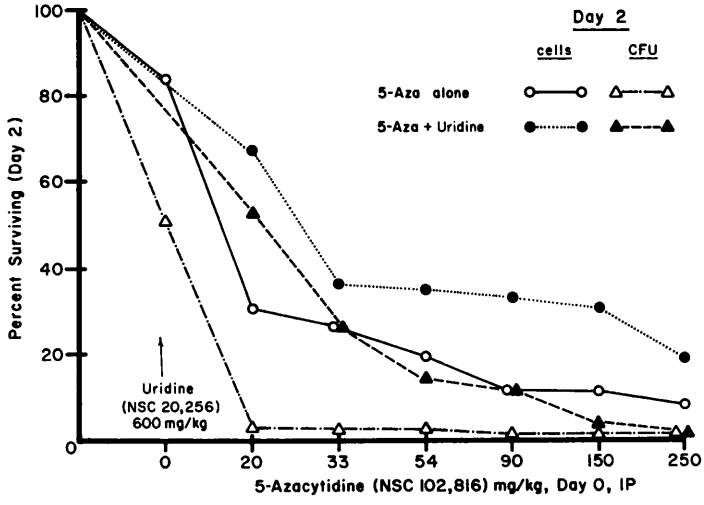

FIG. 1. Influence of concurrent treatment with uridine on the effect of 5-AzaCyd on bone marrow cells and colony-forming units in nonleukemic mice.

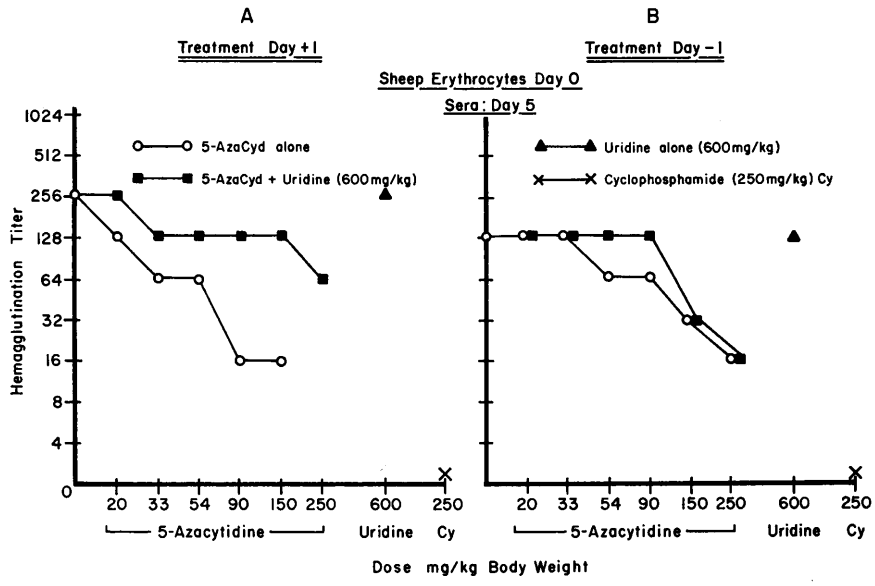

FIG. 2. Influence of combination treatment with uridine on the immunosuppressive effect of 5-AzaCyd in nonleukemic mice.

5-AzaCyd after SRBC (Fig. 2A), the reciprocal HA titers ranged from 256 to 64, indicating the reduction of the effect of 5-AzaCyd on HA synthesis. Although treatment with uridine appeared to reduce the immunosuppression of 5-AzaCyd at all dose levels when the drugs were given 1 day after antigenic stimulation, such reduction was less evident when the drugs were given before SRBC (Fig. 2B).

The observation of suppression of antibody synthesis in mice after treatment with 5-AzaCyd was explored further by employing the PFC method with 3 dose levels of 5-AzaCyd (54, 90, and 150 mg/kg) alone or in combination with 600 mg/kg of uridine.

The results of the effect of treatment with uridine (600 mg/kg) alone, or in combination with 5-AzaCyd, on PFC response at several intervals after the administration of the drug is shown in Fig. 3. The maximum PFC response in the spleens of untreated control mice and the mice treated with uridine (600 mg/kg) was observed on day 4. This effect gradually tapered off by day 11 to the level observed on day 2. It appears that treatment with uridine alone had not markedly influenced the PFC response in mice.

The results of the effect of treatment with 5-AzaCyd alone, or in combination with uridine, on PFC response in mice are summarized also in Fig. 3. Treatment with 5-AzaCyd (54, 90, or 150 mg/kg) greatly reduced PFC on day 4 at the time when a maximum PFC response was observed in the untreated controls. On day 6 the number of PFC in the drug treated animals was still at a lower level than in the controls. On day 8 the number of PFC in the treated mice reached the peak levels and was found to be close to that of the controls. In mice treated with uridine and 5-AzaCyd, the PFC response was evident earlier and the maximum response was observed on day 6 with 54 or 90 mg/kg of 5-AzaCyd, and on day 8 in the groups treated with 150 mg/kg of 5-AzaCyd. Although the number of PFC in animals with the combination treatment was greater than that in animals treated with 5-AzaCyd alone, it was less than in the untreated controls. These results indicate that treatment with uridine (600 mg/kg) had reduced the immunosuppressive effect of 54 or 90 mg/kg of 5-AzaCyd by partially restoring the number of PFC per spleen and shifting the peak of antibody response from day 8 to day 6. This effect was less evident with 150 mg/kg of 5-AzaCyd.

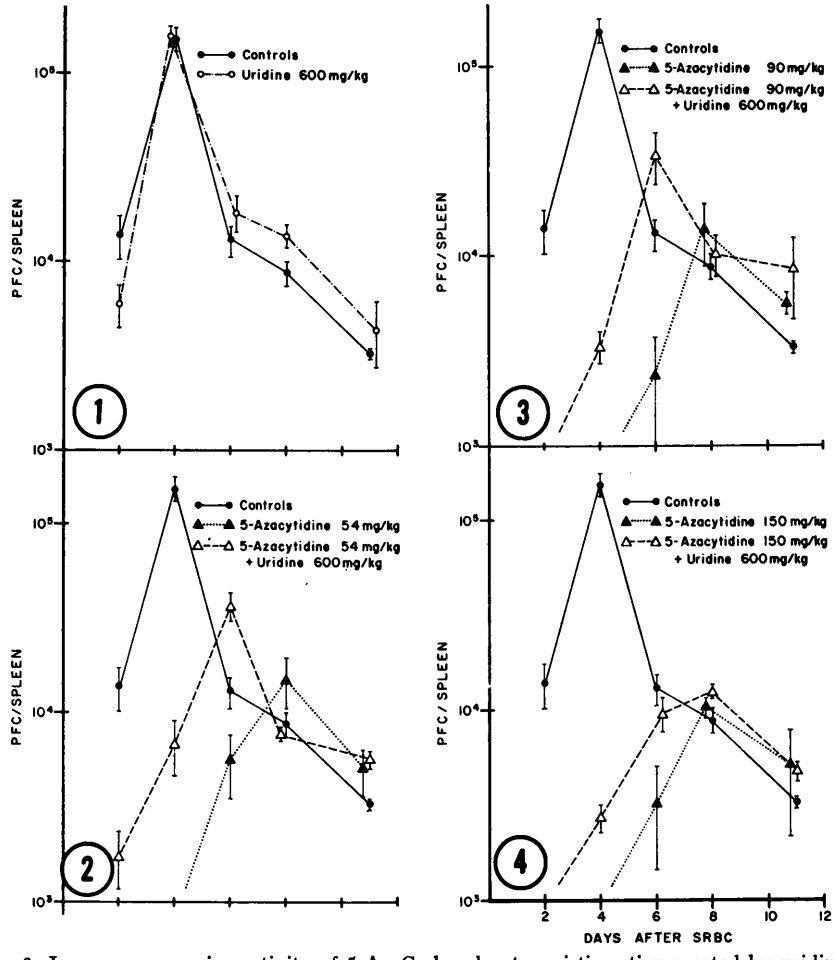

FIG. 3. Immunosuppressive activity of 5-AzaCyd and antagonistic action exerted by uridine: PFC per spleen of BDF_1 male mice immunized with 4.10^8 SRBC, ip, and treated 24 hr later with a single injection of 5-AzaCyd alone, or 5-AzaCyd plus uridine. Each point represents the mean ± SE of four animals on days 2, 6, 8, and 11, and of 8 animals on day 4.

Discussion. Mammalian cells phosphorylate 5-AzaCyd and further convert it into 5′-polyphosphates, which are in turn incorporated into ribonucleic acids (13). The striking biological activity of 5-AzaCyd was attributed to the low stability of the 5′-polynucleotides (14). The antileukemic (L1210) and toxic effect of 5-AzaCyd, and the reversal of these activities by combination treatment with cytidine, have been reported before (5, 7). The data presented here show that treatment with a single dose of 5-AzaCyd increased the life-span of leukemic mice by 79% over the untreated controls. At the same time this treatment diminished the number of surviving bone marrow cells and the colony-forming ability of these cells. Also, treatment with 150 or 250 mg/kg of 5-AzaCyd was lethal to leukemic or nonleukemic mice. Treatment with uridine reduced the toxic activity of 5-AzaCyd (20–90) mg/kg on bone marrow cells and CFU of nonleukemic mice, and also diminished the lethal effect of higher doses of 5-AzaCyd (150 and 250 mg/kg). Only 1/16 mice died in the groups treated with the combination of drugs, whereas 12/16 mice died in the groups treated with 5-AzaCyd alone.

Since 5-AzaCyd is a cytotoxic agent and capable of inhibiting RNA and DNA synthe-

sis, this agent would be expected to suppress the antibody formation. The data reported here indicate that the activity of 5-AzaCyd against normal rapidly dividing cells is accompanied by immunosuppressant action given before or after the injection of SRBC. The immunosuppressive activity of the drug when given before the antigen suggests the possibility that the effect of the drug is on the antigen processing activity of macrophages (14). The incorporation of 5-AzaCyd into the RNA of macrophages with the concurrent formation of fraudulent RNA would prevent the transfer of information from macrophages to the antibody forming cells, mediated by a mRNA (15) or RNA-antigen complex (16). The immunosuppressant activity of the drug given after the antigenic stimulus, as shown in HA and PFC tests, suggest that the antimetabolite may also be able to interfere with the proliferation of immunocompetent cells. In particular, the delay in PFC peak response (from days 4 to 8) is consistent with similar observations with methotrexate by Rivarola *et al.* (17). Furthermore, the total reduction in PFC per spleen suggests the possibility that the majority of the immunocompetent cells were completely inhibited by 5-AzaCyd.

The antagonistic activity of uridine is evident when the drugs are given after SRBC injection, as observed with the HA and PFC tests. In the latter test the influence exerted by uridine may be observed on the number of PFC per spleen and the extent of delay in the peak PFC response. The antagonistic activity of uridine on immunosuppressive activity of 5-AzaCyd was less evident when these drugs were given before the antigen in the HA test, suggesting the possibility that the biochemical pathway involved in the early steps of antibody response is less sensitive to the protective activity of uridine. The control of toxicity and reduction of immunosuppressive activities of 5-AzaCyd by uridine suggests the need to explore the temporal aspects of treatment with a metabolite and an antimetabolite.

Summary. Investigations were carried out to determine the influence of concurrent treatment with uridine on the antileukemic, toxic, and immunosuppressive effects of 5-AzaCyd, a synthetic analog of cytidine. A single treatment on day 1 after tumor implantation with 5-AzaCyd alone (20–90 mg/kg) increased the MST of leukemic mice by 57–79% over the untreated controls. Combination treatment with uridine reduced the antileukemic activity of 5-AzaCyd. Treatment with 150 or 250 mg/kg of 5-AzaCyd was lethal to leukemic as well as nonleukemic mice. Concurrent treatment with uridine reduced the toxic effect of 5-AzaCyd in these mice.

A single treatment with 5-AzaCyd alone (20–250 mg/kg) diminished the surviving bone marrow cells 30 to 6% and CFU to 2% of the untreated control values. Treatment with uridine reduced the toxic effect of 5-AzaCyd on the bone marrow cells and CFU. Similarly, a single treatment with 5-AzaCyd (33–150 mg/kg) inhibited the hemagglutinin synthesis and hemolytic plaque-forming ability of the spleen cells of nonleukemic mice. In hemagglutination tests simultaneous administration of uridine reduced the immunosuppressive effect of 5-AzaCyd to a greater extent when the drugs were administered after, rather than before, SRBC administration. Furthermore, treatment with uridine partially reversed the effect of 5-AzaCyd on hemolytic plaque-forming cells.

The authors gratefully acknowledge the excellent technical assistance of Mrs. M. A. Hamilton and Mr. B. Krishna.

1. Piskala, A., and Sorm, F., Collect. Czech. Chem. Commun. **29**, 2060 (1964).

2. Sorm, F., Piskala, A., Cihak, A., and Vesely, J., Experientia **20**, 202-203 (1964).

3. Vesely, J., Cihak, A., and Sorm, F., Int. J. Cancer **2**, 639–646 (1967).

4. Vesely, J., and Sorm, F., Neoplasma **12**, 3 (1965).

5. Evans, J. S., and Hanka, L. J., Experientia **24**, 922–923 (1968).

6. Vadlamudi, S., and Goldin, A., J. Exp. Hematol. **17**, 47–51 (1968).

7. Vadlamudi, S., Choudry, J. N., Waravdekar, V. S., Kline, I., and Goldin, A., Cancer Res., in press.

8. Fischer, D. S., Cassidy, E. P., and Welch, A. D., Biohem. Phamacol. **15**, 1013 (1966).

9. Vadlamudi, S., Waravdekar, V. S., Choudry, J. N., and Goldin, A., Cancer Res. **28**, 1242–1249 (1968).

10. Stavitsky, A. B., J. Immunol. **72**, 360 (1954).

11. Jerne, N. K., and Nordin, A. A., Science **140**, 405 (1963).

12. Shearer, G. M., Cudkowicz, G., Connell, M. St. J., and Priore, R. L., J. Exp. Med. **128**, 437 (1968).

13. Jurovcik, J., Raska, K., Sorm, F., and Sormova, Z., Collect. Czech. Chem. Commun. **30**, 3370–3376 (1965).

14. Pithova, P., Piskala, A., Pitha., J., and Sorm, F., Collect. Czech. Chem. Commun. **30**, 2801–2811 (1965).

15. Fishman, M., and Adler, F. L., J. Exp. Med. **117**, 595 (1963).

16. Akonas, B. A., and Rhodes, J. M., Nature (London) **205**, 470 (1965).

17. Rivarola, A., Friedman, M., and Lawrence, W., Jr., Transplantation **5**, 1223 (1967).

Received Sept. 5, 1969. P.S.E.B.M., 1970, Vol. 133.