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ALTERATIONS IN [^{3}H]THYMIDINE INCORPORATION INTO DNA AND [^{3}H]URIDINE INCORPORATION INTO RNA INDUCED BY 5-AZACYTIDINE *IN VIVO*

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Abstract—Administration *in vivo* of 5-azacytidine (5-aza-CR) caused suppression of [^{3}H]thymidine ([^{3}H]TdR) incorporation into DNA of bone marrow and gastrointestinal mucosa of mice and a more prolonged suppression of L1210 ascites tumor. Single doses of 5-aza-CR caused a modest and short-lived suppression of incorporation of [^{3}H]uridine ([^{3}H]UR) into nuclear RNA of L1210 ascites tumor cells. No suppression of [^{3}H]UR incorporation into RNA of bone marrow or gastrointestinal mucosa was observed. L1210 tumor cells resistant to the other active cytidine analogue, cytosine arabinoside, demonstrated less disruption of [^{3}H]TdR incorporation after exposure to 5-aza-CR, suggesting some cross resistance in the effects of these two drugs on DNA synthesis. Survival studies carried out in mice bearing both the sensitive and resistant L1210 tumor cell lines confirmed cross resistance of the anti-tumor effects of the two cytidine analogues. Second doses of 5-aza-CR, with the timing of administration based upon the differing patterns of recovery of [^{3}H]TdR incorporation between normal tissues and tumor cells, led to a prolongation of survival in mice bearing the sensitive L1210 ascites tumor.

5-Azacytidine (5-aza-CR) is an analogue of cytidine first synthesized by Cihak [1]. It has been shown to possess anti-tumor effects in the L1210 tumor system [2–4], and further to be active in human adult acute myelogenous leukemia [5, 6]. We have also noted activity in some patients in the blastic phase of chronic myelogenous leukemia (unpublished data).

The mechanism of action of 5-aza-CR is not completely understood, but various studies have shown that it interferes with DNA, RNA and protein synthesis [3, 7–9]. In the L1210 ascites tumor, Li *et al.* [3] showed that DNA synthesis was altered to a greater extent than RNA synthesis even though studies with ^{14}C-labeled 5-aza-CR showed major incorporation into newly synthesized RNA.

Scheduling of therapy with 5-aza-CR has been largely empiric. Presently, 5-aza-CR is usually given to patients as a daily infusion for 5 days. Previous studies of [^{3}H]thymidine ([^{3}H]TdR) incorporation *in vivo* using other chemotherapeutic agents in the L1210 system have shown that following differences in the sensitivity between normal and tumorous tissues allowed sequencing of dosages that increased survival while reducing toxicity [10, 11].

The present study was undertaken to quantitate both degree and duration of alterations in DNA and RNA synthesis in normal and tumorous tissues after exposure to 5-aza-CR; to utilize any differences in the anti-metabolic effects of 5-aza-CR on normal and tumorous tissues in scheduling subsequent drug dosages; and to look at the ability of 5-aza-CR to inhibit the growth of a tumor cell line known to be resistant to the other active cytidine analogue, cytosine arabinoside.

EXPERIMENTAL

BDF_1 female mice of an average weight of 20 g were obtained from Hazelton Labs, Vienna, VA. They were maintained in a constant temperature environment in plastic cages, and fed standard lab chow and water *ad lib.* L1210 murine leukemia carried intraperitoneally (i.p.) was used as the tumor source and transplanted to recipient mice by injecting 1×10^6 cells i.p. Studies were begun on day 5 of tumor growth (which means injections were carried out the beginning of day 6 or after the inoculum had been growing for a full 5 days). BDF_1 female mice of comparable age and weight were used as normal controls. All mice were sacrificed by cervical dislocation. Cytosine arabinoside-resistant L1210 ascites tumor was obtained from Arthur D. Little, Inc., Cambridge, MA. Resistance was induced by continuous exposure of L1210 cells to low doses of ara-C and was confirmed by both survival and [^{3}H]TdR incorporation studies. Wodinsky and Kensler [12] developed the ara-C-resistant cell line which continued to show resistance in transplant generations 10–32. Using doses of ara-C of 10–160 mg/kg, the sensitive L1210 showed 205 per cent increased life span while the resistant L1210 cell line showed only 94 per cent increased life span. The mean day of death in control animals was 9.1 for normal L1210 and 8.0 for ara-C-resistant L1210. [^{3}H]TdR studies performed in our laboratory using a dosage of 1000 mg/kg of ara-C i.p. caused suppression of [^{3}H]TdR incorporation into DNA of the resistant cell line to 11 per cent of control with recovery to baseline values complete by 24 hr. In contrast, [^{3}H]TdR incorporation into DNA of the sensitive

cell line was suppressed to 1 per cent of control with recovery delayed to 96 hr. Using this resistant L1210 cell line, animals were implanted with 2×10^5 cells i.p. and studies were begun on day 5 of tumor growth.

Specific activity of DNA. Six tumor-bearing mice were left untreated and served as controls. Treated animals were given 25, 50 and 100 mg/kg of 5-aza-CR either intraperitoneally or intravenously via the tail vein. At times 1, 6, 12, 24, 36, 48, 72, 96, 144, 168 and 192 hr after drug administration, groups of six animals at each time point were given 100 μCi (0.1 ml) of undiluted [^{3}H]TdR (sp. act. 6.7 Ci/m-mole; New England Nuclear, Boston, MA) i.p. and 1 hr later were sacrificed. This method had been previously found to give the most reproducible results in the L1210 model as compared to the use of other dosages of [^{3}H]TdR and other incorporation times [10, 11]. Ascites tumor was aspirated from the peritoneal cavity with a Pasteur pipette and then washed with iced phosphate-buffered 0.85% saline (P.B.S.). A 3-cm piece of duodenum was removed, washed free of ascites, split lengthwise, and the mucosa stripped free with a clean glass slide. The mucosal tissue was suspended in iced P.B.S. Bone marrow cells were harvested from both tibias of each mouse by removing the tibia, en bloc, stripping the attached muscles, sectioning the bone at each end, and expressing the marrow with a 25-gauge needle and a syringe of iced P.B.S. Samples from three animals were pooled and were spun at 800 *g* for 5 min at 4°. The supernatant was discarded and the resulting cell buttons were frozen for later processing.

The DNA in each sample was extracted using a modification of the technique of Schneider [13]. One 0.5-ml aliquot of the resulting supernatant was added to 10 cm^3 Aquasol (New England Nuclear, Boston, MA) and counted in a Packard Tri-Carb liquid scintillation spectrometer. A second 0.5-ml aliquot was processed by the method of Burton [14] for DNA content. The efficiency of tritium counting was 26 per cent, and the results were expressed as dis./min/μg of DNA. Results are graphed as a percentage of control over time.

Specific activity of RNA. As in the DNA determinations, six tumor-bearing mice were left untreated and served as controls. Groups of six animals were selected at 1, 6, 12, 24, 36, 48, 72, 96, 120 and 144 hr after i.p. injection of 50 mg/kg of 5-aza-CR and injected i.p. with 100 μCi (0.1 ml) of undiluted [^{3}H]uridine ([^{3}H]UR) (sp. act. 40–50 Ci/m-mole; New England Nuclear, Boston, MA). Animals were sacrificed 45 min after injection of the [^{3}H]UR as this was the optimal time for highest levels of [^{3}H]UR incorporation. Bone marrow, duodenal mucosa and ascites tumor were harvested as described above. RNA in each sample was extracted from isolated nuclei only, by methods described by Hogeboom [15] and Schmidt and Thannhauser [16]. A 0.5-ml aliquot of the resulting supernatant was placed in 10 ml Aquasol and counted as before in a liquid scintillation spectrometer. A second 0.5-ml aliquot was analyzed in a Coleman, Jr. spectrometer at 260 μm and compared to a standard curve to determine RNA content. Again, results are graphed as percentage of control over time.

Toxicity studies. BDF_1 female mice were used of approximately the same age and weight as in survival studies; however, these animals were tumor-free. Ten to twenty animals per group were used, and the amount, route and times of drug administration were the same as in survival studies. Results are expressed as percentage of mortality.

Survival studies. BDF_1 female mice bearing L1210 ascites tumor (initial inoculum 1×10^6 cells) were treated with 25, 50 and 100 mg/kg of 5-aza-CR on day 5 of tumor growth. Second doses of 5-aza-CR were administered at 0, 6, 12, 24, 36, 48, 72, 96, 120, 144, 168 and 192 hr after the initial dose. Ten to twenty mice were used in each group. Untreated animals served as controls. Results are expressed as percentage of survivors.

RESULTS

Alterations in DNA synthesis in bone marrow, gastrointestinal mucosa and ascites tumor. Average activities ± standard deviations of control specimens are 70,000 ± 12,500 dis./min/μg of DNA for ascites tumor, 950 ± 100 dis./min/μg of DNA for bone marrow and 500 ± 40 dis./min/μg of DNA for GI mucosa. The simultaneous changes in DNA synthesis, reflected by changes in incorporation of [^{3}H]TdR, are shown in Fig. 1. Shown are alterations induced by 25, 50 and 100 mg/kg of 5-aza-CR, given i.p. on day 5 of tumor growth (LD_{10} = 80 mg/kg as single i.p. dose). Looking at the bone marrow as an example, one can see that: (1) the nadirs of [^{3}H]TdR incorporation occur at 12–24 hr after drug exposure at all three dosages; (2) the magnitude of this inhibition when expressed as percentage of control is 47, 29 and 14 per cent at doses of 25, 50 and 100 mg/kg respectively; and (3) return toward normal levels of [^{3}H]TdR incorporation occurs at 24 hr after drug exposure and is complete by 48–72 hr. For the gastrointestinal mucosa, the nadirs are at 6–12 hr. The magnitude of inhibition is 79, 56 and 7 per cent of control at doses of 25, 50 and 100 mg/kg, respectively, and return toward normal levels occurs between 12 and 24 hr. Tumor cells have nadir inhibition at 12 hr, achieving magnitudes of inhibition of 2, 2 and 1 per cent of control at 25, 50 and 100 mg/kg respectively. Return toward normal levels is prolonged to greater than 96 hr after drug exposure at each of the three doses studied. Apparently, then, dosage escalation causes a more profound inhibition of [^{3}H]TdR incorporation but does not affect the time course at which maximum inhibition or return toward normal levels of [^{3}H]TdR incorporation occurs.

Because of the prolonged period of inhibition of [^{3}H]TdR incorporation seen in the ascites tumor, the study was repeated for a longer period of time after drug exposure. Figure 1 shows the effects of a single 50 mg/kg i.p. dose of 5-aza-CR on the same three tissues followed for a period up to 192 hr. Even as late as 168–92 hr after drug therapy, incorporation of [^{3}H]TdR into tumor cells has not recovered to pretreatment levels, whereas normal tissues display [^{3}H]TdR incorporation levels which exceed baseline values.

To assure that the prolonged low level of [^{3}H]TdR incorporation into tumor cells was due to drug effect and not the persistence of dead ascitic cells in the

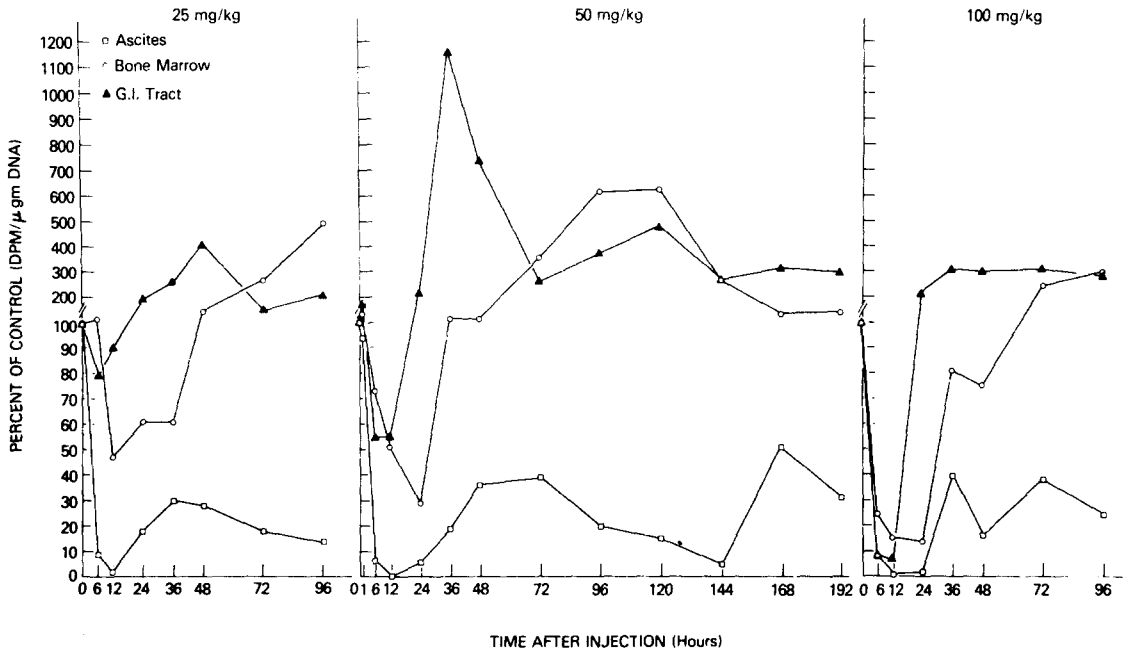

Fig. 1. Alternations in [^{3}H]TdR incorporation into DNA of L1210 ascites tumor, bone marrow and gastro-intestinal mucosa due to 25, 50 and 100 mg/kg of 5-aza-CR i.p. in BDF_1 mice. Control incorporation rates were 70,000 ± 12,500 dis./min/μg of DNA for ascites tumor, 950 ± 100 dis./min/μg of DNA for bone marrow and 500 ± 40 dis./min/μg of DNA for gastrointestinal mucosa.

peritoneal cavity, a Trypan Blue dye exclusion study was carried out. At all time points after drug therapy, the cellular milieu of the peritoneal cavity was 95 + % ascites tumor cells histologically, and Trypan Blue exclusion did not fall below 98 per cent.

Since another explanation for the apparent prolonged inhibition of [^{3}H]TdR incorporation into tumor cells could be the high drug concentration attained in the peritoneal cavity with i.p. administration, a separate experiment was carried out in which the drug was i.v. administered. Figure 2 shows the effects of 50 mg/kg of 5-aza-CR given by the i.v. route. As can be seen, [^{3}H]TdR incorporation into bone marrow and gastrointestinal mucosa is inhibited to a greater extent with i.v. drug than with i.p. drug, suggesting that higher tissue drug levels are attained using the i.v. route. [^{3}H]TdR incorporation into tumor cells is inhibited to a lesser extent with i.v. drug administration than with i.p. administration. Apparently lower i.p. drug levels with i.v. drug administration are reflected by less inhibition of [^{3}H]TdR incorporation into tumor cells, but as before, the time course at which maximum suppression and recovery of [^{3}H]TdR incorporation occur is not dramatically different for the i.p. and i.v. routes of administration. In other words, the magnitude of inhibition of [^{3}H]TdR incorporation into tumor cells is greater with an i.p. drug route than with an i.v. route, but the prolonged time period during which [^{3}H]TdR incorporation is inhibited in tumor cells is not a function of the higher drug level attained with i.p. drug administration.

Alterations of RNA synthesis in bone marrow, gastrointestinal mucosa and ascites tumor. Average activities of control specimens are 13,500 ± 290 dis./min/absorbance unit at 260 nm for ascites tumor, 25 ± 12 dis./min/absorbance unit at 260 nm for marrow and 25 ± 8 dis./min/absorbance unit at 260 nm for GI mucosa. Previous studies had indicated that 5-aza-CR exerted its primary anti-metabolic function through alterations in RNA synthesis [3]. Figure 3 shows the effects of a single 50 mg/kg dose of 5-aza-CR on rates of RNA synthesis as reflected by

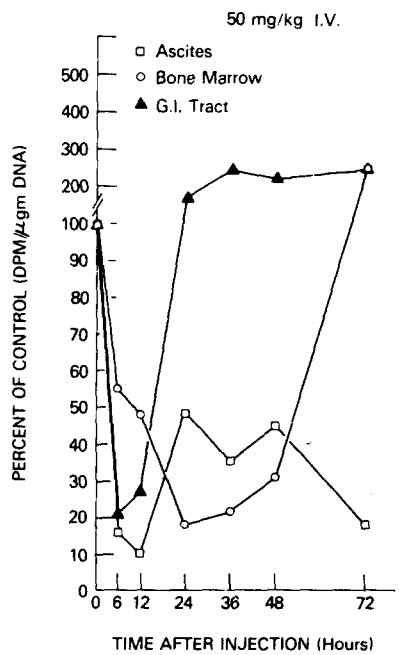

Fig. 2. Alterations in [^{3}H]TdR incorporation into DNA of L1210 ascites tumor, bone marrow and gastrointestinal mucosa due to 50 mg/kg of 5-aza-CR i.v. in BDF_1 mice.

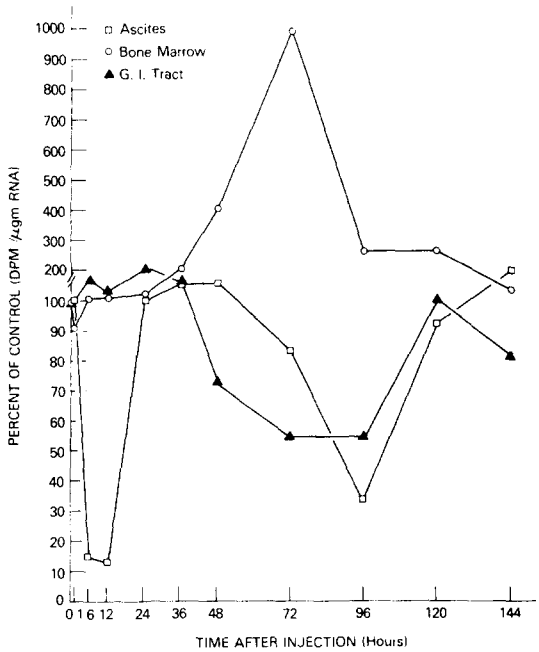

Fig. 3. Alterations in [^{3}H]UR incorporation into RNA of L1210 ascites tumor, bone marrow and gastrointestinal mucosa due to 50 mg/kg of 5-aza-CR i.p. in BDF_1 mice. Control incorporation rates were 13,550 ± 290 dis./min/absorbance unit at 260 nm for ascites tumor, 25 ± 12 dis./min/absorbance unit at 260 nm for bone marrow and 25 ± 8 dis./min/absorbance unit at 260 nm gastrointestinal mucosa.

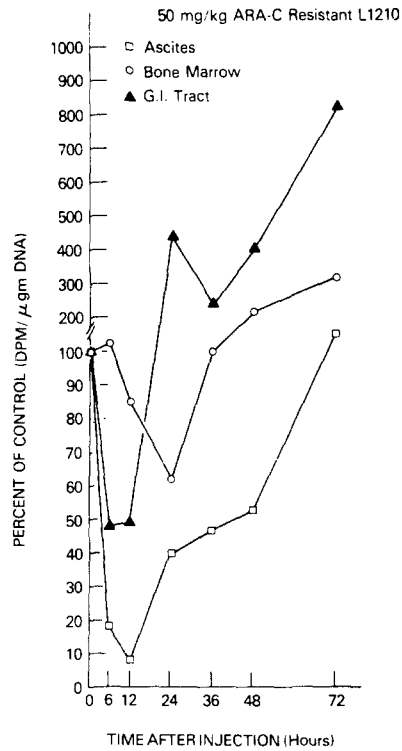

Fig. 4. Alteration in [^{3}H]TdR incorporation into DNA of an ara-C-resistant L1210 ascites tumor due to 50 mg/kg of 5-aza-CR i.p. in BDF_1 mice.

rates of incorporation of [^{3}H]UR into nuclear RNA. A nadir of 13 per cent of tumor control level was reached by 12 hr and recovery to baseline was complete by 24 hr. Gastrointestinal mucosa shows only an initial rise and then a modest fall in [^{3}H]UR incorporation while bone marrow cells shown only enhanced [^{3}H]UR incorporation after 5-aza-CR administration. At least in this model system, 5-aza-CR appears to have some effect on RNA synthesis in tumor cells as reflected by inhibition of [^{3}H]UR incorporation, but the effect on tumor RNA is of much shorter duration than on tumor DNA.

Alterations of DNA synthesis in an ara-C-resistant L1210 tumor. Since 5-aza-CR has been reported to be activated by the enzyme that activates ara-C, dCR-kinase, we wondered if an L1210 tumor cell known to be resistant to ara-C would also be resistant to 5-aza-CR [17]. Figure 4 shows the effects of a single 50 mg/kg i.p. dose of 5-aza-CR on incorporation of [^{3}H]TdR into DNA. The normal cell line has a nadir of 2 per cent of control at 12 hr with recovery prolonged to greater than 192 hr. The resistant tumor cell line has a nadir of 8 per cent at 12 hr and recovery to baseline occurs between 48 and 72 hr.

Toxicity studies. Figure 5 indicates the percentage mortality in groups of ten animals given a second i.p. dose of the same size. Mortality is virtually 100 per cent when a second dose is administered during the period of recovery of [^{3}H]TdR incorporation in normal tissues. Toxicity is reduced to 50 per cent if the second dose is administered at the nadir of [^{3}H]TdR incorporation in normal tissues. Toxicity is also reduced to 20–40 per cent when the second dose is administered at 168–92 hr when [^{3}H]TdR incorporation in normal tissues is returning to baseline levels.

Survival studies. Figure 6 indicates the effect of second doses of 50 mg/kg of 5-aza-CR on mice bearing a 5-day growth of L1210 ascites tumor. Toxicity is the limiting factor with second doses of 5-aza-CR administered during recovery of incorporation in normal tissues (0° + 36°). Second doses at 168–92 hr, however, when [^{3}H]TdR incorporation into bone marrow and GI tract is returning to baseline levels, prolong survival. This may also be due to possible onset of recovery of [^{3}H]TdR incorporation into tumor tissues, making them more susceptible to the cytotoxic action of 5-aza-CR. Of note, a second dose

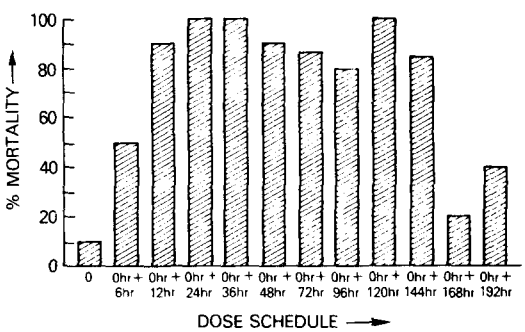

Fig. 5. Percentage mortality in groups of from ten to twenty normal BDF_1 mice given 50 mg/kg i.p. of 5-aza-CR at time 0 and a second i.p. dose of 50 mg/kg at times noted.

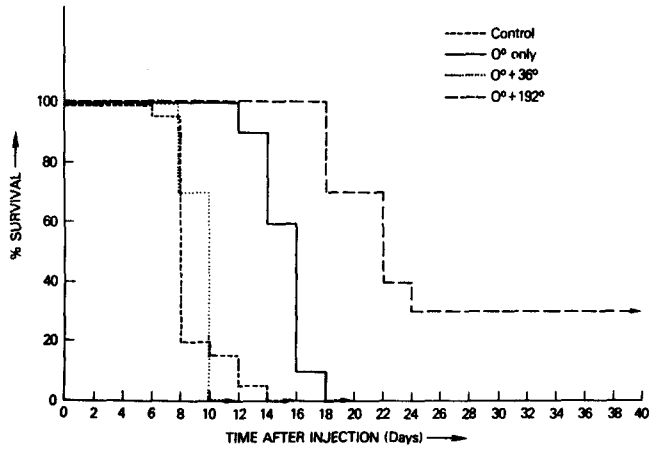

Fig. 6. Percentage survival in groups of from ten to twenty tumor-bearing BDF_1 mice given 50 mg/kg i.p. of 5-aza-CR at time 0 and a second i.p. dose of 50 mg/kg at times noted.

of 5-aza-CR administered at 72 hr, when there appeared to be recovery of [^{3}H]TdR incorporation into tumor cells, was not effective, possibly due to normal tissue toxicity.

DISCUSSION

Several studies from this laboratory [10, 11, 18–20] have emphasized the facility, accuracy and speed of following [^{3}H]TdR incorporation into DNA as a means of exploiting kinetic differences between normal and tumorous tissues in the scheduling of chemotherapy. An extension of this method employing [^{3}H]UR incorporation into RNA allows simultaneous observations on the alterations induced in ribonucleic acid synthesis by such chemotherapy *in vivo*.

Since 5-aza-CR has been reported to alter synthesis of both DNA and RNA [3, 8, 9], we were interested in determining in this system which of these alterations would more accurately reflect kinetically governed chemotherapy. Utilizing these incorporation techniques, we have shown that in the L1210 tumor system, [^{3}H]TdR incorporation into DNA is more significantly altered than [^{3}H]UR incorporation into RNA. Further, the recovery patterns in DNA synthesis between normal and tumorous tissues were more disparate than were patterns of recovery of RNA synthesis.

A drug which disrupts synthesis of DNA should have its greatest effect during a period when DNA is being actively produced [3]. We attempted to exploit the differences in patterns of recovery of DNA synthesis between normal tissues and tumor cells. If a cell were exposed to a drug which alters DNA synthesis during a period when the DNA synthetic rate is high, it should be more profoundly affected than during a period when the DNA synthetic rate is low. Consequently, a second dose of 5-aza-CR given during the recovery phase of DNA synthesis in normal tissues should be highly toxic, and this was confirmed in toxicity studies. Second 50 mg/kg doses of 5-aza-CR caused virtually 100 per cent lethal toxicity except when given at the nadir of normal tissue DNA synthesis (i.e. 12 hr) or after the recovery phase of DNA synthesis, when there is again a near basal rate of [^{3}H]TdR incorporation into DNA from GI mucosa and bone marrow. Employing the converse of this reasoning, one should be able to exert a selective toxic effect on tumor cells if second doses of 5-aza-CR are given during the recovery phase of [^{3}H]TdR incorporation into DNA of the tumor cells. Confirming this are survival studies showing improved survival in animals given a second 50 mg/kg dose of 5-aza-CR at 168–92 hr. Earlier doses proved either too toxic or ineffective.

Although the highest recovery level of DNA synthesis during this study occurs at 168–92 hr and second doses at this time produce maximal prolongation of survival, a similar but lesser level of DNA recovery occurs at 48 and 72 hr in this tumor. It is probable that enhanced therapeutic efficacy is not achieved with second doses administered at this time because of the enhanced toxicity, indicated by the rapid recovery of DNA synthesis in the bone marrow and GI mucosa and confirmed by the toxicity studies (Fig. 5).

Studies comparing rates of [^{3}H]TdR incorporation into normal and tumor cells using intravenous and intraperitoneal 5-aza-CR confirm that the disparity between the recovery phases of this incorporation in normal tissues and tumor tissue is a real and reproducible phenomenon due to the effects of 5-aza-CR and not due to a higher 5-aza-CR concentration because of direct contact of the drug with tumor cells after i.p. drug administration.

Further, the high degree of Trypan Blue dye exclusion in ascites tumor cells at all times after drug administration makes it unlikely that the prolonged period of suppression of [^{3}H]TdR in tumor cells is due to persistence of dead cells in the peritoneal cavity. Under conditions *in vivo* apparently dead cells are rapidly cleared from the intraperitoneal space [21].

We must emphasize that we are not suggesting that inhibition of [^{3}H]TdR incorporation into the DNA of any tissue is the cause of the toxicity to the normal tissues or the therapeutic effect on the tumor tissue. Rather, we wish to suggest that patterns of suppression and recovery reflect these events and in so doing

may be used in a predictive manner in scheduling subsequent doses of a nucleic acid anti-metabolite.

If in fact the degree and/or duration of suppression of DNA synthetic rate is an accurate reflection of a cell's disruption by 5-aza-CR, one would predict that the rapid recovery of tumor cell DNA synthesis in the ara-C-resistant L1210 tumor line after initial exposure to 5-aza-CR should indicate a partial resistance to the cytotoxic action of the drug. Survival studies carried out in the present studies using the partially resistant cell line confirmed this prediction, as single 50 mg/kg doses of 5-aza-CR in animals containing the normal L1210 cell line led to a 156 per cent increased life span while animals containing the ara-C-resistant cell line had only a 121 per cent increased life span ($P < 0.05$).

Using doses of 5-aza-CR equal to the amount used in these studies, Presant *et al.* [22] showed, using the technique of repopulation of normal and leukemic colony-forming units, that the cytotoxic action of 5-aza-CR on leukemic cells was prolonged to greater than 6 days. Further, they showed with doses of approximately 25 mg/kg that the leukemia cell population was 100 times more sensitive than normal cell populations. Our data of recovery of [^{3}H]TdR incorporation in ascitic tumor cells correlate well with the time at which Presant *et al.* saw repopulation of the leukemia colony-forming units using the spleen colony assay technique. The technique of following [^{3}H]TdR incorporation is faster and easier than the spleen colony assay technique, which, in addition, cannot be used in man. Also, Rosenoff *et al.* [19] showed that the technique of following suppression of [^{3}H]TdR incorporation into DNA is as good or better than the C-FUC spleen colony assay method in assessment of early cytotoxic activity and recovery from a drug.

This technique could, with some modifications, be applicable to clinical situations in which 5-aza-CR would be administered in responsive human tumors using a schedule dictated by following [^{3}H]TdR incorporation into the tumor cell rather than by the present empiric daily infusion for 5 days. For example, we have been able to show suppression of [^{3}H]TdR incorporation in human myeloblasts isolated from a patient with the blastic phase of CML after incubation *in vitro* with 5-aza-CR. In fact, this patient had a sustained partial remission on 5-aza-CR therapy. Such studies have been done in patients with ovarian ascites tumor after i.v. methotrexate therapy also [21].

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