Inhibition and Recovery of DNA Synthesis in Host Tissues and Sensitive and Resistant B16 Melanoma after 1-(2-Chloroethyl)-3-(trans-4-methylcyclohexyl)-1-nitrosourea, a Predictor of Therapeutic Efficacy

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SUMMARY

Single doses of 1-(2-chloroethyl)-3-(trans-4-methylcyclohexyl)-1-nitrosourea caused transient suppression of [3H]thymidine incorporation into DNA in bone marrow and gastrointestinal mucosa and more prolonged inhibition of such incorporation in B16 melanoma. A single dose of 1-(2-chloroethyl)-3-(trans-4-methylcyclohexyl)-1-nitrosourea, 16 mg/kg, doubled the mean life-span after treatment of C57BL x DBA/2 F1, male mice bearing 12-day-old B16 melanomas. Subsequent doses timed to minimize toxicity and maximize antitumor effect, however, produced no further prolongation of survival, and studies with B16 melanoma previously exposed to 1-(2-chloroethyl)-3-(trans-4-methylcyclohexyl)-1-nitrosourea demonstrated that the suppression of [3H]thymidine incorporation into DNA was no longer prolonged beyond that seen with normal host tissues. The loss of clinical efficacy was accompanied by a loss of differential suppression of [3H]thymidine incorporation into DNA between the tumor and host tissues.

INTRODUCTION

From this laboratory indicate that, under some circumstances, alterations in DNA synthesis in tumors and normal target tissues in vivo, although not directly measured, can be estimated by measuring alterations in the rate of [3H]thymidine incorporation into DNA after drug treatment (11, 12). With several drugs studied previously, including 1,3-bis(2-chloroethyl)-1-nitrosourea, an initial depression in [3H]thymidine incorporation into DNA in both tumor and normal target tissues occurs. However, the pattern of recovery of [3H]thymidine incorporation into DNA in the tumor is different from normal target tissues. This differential pattern of recovery can suggest optimal intervals for subsequent treatment. The studies reported here were undertaken to apply this technique in a solid tumor and to evaluate the simultaneous alterations in DNA synthesis, reflected by changes in the incorporation of [3H]thymidine into DNA caused by MeCCNU in B16 melanoma, murine bone marrow, and duodenal mucosa in vivo and to use this information to suggest useful schedules of drug administration with minimal toxicity.

MATERIALS AND METHODS

Male mice (C57BL x DBA/2 F1, hereafter called BD2F1) weighing approximately 20 g were used for these experiments. They were kept in a constant temperature environment in plastic cages and fed laboratory chow and water ad libitum. B16 melanoma (Hazelton Laboratories, Rockville, Md.) was carried i.m. in the hind limb and transplanted to recipient mice as a 0.1-ml suspension of approximately 1 x 10^6 melanoma cells. Viability was determined by trypan blue exclusion, and cell counts were performed using a hemocytometer. BD2F1 male mice of comparable age and weight were used as controls. Experiments were carried out at the same time each day, and all mice were sacrificed by cervical dislocation.

Specific Activity of the DNA (dpm/µg DNA). Studies involving tumor-bearing mice were begun on Day 12 of tumor growth, and MeCCNU was administered i.p. as an emulsion using polyoxyethylated vegetable oil (Emulphor EL-620; GAF Corp., New York, N. Y.) as a vehicle. Six mice received only the polyoxyethylated vegetable oil vehicle and served as controls for each experiment. One, 6,
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12, 24, 36, 48, 72, 96, 120, and 144 hr after receiving injections of MeCCNU, 6 mice were given 100 μCi of \(^{[3]H}\)thymidine i.p. (specific activity, 6.7 Ci/mmmole; New England Nuclear, Boston, Mass.) and were sacrificed 1 hr later. A small portion of tumor was excised from the periphery of the tumor mass in each animal and homogenized in iced phosphate-buffered 0.85% NaCl, pH 7.4. Duodenal mucosa and bone marrow were also obtained from each animal as previously described (5). Separate samples of B16 melanoma, duodenal mucosa, and bone marrow from the 6 mice sacrificed for each time point were pooled into 2 groups of 3. The pooled samples were centrifuged at 800 x g for 5 min at 4° and the supernatant was discarded. The DNA of each of the pooled specimens was then extracted using a modification of the Schneider method (7). A 0.5-ml aliquot of the final supernatant was added to 15 ml of Aquasol (New England Nuclear, Boston, Mass.) and counted in a Packard Tri-Carb liquid scintillation spectrometer. A 0.5-ml aliquot of the final supernatant was also processed by the Burton method (1) for DNA determination. The efficiency of counting tritium was 26%, and the results were expressed as dpm/μg DNA and graphed as percentages of control over time.

Survival Studies in Tumor-bearing Animals. BD2F, male mice weighing approximately 20 g were given i.m. injections of approximately 1 x 10⁶ viable tumor cells in the hind limb. Tumor cell viability prior to implantation was determined by trypan blue exclusion. Twenty mice were used in each study; the mice were inspected daily and deaths were recorded.

Toxicity Studies in Normal Mice. Normal BD2F, male mice weighing approximately 20 g were used to determine the toxicity of MeCCNU in these experiments. Twenty mice were used for each drug dose or schedule and the drug was administered i.p. as previously described. The mice were inspected daily and deaths were recorded.

RESULTS

Determination of Optimum Dose of MeCCNU in Tumor-bearing Mice. To determine the efficacy of MeCCNU in this animal tumor model, 3 different doses of the drug were administered i.p. MeCCNU as a single dose, 8, 16, and 32 mg/kg, was given to 3 groups of 20 mice bearing 12-day-old B16 melanoma, and the survival of the 3 groups was compared.

The mean survival of these 4 groups of animals after treatment was as follows: control animals, 14 days; animals given injections of MeCCNU, 32 mg/kg, 13 days; animals given injections of MeCCNU, 8 mg/kg, 21 days; and animals given injections of MeCCNU, 16 mg/kg, 40 days. Animals that were treated with MeCCNU, 8 or 16 mg/kg, survived significantly longer than did control animals that received only vehicle (p = 0.005 and 0.0005, respectively; generalized Wilcoxon method).

Alteration in Pattern of DNA Synthesis in B16 Melanoma, Gastrointestinal Mucosa, and Bone Marrow following MeCCNU. The simultaneous changes that occur in \(^{[3]H}\)thymidine incorporation into DNA in tumor, duodenal mucosa, and bone marrow for 3 different doses of MeCNU are depicted in Chart 1. Following MeCCNU, 4 mg/kg i.p., the incorporation of \(^{[3]H}\)thymidine into DNA fell rapidly to a nadir for all tissues within 24 hr. Bone marrow and duodenal mucosa recovered quickly and incorporated \(^{[3]H}\)thymidine into DNA at the pretreatment level by 24 and 48 hr. respectively; there is a slight overshoot phase of DNA synthesis for both of these normal target tissues. Incorporation of \(^{[3]H}\)thymidine into tumor DNA, however, remained depressed to 24% of base line for 24 hr and gradually returned to base line by the 4th day. Following MeCCNU, 16 mg/kg i.p., the nadir of DNA synthesis of the duodenal mucosa and bone marrow occurred at 24 hr and was 20 and 50% of control values.

Chart 1. \(^{[3]H}\)Thymidine incorporation into DNA in bone marrow, tumor, and gastrointestinal mucosa (GI tract) after MeCCNU, 4 mg/kg (left), 16 mg/kg (center), and 32 mg/kg (right).

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respectively. These tissues recovered to base line by 36 hr and reached maximum rates of $[^{3}H]$thymidine incorporation into DNA at 72 hr of 300% base line for duodenal mucosa and 400% of base line for bone marrow. At 24 hr after treatment, $[^{3}H]$thymidine incorporation into DNA in the tumor had fallen to 30% of base line, and the nadir of 20% was reached between 36 and 72 hr posttreatment. $[^{3}H]$Thymidine incorporation into DNA in the tumor then rose toward pretreatment levels over Days 3 to 6. Following MeCCNU, 32 mg/kg, $[^{3}H]$thymidine incorporation into DNA was depressed to the lowest level in all tissue examined, approximately 15% of control levels by 24 hr. Bone marrow and gastrointestinal mucosa began recovery by 48 hr and reached supranormal levels of 400% at 72 hr and 300% at 96 hr, respectively. $[^{3}H]$Thymidine incorporation into DNA then returned toward base line by Day 6. During this period of recovery and overshoot of the normal target tissues, the tumor remained depressed throughout the period of observation to approximately 15% of control levels.

Although the 32-mg/kg dose would appear to produce the optimal differential effect between the tumor and normal tissues, it is nevertheless still too toxic to normal tissues and is a lethal dose to approximately 100% of BD2F1 male mice. The dose producing the greatest differential effect between tumor and normal tissues but still acceptable toxicity to the normal tissues was 16 mg/kg.

**Toxicity Studies with 2 Sequential Doses of MeCCNU in Normal Mice.** Normal mice (140) were treated with MeCCNU, 16 mg/kg i.p., and were divided into 7 groups of 20 mice each. These animals were treated with an additional dose of MeCCNU, 16 mg/kg i.p., on Day 0, 1, 2, 3, 4, 5, or 6. The percentage of mice dying as a function of the time interval between doses is depicted in the bar graph in Chart 2B. The toxicity of 2 sequential doses is compared with the alterations induced in DNA synthesis in duodenal mucosa and bone marrow by MeCCNU in Chart 2A.

As depicted in Chart 2B, no mouse treated with 2 doses of MeCCNU, 16 mg/kg i.p., on Day 0 survived. When the 2 doses were separated by 1 day, at a time when incorporation of $[^{3}H]$thymidine into DNA in bone marrow and duodenal mucosa had reached a nadir, 80% of the mice survived. When the interval between doses was increased to 2 days, at a point when recovery of $[^{3}H]$thymidine incorporation into DNA was proceeding most rapidly in the normal target tissues, only 60% of the animals survived. Thereafter, as the interval between doses was increased, and $[^{3}H]$thymidine incorporation into DNA gradually returned to base line, the percentage of animals surviving increased, so that when 6 days separated the doses, 90% of the animals survived. The difference in survival between animals treated with a 2nd dose on Day 0 and animals treated with a 2nd dose at any other time interval shown is significant ($p < 0.005$); and the difference in survival between animals treated with a 2nd dose on Day 6 and the group treated with the 2nd dose on Day 2 is significant ($p < 0.05$).

**Survival and Kinetic Studies following 2 Doses of MeCCNU Given 6 Days Apart in Tumor-bearing Animals.** On the basis of the preceding studies, it appeared that the toxicity of a 2nd dose of MeCCNU could be minimized by giving it either on Day 1 or Day 6 following the initial dose. However, the potential for antitumor effect would theoretically be better on Day 6 since $[^{3}H]$thymidine incorporation into DNA in the tumor had largely recovered by that time, and MeCCNU, a cycle-dependent agent, would presumably be more active in a population of cells more rapidly synthesizing DNA. Thus, an interval of 6 days between doses was selected, and the survival of tumor-bearing mice treated with 2 doses of MeCCNU was contrasted, in Chart 3, with the survival of tumor-bearing mice treated with a single dose and a control group.

The mean survival of untreated animals bearing B16 melanoma was 12 days. A mean survival of 32 days was noted after administration of either a single dose of MeCCNU, 16 mg/kg i.p., on Day 12 of tumor growth or doses of MeCCNU, 16 mg/kg i.p., on Days 12 and 18 of tumor growth. The failure of a properly spaced 2nd dose to prolong survival suggested an altered sensitivity of the B16 melanoma to MeCCNU.

Kinetic studies of $[^{3}H]$thymidine incorporation into DNA in tumor, duodenal mucosa, and bone marrow, as previously described, were repeated following a 2nd dose of MeCCNU. These data are contrasted, in Chart 4B, to the same information obtained following only a single dose of
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following the 2nd dose of MeCCNU (Chart 4B), patterns of suppression and recovery similar to those seen after 1 dose are seen in the normal tissues. $[^3H]$Thymidine incorporation into DNA in the bone marrow fell to a nadir of 40% of control by 24 hr, rose to a maximum of 200% of control by 72 hr, and returned to base line by Day 5. $[^3H]$Thymidine incorporation into DNA in duodenal mucosa reached a nadir of 25% of control by 6 hr, rose to a maximum level of 175% of base line by 72 hr, and returned to base line by Day 5. In contrast to the normal tissues, alteration of DNA synthesis in the tumor is strikingly different after the 2nd dose of MeCCNU. Tumor DNA synthesis reached a nadir at 12 hr of 50% of control and then returned to 70% of control by 48 hr and 90% of control by 96 hr.

The data presented in Charts 2, 3, and 4, A and B, demonstrate that a 2nd dose of MeCCNU timed to minimize toxicity and maximize antitumor effect in reality minimized toxicity but produced no further prolongation of survival than did a single dose of MeCCNU and that the loss of therapeutic efficacy is accompanied by a loss of differential suppression of $[^3H]$thymidine incorporation into DNA in tumor and normal target tissues.

Studies to Determine the Persistence of Altered Sensitivity Induced in B16 Melanoma by MeCCNU in Vivo. Tumor-bearing mice were treated with MeCCNU, 16 mg/kg i.p. These mice were sacrificed 30 days following treatment, the tumors were removed and homogenized, and $1 \times 10^6$ cells were introduced i.m. into the hind limb of a 2nd group of animals. This 2nd group was sacrificed after 20 days, the tumors were removed and homogenized, and $1 \times 10^4$ tumor cells were introduced i.m. into the hind limb of a 3rd group of mice. On the 12th day of tumor growth, these animals were treated with MeCCNU, 16 mg/kg i.p., and kinetic studies of $[^3H]$thymidine incorporation into tumor, duodenal mucosa, and bone marrow DNA, as previously described, were performed (Chart 4C); the studies are contrasted to identical studies carried out following MeCCNU, 16 mg/kg i.p., with mice bearing 12-day-old tumors previously unexposed to MeCCNU (Chart 4A). Although the level of depression and overshoot of $[^3H]$thymidine into DNA in gastrointestinal mucosa and, particularly in bone marrow varied in these experiments, the pattern of inhibition of $[^3H]$thymidine incorporation into DNA, subsequent recovery, overshoot, and return to base line for duodenal mucosa and bone marrow was similar to the pattern previously described following a single dose of MeCCNU, 16 mg/kg i.p.

Tumor previously unexposed to MeCCNU demonstrated a depression of $[^3H]$thymidine incorporation into DNA to a nadir 12 hr after exposure to MeCCNU, 16 mg/kg, with gradual recovery toward base line by 120 hr. Tumor exposed to MeCCNU and subsequently implanted serially through 3 generations of mice showed a reduction in $[^3H]$thymidine incorporation into DNA to a nadir 24 hr after exposure to MeCCNU, 16 mg/kg i.p., but with a return to 80% of base line by 36 hr and a maximum of 150% of base line by 96 hr. This lack of significant tumor inhibition resembled that seen in Chart 4B that had occurred immediately after 2 doses of MeCCNU. The important difference between the studies of a single dose of MeCCNU and multiple subsequent doses is that the loss of differential suppression of $[^3H]$thymidine incorporation into DNA of tumor, first noted after a 2nd dose of MeCCNU
given 6 days after an initial dose, persists, even though the 2 doses are 62 days apart and the tumors have been passed through 3 successive groups of animals.

DISCUSSION

MeCCNU produces alterations in the [H]thymidine incorporation into DNA in normal target tissues that are different in duration and magnitude from those produced in B16 melanoma in vivo. This phenomenon has been described by others following single doses of drug in other animal tumor systems. Wheeler and Alexander (9), Young and Goldberg (11), Rosenoff et al. (5), Mizuno and Humphrey (3), and Schein et al. (6) have shown that nitrosoureas may damage both normal host tissues and neoplastic tissue but that normal host tissues recover more readily. In the studies reported here, [H]thymidine incorporation into DNA following MeCCNU, 4, 16, and 32 mg/kg i.p., were performed in BD2F1, male mice bearing B16 melanoma. In the normal target tissues, gastrointestinal tract, and bone marrow, there was a prompt initial depression of [H]thymidine incorporation into DNA within hr of the administration of MeCCNU. This depression was followed by a rapid recovery of DNA synthesis within 1 to 3 days, followed by a phase of DNA synthesis of 200 to 400% of control, in turn followed by a gradual return to pretreatment levels by 4 to 7 days. With the larger dose of MeCCNU, the initial depression of [H]thymidine incorporation into DNA was more profound and prolonged, the overshoot was greater, and the return to pretreatment levels was more prolonged. With each dose, the tumor also demonstrated a consistent pattern of rapid initial depression of [H]thymidine incorporation into DNA but, unlike the normal target tissues, demonstrated only a gradual and prolonged return to pretreatment levels. These gradual returns took 4 days following MeCCNU, 4 mg/kg, 6 days following MeCCNU, 16 mg/kg, and had not begun by 7 days following MeCCNU, 32 mg/kg. In each case, a differential suppression of [H]thymidine incorporation into DNA between normal target tissues and tumor tissue was noted. This phenomenon was dose related, as the larger doses of MeCCNU produced the greater and more prolonged differential between tumor and normal tissue. Although both the bone marrow and the gastrointestinal tract recover the ability to incorporate [H]thymidine into DNA after MeCCNU, 32 mg/kg, this recovery does not reflect the state of health of the animal but shows only that the surviving cells, which may not be enough to support life, have recovered the ability to incorporate [H]thymidine into DNA. Excessive toxicity to normal tissues precluded the use of high doses of MeCCNU, even though they showed optimal differential effect of the doses examined. The best single dose was 16 mg/kg, and this more than doubled the mean survival of BD2F1 mice bearing 12-day-old B16 melanoma.

To determine the appropriate interval between 2 doses of drug, the hypothesis was presented that the toxicity of a 2nd dose would depend on the level of recovery of [H]thymidine incorporation into DNA in the normal target tissues following the 1st dose. This indeed proved to be true, for a 2nd dose given when [H]thymidine incorporation into DNA in duodenal mucosa and bone marrow was lowest was less toxic than a 2nd dose given when [H]thymidine incorporation into DNA in these tissues was rapidly recovering toward base line. A 2nd dose was least toxic after [H]thymidine incorporation into DNA in duodenal mucosa and bone marrow had stabilized at base line levels. This interval between doses was 6 days. Survival studies performed in tumor-bearing animals following 2 doses of MeCCNU, 16 mg/kg i.p., given 6 days apart, and thus optimally timed to reduce toxicity, however, gave the unexpected result of no additional benefit beyond that achieved with a single dose. This phenomenon was investigated by performing studies of [H]thymidine incorporation into DNA following 2 doses of MeCCNU given 6 days apart. These studies demonstrated that, following the 2nd of 2 doses of MeCCNU, the depression of [H]thymidine incorporation into tumor DNA lasted a much shorter time than after a single dose and that the pattern of suppression and recovery of [H]thymidine incorporation into DNA paralleled that of the normal target tissues except for lacking a significant overshoot phase. The differential effect of MeCCNU on [H]thymidine incorporation into DNA in normal vs. get tissues and tumor had been lost, and this loss correlated with a loss of antitumor effect. The rapid induction of resistance of B16 melanoma to MeCCNU has been reported previously by Griswold (2). Those studies revealed a decreasing effect of MeCCNU, 20 mg/kg, on the growth of B16 melanoma in vivo as a function of the number of doses given and compared the growth of treated and untreated tumors following each of 4 passages of previously treated tumors. Complete resistance of B16 melanoma to MeCCNU was demonstrated after only 3 passages. Our data correlate well with these earlier studies and also suggest that a loss of differential suppression of [H]thymidine uptake between tumor and normal target tissues may predict a loss of clinical usefulness. This latter phenomenon has also been examined by Wheeler (8) in hamsters bearing cyclophosphamide-sensitive and -resistant plasmacytomas; in this instance, there was a differential suppression of in vivo fixation of [H]thymidine in acid-insoluble fraction of tissues between normal target tissues and tumor sensitive to cyclophosphamide. This differential suppression was absent with cyclophosphamide-resistant tumor.

To examine the persistence of these drug-induced alterations in tumor kinetics, tumors previously exposed to MeCCNU were carried for 3 generations of animals (approximately 62 days) before being reexposed to the drug. Despite the prolonged interval between doses, the depression of [H]thymidine incorporation into DNA in the tumor following the 2nd dose of MeCCNU was less than after a single dose alone, and the recovery to base line was accomplished sooner than after a single dose. The mechanism of this kinetic change is unknown. It is possible that, under the influence of MeCCNU, a clonal selection process allowed a resistant strain of cells to evolve quickly, but the precise mechanism of resistance to the drug is unknown. In
any event, the loss of therapeutic efficacy of this drug is correlated with a loss of differential sensitivity in suppression of [3H]thymidine incorporation into DNA between normal target tissues and tumor in vivo.

As more drugs are examined with these techniques, it is possible that the presence of a differential suppression of [3H]thymidine incorporation into DNA between normal host target tissues and tumor will predict the clinical usefulness of a particular drug early in the therapeutic course and that the lack of this differential sensitivity or its loss with subsequent treatments would suggest tumor resistance and provide a more rational basis for the selection of a different therapeutic agent.

REFERENCES

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