[CANCER RESEARCH 47, 26-30, January 1, 1987]

Prevention of Dacarbazine Damage of Human Neoplastic Cell DNA by Aphidicolin[1]

Ulf Lönn[2] and Sigrid Lönn

Department of Histology, Karolinska Institutet, P. O. Box 60 400, and Radiumhemmet, Karolinska Hospital, S-104 01 Stockholm, Sweden

ABSTRACT

Treatment of human neoplastic cells with dacarbazine both inhibits DNA synthesis and induces damage in the DNA. Lysis of cells in dilute alkali and subsequent electrophoretic analysis of the isolated DNA show that the DNA of treated cells includes a high molecular weight component and a population of 2-10-kilobase single-stranded DNA fragments while untreated cells contain only high molecular weight DNA. When DNA is pulse-labeled at the beginning of the dacarbazine treatment high amounts of small DNA fragments are seen but no labeled high molecular weight DNA. Moreover the DNA fragments are not formed in cells which are treated with aphidicolin before the addition of dacarbazine. Aphidicolin is a specific inhibitor of DNA polymerase α, the enzyme responsible for the replicative synthesis of DNA. We conclude that dacarbazine damages DNA only in cells which are synthesizing new DNA.

INTRODUCTION

Dacarbazine (DTIC-Dome) is an antineoplastic agent used in the clinical treatment of malignant melanoma, various lymphomas, and sarcomas. At present we have an incomplete understanding of how dacarbazine exerts its cytotoxic effect. Thus far it has been believed that dacarbazine is an inactive molecule and that the cytotoxic effect is generated by metabolites formed in the liver by microsomal enzymes. Several complex enzyme systems are needed in order to allow the formation of metabolites showing cytotoxic effects (1–4).

However, dacarbazine is a purine analogue, suggesting that it may affect DNA synthesis and/or the stability of the DNA and consequently show an antineoplastic effect in its own right. In agreement with this idea it has been found that during isolated extremity perfusion with dacarbazine for treatment of malignant tumors there is no evidence for formation of metabolites (5). In this paper we have investigated whether dacarbazine shows cytotoxic activity in cultured human neoplastic cells and if so whether dacarbazine induces damage in the DNA.

During the last years we have obtained a better knowledge of the structure of the chromosome and how DNA is synthesized (6–8). It is, *e.g.*, now well established that DNA polymerase α is responsible for semiconservative DNA synthesis. Aphidicolin is a specific inhibitor of DNA polymerase α (7) and has been shown to inhibit the formation of DNA replication intermediates (7, 9).

We have used aphidicolin to analyze the effect of dacarbazine on cells containing either functioning or aphidicolin-inhibited DNA polymerase α. Also we have examined the effect of dacarbazine on the synthesis of DNA replication intermediates as well as the stability of steady-state and pulse-labeled DNA during treatment with dacarbazine. We analyze the DNA after cell lysis in dilute alkali in order to partially denature the DNA and induce release of DNA fragments. The technique has earlier been used to analyze the formation of DNA replication intermediates (9–11).

MATERIALS AND METHODS

Cells, Culture Methods, and Labeling with [^{3}H]Thymidine. A human melanoma cell line (CRL 1424) and a human colon adenocarcinoma cell line (CCL 218), both obtained from Flow Laboratories, United Kingdom, were grown as monolayers as described earlier (9). The culture medium was minimal essential medium (Eagle's) with Earle's salt mixture, 2 mM L-glutamine, 10% fetal calf serum, and antibiotics. The culture medium was changed twice weekly and the cells were passaged every 4–6 days. The number of cells per culture dish was determined using an Analys Instrument Cell Counter 134.

For experiments involving prelabeled DNA the cells were seeded in small culture dishes (35 x 10 mm) containing 3 ml medium. Tritiated thymidine (50 μCi; 22 Ci/mmol; Amersham, Inc.) was added to the culture medium. After 24 h the medium was changed to fresh medium without thymidine and after another 24 h the cells were used for drug treatment experiments. For pulse-labeling experiments 100 μCi tritiated thymidine were added to the culture medium for the desired length of time.

Treatment with Dacarbazine (DTIC-Dome). Dacarbazine was obtained from a local pharmacy. Fresh solutions were always made up immediately before experiments. All our experiments were performed in the dark since degradation of dacarbazine may occur during light conditions (4). Treatments with dacarbazine were terminated 60 min after the addition of the drug to the culture medium. We tested the stability of dacarbazine under the incubation conditions by high pressure liquid chromatography (5). Tritium-labeled dacarbazine cannot at present be obtained without degrading the molecule, according to information from NEN, Inc., and Amersham, Inc.

Aphidicolin was a gift from ICI, United Kingdom. It was dissolved in ethanol prior to the addition to the cell cultures.

Cell Lysis. For cell lysis the incubation medium was drained off and the cells were rinsed twice in cold phosphate-buffered saline. Cell lysis was performed in the dark at 0°C by the addition of 2.25 ml of 0.03 M NaOH. After 30 min the solution was neutralized by the addition of 0.9 ml of 0.067 M HCl-0.02 M NaH_2PO_4. Finally the solution was made 1% with regard to SDS.[3]

Gel Electrophoresis. The labeled DNA was separated in 0.75% agarose gels (12) using a LKB Multiphor electrophoretic system. After the separation was terminated the gels were sliced in 1-mm-thick slices. The slices were incubated in scintillation fluid containing 3% Soluene 100 (Packard) and the radioactivity was measured in a Packard scintillation counter.

RESULTS

The maximum concentration of dacarbazine in plasma of human melanoma patients receiving conventional treatment with dacarbazine is 10 μg/ml (5). Moreover 60 min after i.v. injection of the drug, 90% of the dacarbazine has been removed from the plasma. We have therefore chosen as a standard incubation procedure to treat cells with 10 μg/ml for 60 min.

First we analyzed the effect of dacarbazine on the ability of melanoma and colon adenocarcinoma cells to divide. The results showed that immediately after the cells were treated with

Received 2/24/86; revised 5/13/86; accepted 9/10/86.

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[1] This work was supported by grants from the Swedish Cancer Society, T. R. Söderbergs Foundation, Swedish Society of Medicine, and Karolinska Institutet.

[2] To whom requests for reprints should be addressed.

[3] The abbreviation used is: SDS, sodium dodecyl sulfate.

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dacarbazine (10 μg/ml) for 60 min there was no increase in cell number after the drug was removed. However after 12–24 h the cells started to divide. Control measurement with dextran blue showed that 95% of the cells were viable. In contrast, cells treated with aphidicolin (to inhibit DNA polymerase α) before and during the addition of dacarbazine start to divide shortly after the period of drug treatment. This suggests that aphidicolin prevents dacarbazine from damaging the cells.

Cell Lysis. Although DNA-damaging agents can give rise to many different types of modifications in DNA, the damage often results in alkali-labile regions (13). The regions arise either through enzymatic strand scission as part of a repair process or through direct chemical alterations in the DNA molecule; *e.g.*, it has been shown that 1-β-D-arabinofuranosylcytosine and 5-fluorouracil are incorporated into DNA and that DNA-containing drug molecules are alkali labile (14, 15).

To examine DNA we lysed cells in dilute alkali which removes macromolecules from the DNA and disrupts the base pair structure of the DNA (16–18). However, the DNA strands cannot separate before enough time has elapsed to allow unwinding. The unwinding is initiated at gaps present in the DNA chains. Such gaps are known to exist during the process of synthesis of new DNA chains (10, 11). However, the time needed to unwind the DNA is shortened when alkali-labile bonds are present in the DNA, since the cell lysis in alkali induces additional gaps in the DNA chains. Treatment of cells with X-irradiation or drugs as 5-fluorouracil or 1-β-D-arabinofuranosylcytosine introduces gaps and/or alkali-labile regions in the DNA. This results in an increased number of points to initiate unwinding of the DNA. The amount of DNA that may be unwound at each point has been estimated as 20 kilobases (17).

When the alkaline solution is neutralized the high molecular weight DNA renatures and forms double-stranded DNA. Small DNA fragments which are released during the unwinding remain in solution as single-stranded DNA molecules. The fragments can then be separated from the high molecular weight DNA by agarose gel electrophoresis. We have earlier shown that the 10-kilobase DNA replication intermediate and Okazaki fragments remain as single-stranded DNA (10, 11). Also we have found that treatment of cells with 5-fluoropyrimidines results in release of DNA fragments (15). Consequently it should be possible to detect whether dacarbazine induces alkali-labile regions in the DNA, since such damage should result in the generation of DNA fragments.

Treatment of Cells with Dacarbazine Induces DNA Fragmentation. Initial experiments, in which the dacarbazine-treated cells were lysed at neutral pH, did not reveal fragmentation of the DNA. Therefore we analyzed whether dacarbazine induces alkali-labile regions in the DNA, because the presence or absence of fragments may serve as a marker for the cytotoxic effect of dacarbazine.

Cells with steady-state labeled DNA were treated with dacarbazine (10 μg/ml) for 60 min, cultivated in fresh medium for 24 h, and then lysed in dilute alkali. When the labeled DNA was then separated in 0.75% agarose gels, the results showed that there was a peak of labeled high molecular weight DNA at slices 3–6, which coincides with the labeled DNA in untreated cells cultured in parallel. However, in the dacarbazine-treated cells there was also a second peak of labeled DNA located at slices 23–30. This peak could not be detected in control cells, which implies that it is formed as a result of the treatment with dacarbazine.

The location of the fragments was not changed when the DNA was treated with 90% formamide at 80°C prior to gel electrophoresis, indicating that this is a single-stranded DNA (size range, 2–10 kilobases). Fig. 1*A* shows the results obtained with melanoma cells and Fig. 1*B* those obtained with colon adenocarcinoma. The results are very similar in both cell types.

Fig. 1*C* shows that fragmentation is not extensive at first and that it increases to a maximum 12–24 h after dacarbazine treatment. Furthermore with time the fragments increase somewhat in size although the size does not exceed 10 kilobases.

Pretreatment with Aphidicolin Prevents the Dacarbazine-induced Fragmentation of DNA. Melanoma cells were treated with aphidicolin (10 μg/ml) for 60 min and then treated with dacarbazine (10 μg/ml) for 60 min in the presence of aphidicolin. After the treatment the cells were washed free of the drugs and analyzed after cultivation for 24 h in fresh medium. As can be seen in Fig. 2*A* the results showed that a pretreatment with

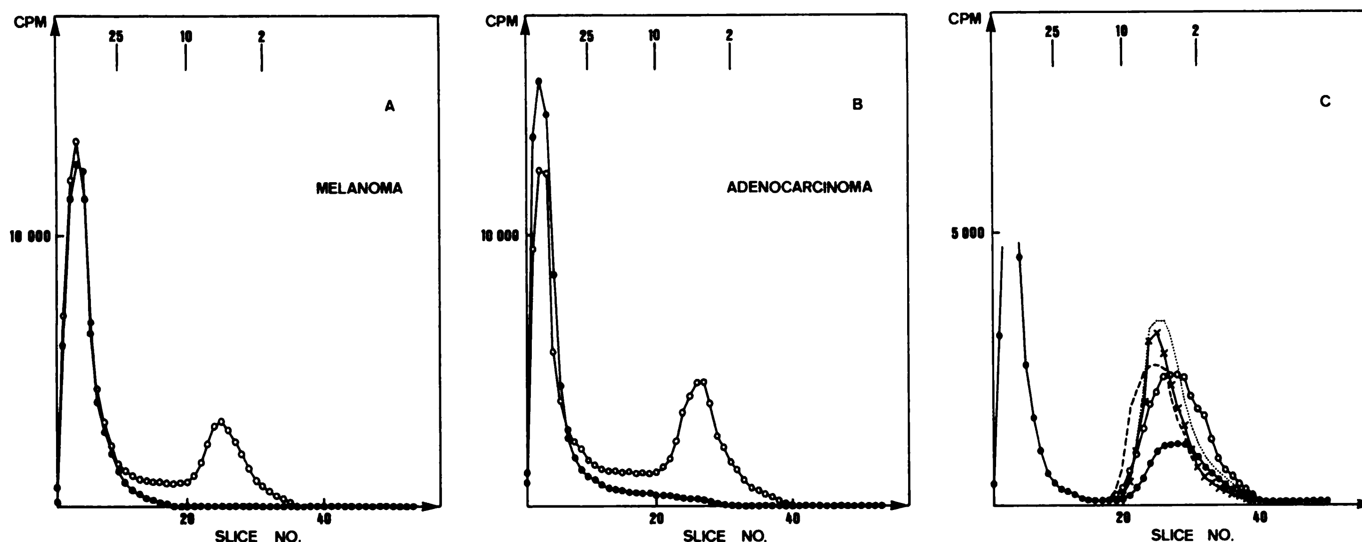

Fig. 1. In *A*, melanoma cells with steady-state labeled DNA were treated with dacarbazine (10 μg/ml) for 60 min and then cultured in fresh medium for 24 h (○). Untreated cells (●) and the dacarbazine-treated cells were lysed in dilute alkali and the labeled DNA was then separated by electrophoresis in 0.75% agarose gel. *Numerals* across the *top* (*25, 10*, and *2*) denote the size (in kilobases) and location of single-stranded DNA markers. In *B* colon adenocarcinoma cells were treated the same way as were melanoma cells in *A*. Cell lysis and agarose gel electrophoresis were performed as described in *A*. (○) dacarbazine-treated cells, (●) untreated cells. In *C* melanoma cells were treated with dacarbazine for 60 min. The cells were then either lysed immediately (●) or lysed after cultivation in fresh medium for 6 h (○), 12 h (·····), 24 h (×), or 48 h (– – –).

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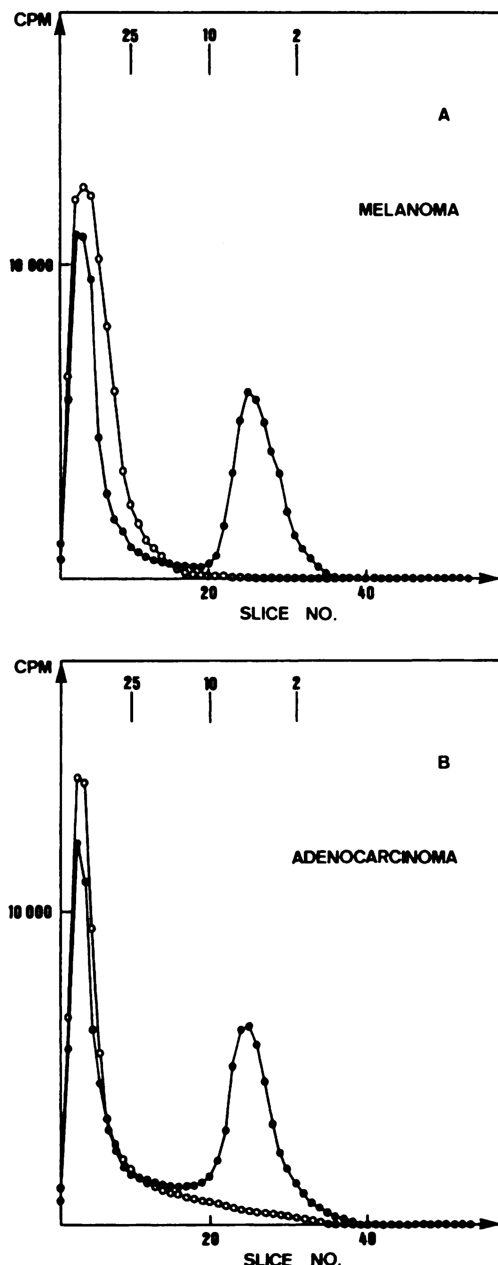

Fig. 2. In *A*, melanoma cells with steady-state labeled DNA were treated with aphidicolin (10 µg/ml) (O) for 60 min before the addition of dacarbazine (10 µg/ml). The subsequent treatment with dacarbazine and aphidicolin was performed for 60 min. The aphidicolin-treated cells as well as cells that received treatment with dacarbazine only (●) were then cultivated for 24 h in fresh medium before cell lysis. The labeled DNA was separated in 0.75% agarose gels. *Numerals across the top* (*25*, *10*, and *2*) denote the size (in kilobases) and location of single-stranded DNA markers. In *B* colon adenocarcinoma cells were incubated as described in *A* but at an aphidicolin concentration of 25 µg/ml. (O) cells treated with aphidicolin and dacarbazine, (●) cells treated with dacarbazine only. The labeled DNA was separated in 0.75% agarose gels as described in *A*.

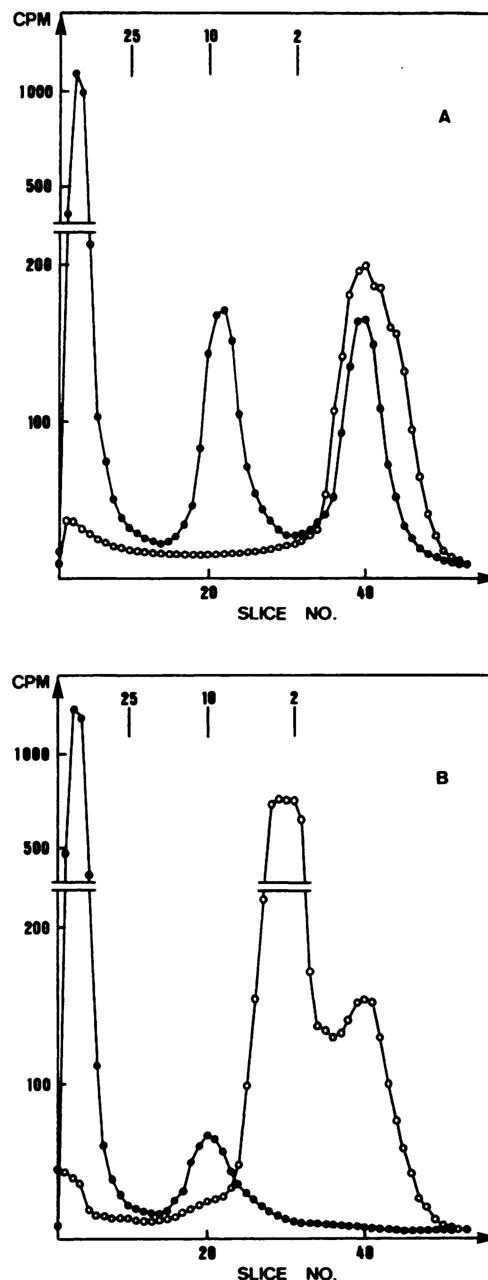

Fig. 3. In *A*, melanoma cells were treated with dacarbazine (10 µg/ml) for 60 min and pulsed with tritiated thymidine during the last 5 min (O). Control cells were incubated in parallel with tritiated thymidine for 5 min (●). The cells were lysed in dilute alkali and the DNA then separated in 0.75% agarose gels. *Numerals across the top* (*25*, *10*, and *2*) denote the size (in kilobases) and location of single-stranded DNA markers. In *B* melanoma cells were pulsed with tritiated thymidine during the first 5 min of the 60-min treatment with dacarbazine (10 µg/ml) (O). Control cells were pulsed with tritiated thymidine for 5 min and then incubated for 55 min in medium without thymidine (●). The labeled DNA was separated in 0.75% agarose gels as in *A*.

aphidicolin completely prevented dacarbazine from generating DNA fragments. The results indicate that functioning DNA polymerase α is needed in order for dacarbazine to fragment the DNA.

Similar results were obtained with colon adenocarcinoma cells as shown in Fig. 2*B*.

Pulse-labeling of DNA in the Presence of Dacarbazine. Our results indicate that dacarbazine interferes with the synthesis of DNA. We have earlier examined the synthesis of DNA replication intermediates by the technique used in the present work and have detected two distinct replication intermediates, Okazaki fragments and 10-kilobase DNA replication intermediates (10, 11). The Okazaki fragments, the smallest intermediates, give rise to the 10-kilobase DNA intermediates which later are joined together to form high molecular weight DNA (11).

In order to investigate whether dacarbazine interferes with the formation of DNA replication intermediates we performed the following experiments. Melanoma cells were treated with dacarbazine (10 µg/ml) for 60 min and pulsed with tritiated thymidine during the last 5 min of the drug treatment. The cells were lysed in dilute alkali and the labeled DNA was analyzed by agarose gel electrophoresis. The results show that the label was incorporated into Okazaki fragments (Fig. 3*A*).

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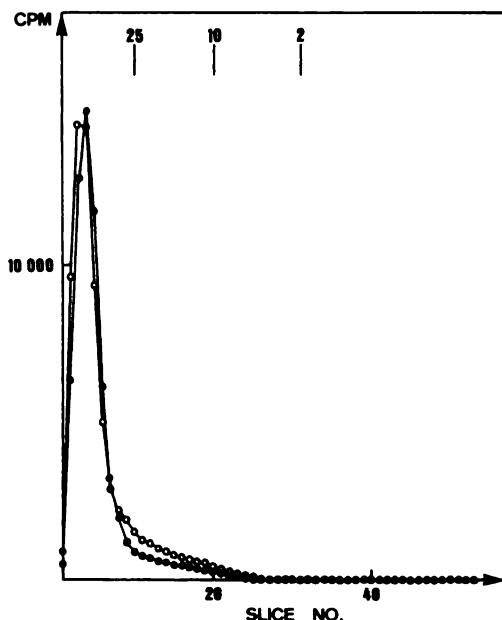

Fig. 4. Melanoma cells with steady-state labeled DNA, with (○) or without (●) aphidicolin treatment (10 μg/ml) for 60 min, were lysed in 0.5% SDS in 0.01 M Tris-0.001 M EDTA, pH 8. The lysates were made 10 μg/ml with respect to dacarbazine and incubated for 60 min in the dark at 37°C. Dilute alkali was then added according to our routine procedure and after 30 min at 0°C the solution was neutralized. The DNA was then separated in 0.75% agarose gels. *Numerals across the top* (*25, 10,* and *2*) denote the size (in kilobases) and location of single-stranded DNA markers.

Neither high molecular weight DNA nor 10-kilobase DNA replication intermediates were labeled. In control cells incubated in parallel we can detect labeled high molecular weight DNA (slices 3–6), 10-kilobase DNA (slices 19–25) and Okazaki fragments (slices 37–45). Since there is no labeling of high molecular weight DNA or 10-kilobase DNA the results imply that there is no movement of the replication fork in cells treated with dacarbazine for 60 min.

We have measured the rate of DNA synthesis by pulse-labeling with tritiated thymidine during the last 5 min of drug treatment. The results showed that a 30-min treatment with dacarbazine is needed in order to reduce the incorporation of label to background level. Gel electrophoretic separation of DNA from cells treated with dacarbazine for periods shorter than 30 min showed labeled high molecular weight DNA, 10-kilobase DNA as well as Okazaki fragments indicating the movement of replication forks.

Next we analyzed cells pulsed with tritiated thymidine during the first 5 min of the treatment with dacarbazine. When the DNA was analyzed in agarose gels the results showed that the majority of the labeled material was located at slices 23–30 (Fig. 3*B*), *i.e.*, the location for the 2–10-kilobase DNA fragments released from steady-state labeled DNA by dacarbazine, as described in Fig. 1. The fragments are smaller than those in Fig. 1. There was also some label in the Okazaki fragments but almost no labeled high molecular weight DNA or 10-kilobase DNA.

Hence the DNA synthesized during the first 5 min of the treatment with dacarbazine is almost completely fragmented. The results of Figs. 1 and 2 show that both pulse-labeled and prelabeled DNA of drug-treated cells contain alkali-labile regions.

Control Experiments. To exclude the possibility that dacarbazine induces alkali-labile regions in isolated DNA we have performed experiments in which melanoma cells with steady-state labeled DNA were lysed at pH 8 in SDS. Dacarbazine (10 μg/ml) was added to the lysate which was kept at 37°C in the dark for 60 min. The lysate was then made alkaline according to our routine procedure. After neutralization of the solution we performed gel electrophoretic analysis of the DNA. As shown in Fig. 4 this experimental protocol does not allow dacarbazine to induce damage in the DNA. The same result was obtained with cells that had been treated with aphidicolin (10 μg/ml) for 60 min before the initial cell lysis in SDS.

DISCUSSION

We report in this communication the effect of dacarbazine on growth and DNA synthesis of cultured human neoplastic cells. When the cells are treated with dacarbazine for 60 min cell division is inhibited for 12–24 h. In contrast, in cells treated with aphidicolin prior to the addition of dacarbazine cell divisions are resumed almost immediately after the drug treatment. Aphidicolin is a specific inhibitor of DNA polymerase α (7). The results therefore indicate a correlation between DNA synthesis and the ability of dacarbazine to stop cell divisions.

This prompted us to investigate whether dacarbazine may damage the DNA and if so to characterize the interaction of dacarbazine with the DNA. As before we have used a technique of alkaline cell lysis to study DNA synthesis (9–11).

The results show that treatment with dacarbazine of cells containing prelabeled DNA results in the generation of a single-stranded population of DNA fragments with a size between 2 and 10 kilobases. The DNA fragments are not present in untreated cells. In contrast we have not been able to detect DNA fragments in cells treated with aphidicolin prior to dacarbazine treatment. The size of the fragments increase somewhat when the cells are examined 24–48 h after the drug treatment.

Our results show also that dacarbazine inhibits the synthesis of DNA replication intermediates. This effect develops gradually, allowing some movement of the replication fork during the beginning of the drug treatment. When DNA is pulse-labeled at the beginning of the drug treatment, almost all of the label is located in the 2–10-kilobase DNA fragments. No labeled high molecular weight DNA can be detected. The results support the interpretation that dacarbazine has the ability to damage DNA in cells synthesizing DNA. Furthermore they agree with the finding that dacarbazine, and not its metabolites, may be effective in melanoma chemotherapy as found in isolated extremity perfusions (5).

Hence our results show that dacarbazine damages the DNA only in the presence of functioning DNA polymerase α. It is well established that the main function of DNA polymerase α is in semiconservative DNA synthesis (7). Therefore a likely explanation is that dacarbazine induces damage in the DNA during semiconservative DNA synthesis. Some findings indicate that there may exist more than one mechanism for the induction of damage. The sizes of the released fragments increase with time after drug treatment. Furthermore when we compare the level of labeled fragments in prelabeled cells and pulse-labeled cells, we find a higher level in the pulse-labeled cells.

The data are compatible with the incorporation of dacarbazine into DNA as one pathway to induce DNA damage. Other pathways involving the replicon are also possible, *e.g.*, attack of the drug on the single-stranded DNA transiently present during DNA synthesis. Whatever mechanism is operative it shows up as the fragmentation of both parental and newly synthesized DNA strands. DNA with alkali-labile regions arises

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either through enzymatic strand scission as part of a repair process or through the direct chemical alteration of the DNA.

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