Hyperthermia Can Reduce Cytotoxicity from Etoposide without a Corresponding Reduction in the Number of Topoisomerase II-DNA Cleavable Complexes

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ABSTRACT

The chemotherapeutic drug etoposide (VP-16) causes the equilibrium reaction between noncleavable and cleavable topoisomerase II-DNA complexes to shift in favor of the cleavable complex [H. Zang, P. D’Arpa, and L. F. Liu, Cancer Cells (Cold Spring Harbor), 2: 23–27, 1990]. Pulsed-field gel electrophoresis was used to study induction and removal of cleavable complexes in cells heated before, during, or after VP-16 treatment. Pulsed-field gel electrophoresis results were evaluated both as the fraction of activity (DNA) released from the plug and as the number of double-strand breaks (DSBs) calculated from molecular weight distributions; both end points led to the same conclusions. When cells were heated at 42°C during treatment with VP-16 (12 μg/ml up to 60 min), a slight decrease in cleavable complexes (from 36 to 20 DSBs/100 megabase pairs) was detected immediately after treatment when compared with cells treated with the drug at 37°C. Furthermore, heating at 42°C caused a slight decrease in drug cytotoxicity as measured by less than a 2-fold increase in clonogenic survival. When cells were heated for 10 min at 45.5°C prior to or after treatment with the drug, there was a reduction (~50%) immediately after treatment in the number of DSBs/100 megabase pairs compared with unheated cells. The rate of removal of cleavable complexes was decreased slightly by heat. After 120 min at 37°C, the number of DSBs/100 megabase pairs decreased to ~6 for both unheated cells and those heated prior to drug treatment and to ~8 for cells heated after drug treatment. In agreement with a low effect of heat on the number of cleavable complexes after drug treatment, there was no significant effect of this heating protocol on drug cytotoxicity. However, heating at 45.5°C prior to drug treatment at 37°C protected cells from drug cytotoxicity (e.g., increased survival after 12 μg/ml for 60 min by ~100-fold) despite the similarity in the induction and rate of removal of cleavable complexes when compared with nonheated cells. Thus, when cells are heated prior to administration of VP-16, drug cytotoxicity does not correlate with the number of cleavable complexes measured either immediately after treatment or 180 min later when ~75% of the initial number have been removed. Finally, since hyperthermia can actually decrease drug cytotoxicity, the use of hyperthermia as an adjuvant to chemotherapy involving topoisomerase II poisons, such as VP-16, should be approached with caution.

INTRODUCTION

Topoisomerase II transiently breaks and rejoins both DNA strands either simultaneously or coordinately (1, 2) to allow the passage of a second double-stranded DNA molecule through the site of breakage. During this reaction, topoisomerase II molecules bind to the cleavage sites via covalent attachment at the 5' ends (phosphotyrosine linkage). This strand breakage, strand passage, and religation procedure is thought to play a fundamental role in chromosomal organization, especially by unwinding DNA supercoils during DNA synthesis and transcription (3) and by allowing the chromosomes to segregate during mitosis, because topoisomerase II is a major component of the mitotic scaffold (4). Thus, topoisomerase II is critical for cell function and represents an ideal target for growth inhibition and cell killing. Several reviews describe DNA topoisomerases as chemotherapeutic targets (2, 5–7).

VP-16 is a non-intercalative antitumor agent proposed to inhibit strand-passing activity (2, 5, 3, 8, 9) by stabilizing a cleavable complex between topoisomerase II and DNA, thus interfering with the normal breakage-religation reaction catalyzed by the enzyme. Protein denaturation of the trapped cleavable complexes yields DNA breaks and a linkage of a topoisomerase subunit to 5' ends (2, 10). Thus, exposure to strong detergents or proteinase K allows analysis of cleavage patterns and DNA fragmentation by pulsed-field gel electrophoresis (PFGE) [field inversion gel electrophoresis (11)], neutral elution (12), and alkaline unwinding techniques (13). However, in vitro experiments reveal that breaks disappear after removal of the drug, indicating that the lesions are reversible (8, 14). Nevertheless, a cytotoxic lesion may result either from a cleavable complex itself or if the complex is translated into a form of DNA damage (5, 12–14).

Evidence has been reported that these trapped cleavable complexes are indeed cytotoxic. Charron and Hancock (11) reported that when CHO cells were treated with teniposide (also an epipodophyllotoxin), DNA synthesis was not affected by the drug, prompting them to suggest that molecules of topoisomerase II are not associated with DNA replication. However, these molecules showed a delay in the formation of mitotic chromosomes which, when formed, may be incompletely condensed. Furthermore, examination of mitotic chromosomes of teniposide-treated cells revealed breaks and several types of aberrations (15), and a correlation existed among DNA strand breaks, chromosomal aberrations, sister chromatid exchanges, and cytotoxicity (16, 17). Also, Loike and Horwitz (18) observed that VP-16 caused fragmentation of DNA in HeLa cells but did not observe degradation of purified DNA. Markovits et al. (19) reported a higher frequency of protein-DNA cross-links in nuclei isolated from proliferating cells treated with VP-16 than in treated quiescent cells and noted that the yield of cross-links increased concomitantly with the peak of DNA synthesis. This was in agreement with their findings of the increased cytotoxicity of VP-16 on proliferating (especially S-phase) cells versus quiescent cells. In addition, Kaufmann (20), using human HL-60 or KG1A cells, observed irreversible DNA degradation (into nucleosomal ladders) within 4 h after treatment with etoposide. Since fragmentation was inhibited by exposing cells to dinitrophenol, Kaufmann (20) suggested the possibility that intervening metabolic processes may be the cause of the extensive fragmentation observed rather than the direct action of topoisomerase II and/or VP-16 on DNA. Consistent with this observation, Jaxel et al. (21) demonstrated that prolonged exposure of splenocytes (20 h) to etoposide induced a secondary and irreversible fragmentation of DNA into low molecular weight molecules. They postulated that the mechanism of action does not involve topoisomerase II directly, but instead it must be similar to the action of novobiocin, which does not produce topoisomerase II-mediated DSBs. Based on these observations of
DNA fragmentation, an attractive hypothesis is that one mechanism of cell killing by etoposide may involve apoptosis. Caldecott et al. (12) used a repair-deficient CHO cell line (xrs-1) that is hypersensitive to \( \gamma \)-radiation to demonstrate that some DSBs induced by etoposide and m-AMSA are frank breaks and not cleavable complexes, since many DSBs were observed 2 h after treatment when levels of DNA-protein cross-links had returned to background levels. These researchers and others (22-24) believe that multiple mechanisms of action may be involved in cell killing, depending on drug concentration, and they suggest that not all DSBs in cells treated with VP-16 or with m-AMSA remain associated with topoisomerase II-DNA complexes.

Nevertheless, in their studies (12, 25) they observed that after drug treatment the relative level of un repaired DSBs correlated with drug-induced cell killing. All of the studies above support but do not prove the hypothesis that DSBs resulting directly or indirectly from poisons of topoisomerase II are cytotoxic lesions.

An attractive clinical application of hyperthermia has been to combine it with chemotherapeutic agents to synergistically enhance cytotoxicity of the drugs, especially in tumor cells. For example, heat has been shown to potentiate cytotoxicity of several drugs, including cis-platinum (26) and local anesthetics (27), and to sensitize cells to radiation, probably by inhibiting one or several critical processes involved in repair of DNA damage (28, 29).

Heat, which was shown to enhance VP-16 cytotoxicity in a multidrug-resistant mutant leukemia cell line (30), failed to enhance cytotoxicity in human T-cell lymphoblasts when heat (41.8°C) was administered during drug treatment (31). Also, heat was observed to protect human and rodent cell lines (32) from DNA damage and cytotoxicity when heat was administered before m-AMSA (an inhibitor of topoisomerase II). Therefore, in the present study, we wished to examine the effects of VP-16 on cytotoxicity and the induction and removal of cleavable complexes by using PFGE to quantify DSBs when CHO cells were treated prior to, during, or after hyperthermic exposures.

MATERIALS AND METHODS

Culture and Labeling Conditions. In all experiments, asynchronous CHO cells were used. Stock cultures were routinely grown in monolayer in tissue culture flasks (Falcon) containing McCoy's 5A medium supplemented with 10% iron-supplemented calf serum (Hyclone Laboratories, Logan, UT) and maintained in a humidified incubator (95% air, 5% CO\(_2\)) at 37°C. In all PFGE experiments, 1.0-2.5 x 10\(^6\) cells were plated into T-25 flasks approximately 20-48 h prior to radiolabeling for 18 h with 2 \( \mu \)Ci/ml of \( ^{3}H \)-thymidine (5 \( \mu \)Ci/\( \text{mmol} \); Amersham) and 3.2 \( \mu \)g/ml stable thymidine. Cells were chased with fresh medium for 1 h at 37°C.

Hyperthermia, Drug, and Radiation Treatments. Cells were heated either prior to, during, or after incubation with various concentrations of VP-16 (or Vepesid; Bristol-Myers, Princeton, NJ) for various times. Cells receiving hyperthermia treatments were heated at 42.0°C or 45.5°C in precision water baths regulated to 0.1°C. Samples heated prior to drug treatment were transferred to a 45.5°C bath for 10 min. After heating at 45.5°C, cells were transferred to a 37°C water bath for 5 min, the medium was aspirated, and 5 ml of medium (prewarmed to 37°C) containing the appropriate concentrations of VP-16 were added. Flasks were then transferred into a 37°C water bath for the duration of drug treatment, after which cells were washed twice with drug-free medium. Cells to be heated after drug treatment were allowed to incubate in a 37°C bath for appropriate times with the drug, after which the cells were washed twice. Drug-free medium (5 ml) equilibrated at 37°C was then added back to flasks, which were then immersed in a 45.5°C bath for 10 min. Cells heated during drug treatment were immersed in a 42°C bath for up to 1 h and then washed free of the drug. Cells were then trypanized for survival analysis, or flasks were returned to the 37°C bath for various lengths of time to allow for removal of lesions, after which, flasks were cooled on ice at least 15 min prior to preparation of PFGE samples for DSB analysis (see below). Cells to be irradiated were placed on ice for at least 15 min prior to receiving a 60-Gy dose of X-rays. A Westinghouse Quadrocondex X-ray machine was used for irradiation, operating at 15 mA and 250 kVp (unfiltered beam) and at a dose rate of 10 Gy/min. All irradiations were done with the T-25 flasks kept on ice.

Preparation of DNA for PFGE. Normal detergent lysis (to remove proteins from DNA) results in the formation of single- and double-strand breaks at the site of the complex; thus, procedures used for embedding DNA in agarose plugs, followed by PFGE, can be used to detect the presence or removal of cleavable complexes. Preparation of agarose plugs for PFGE has been described in detail elsewhere (33).

Briefly, cells were trypanized on ice with a 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered solution containing 0.01% trypsin (pH 7.85) and resuspended in 5 ml cold McCoy's medium containing 2 mg/ml chick egg white albumin. Cells were centrifuged, resuspended in cold Hanks' balanced salt solution, counted, and washed twice in cold buffer L (0.1 M disodium EDTA, 0.01 MTris, and 0.02 M NaCl, pH 8.0). After the last wash, buffer L was aspirated, and an equal volume of 1% low-melting point agarose was added to the cell suspension in a small amount of buffer L. An agarose “snake” was formed by immediately pipetting this mixture into Tygon tubing, clamping the tubing tight at both ends, and allowing at least 30 min for the agarose to solidify while chilled on ice. The solidified agarose snake was then extruded, cut into 0.5-mm plugs, and lysed overnight in lysis buffer (buffer L with proteinase K and Sarkosyl) in a 50.0°C water bath. Plugs were then washed 4 times (once per h) with 10 mM Tris-1 mM disodium EDTA buffer and inserted into 0.56% agarose gels. Gels were subjected to transverse alternating field electrophoresis (Geneline; Beckman Instruments, Fullerton, CA; 144 h, 40 V, 60-min pulse, 13-14°C), stained with ethidium bromide, and cut into lanes. Lanes were subsequently cut into 5-mm segments and transferred to scintillation vials for assessment of radioactivity per fraction of gel.

Determination of Number of Cleavable Complexes by PFGE. DNA damage has been evaluated from FAR (33, 34). Also, previous calibration of our PFGE system with \[^{125}\text{I}]\text{IrdUrd labeled DNA (35) has related FAR data to the number of DSBs. However, since no }^{125}\text{I}]\text{IrdUrd calibration was done in the present experiments with VP-16, the numbers of DSBs were calculated from molecular weight distribution data like those plotted later in Fig. 2B. Distributions were evaluated by integration to obtain a plot (shown later in Fig. 2C) of the fraction of the DNA (Q) less than \( X_{n} \), which means that }\approx 19% of the DNA is trapped in the plug and not available for analysis by PFGE. This trapping phenomenon has been shown to be due to cells in S phase for which the magnitude of the effect was the same for cells X-irradiated (39) or cells treated with etoposide (40). Therefore, the Q values were normalized by dividing by 0.81 to correct for the DNA trapped in the plug.

The relationship between a, the fraction of DNA less than a certain size, \( X_{n} \), the average chromosome size, \( S \), and the number of DSBs/mbp for a random distribution \( \mu_{s} \) (36-38) is given as

\[
Q = 1 - e^{-\alpha X_{n}} \left( 1 + \mu X_{n} - \frac{\mu X_{n}^{2}}{S} \right)
\]

A computer program was written so that the value of \( \mu_{s} \), the number of DSBs/mbp, could be estimated by least squares fitting of Equation A to the 5 pairs of \( Q \) values and molecular weights, \( X_{n} \), with 230 Mbp used for S. Cell Survival Experiments. After treatments, cells were trypsinized at room temperature (0.05% trypsin with EDTA), resuspended in medium, and counted. Appropriate numbers of cells were plated into T-25 flasks (3/sample) containing 10\(^{5}\) lethally irradiated CHO feeder cells. Flasks were transferred to a 37°C incubator for 7-11 days to allow for colony formation. Then, colonies were fixed with methanol:acetic acid (3:1) and stained with crystal violet. Only colonies with 50 or more cells were included in the survival analysis. The plating efficiency was consistently greater than 90% in all experiments.

RESULTS

We have examined the effect of hyperthermia before, during, and after treatment with VP-16 to determine if heat potentiates drug cytotoxicity and induction of cleavable complexes manifested as...
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Incubation at 37°C or 42°C during Drug Treatment. The effect of hyperthermia during VP-16 treatment (12 μg/ml) on the surviving fraction is shown in Fig. 1. A slight reduction in cell killing was observed when cells heated at 42°C during drug treatment for various times were compared with cells treated with the drug at 37°C. The heat dose itself killed less than 10% of the cells. Also, when cells treated with the drug at 37 or 42°C were examined microscopically, no gross morphological differences were noted (data not shown).

When PFGE was used to determine the effects of heating during drug treatment on the formation of cleavable complexes, the induction curves, based on FAR, were superimposable for VP-16 treatment at 42°C or 37°C (Fig. 2A). The induction of cleavable complexes reached a plateau after 15—30 min of treatment; when the cells were washed free of the drug and returned to 37°C for up to 2 h, no difference in removal of lesions was noted (data not shown). In Fig. 2B, DNA distributions (percentage of radioactivity/5-mm fractions of gel in the lane as a function of migration distance) indicated that migration of DNA through the agarose gel was about the same for cells treated for 30 min with drug at 37°C or 42°C. Fig. 2C was constructed from Q values, the fraction of DNA mass with molecules smaller than molecular weight (X_m) markers; the curves are shown for protein-linked DSBs measured by PFGE. Further, we sought to detect whether heating would result in the inhibition of removal of cleavable complexes. Several investigators have used sodium dodecyl sulfate or proteinase K treatments in conjunction with alkaline unwinding assays (13), neutral elution (12), or PFGE (11) to detect cleavable complexes.

Fig. 1. Survival of cells treated with VP-16 during hyperthermia. Cells were treated with 12 μg/ml VP-16 (20.4 μM) at either 42°C (•) or 37°C (○) for various times. The SEM (bars) for the data was calculated from 2 experiments. A representative survival curve for cells heated at 42°C without the drug (△) is shown for reference.

Fig. 2. Use of PFGE to measure the effect of hyperthermia on the formation of trapped DNA-topoisomerase II cleavable complexes in cells heated for various times at either 42°C (•, ○) or 37°C (△, ○) during treatment with 12 μg/ml of VP-16. Data are from one representative experiment. (A) Percentage of DNA released from the plug as a function of time at 37°C or 42°C. In other experiments, heat alone (up to 60 min at 42°C) did not cause a significant amount of DNA to migrate out of the plug and into the gel (data not shown) compared to the untreated control (2%, see figure). Data represent the total percentage of DNA released from the plug and were not corrected for background. In similar experiments, heated (1 h at 42°C) and unheated samples not treated with VP-16 had 2—5% DNA released from the plug. (B) DNA size distribution profiles for cells treated with VP-16 for 30 min at either 37°C (○) or 42°C (•); △, unheated control cells not treated with VP-16 (heat alone did not alter the DNA size distribution profile, not shown). Abscissa, distance migrated by the DNA fragments in the agarose gel (in cm). Arrows suspended from the top abscissa, distances that the molecular weight standard chromosomes from Saccharomyces pombe (5.7, 4.7, and 3.0 Mbp) and Saccharomyces cerevisiae (2.2 and 1.6 Mbp) migrated into the gel. (C) Q value, the fraction of molecules smaller than a given yeast molecular weight (X_m) chromosomal marker, is plotted as a function of X_m for the two 30-min samples shown in B. (D) Induction of cleavable complexes represented by DSBs/100 Mbp were calculated from the best fits to the Q values shown in C. Data represent total DSBs (uncorrected for control samples). The unheated sample not treated with VP-16 yielded a value of 1.9 DSB/100 Mbp (shown). Heat alone (60 min at 42°C) did not cause a significant increase in DSBs/100 Mbp compared to the untreated control (heated and unheated controls ranged from 1.9 to 3.1 DSBs/100-Mbp in all experiments).
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Fig. 3. Survival of cells treated for 1 h at either 37°C (○) or 42°C (●) with various concentrations of VP-16. Data are from one representative experiment.

30 min of treatment, but other curves not shown were obtained for 15 and 60 min of treatment. From "best fits" to the Q value curves, the number of DSBs per 100 Mbp was calculated and used to construct Fig. 2D. The data, which are from a representative experiment, show a result similar to that for FAR (Fig. 2A); i.e., no significant differences were noted between treating with the drug (12 μg/ml) at 37°C or 42°C.

Survival curves and PFGE results for cells heated at 42°C during treatment with various concentrations of VP-16 (Figs. 3 and 4A) are consistent with the results shown in Figs. 1 and 2A. Heating at 42°C in the presence of the drug protected it only slightly, if at all, from both cytotoxicity and the increase in percentage of DNA migrating from the plug into the lane. Fig. 4B shows examples of DNA distributions as a function of drug concentration (1 h treatment) at 37°C or 42°C, as well as for a 60-Gy dose of X-irradiation. The data for the 60-Gy dose were similar to data for ~12 μg/ml of VP-16 (50–60% FAR values and similar distributions in Fig. 2, B and C). Thus, the molecular weight distributions for either X-irradiation or VP-16 treatment fit reasonably well to the curves for Q values which are generated for a random Poisson distribution of DNA double-strand breaks (38, 41). The survival values resulting from the same number of DSBs (20–30 DSBs/100 Mbp) induced by 60-Gy or 12 μg/ml VP-16, however, are greatly different, i.e., $10^{-20}$ versus $10^{-5}$, respectively. The main point is that heating at 42°C during drug treatment does not cause significant changes in the size distributions of DNA molecules and that the number of DSBs/100 Mbp as a function of drug concentration (Fig. 4D) is only slightly lower for cells treated at 42°C than for those treated at 37°C. The statistical significance of any real divergence between 37°C and 42°C would have to be confirmed in additional experiments. Additionally, cleavable complexes induced by VP-16 appear to be produced at random, as with radiation.

Incubation at 37°C or 45.5°C After Drug Treatment. Cells also were not sensitized to drug cytotoxicity when heat at 45.5°C was administered immediately after 1 h of VP-16 treatment (Fig. 5). The survival curve for cells heated after drug treatment (12 μg/ml = 20.4 μM) was parallel to the curve for cells that received only a heat dose, and when the curve for cells heated after VP-16 treatment is normalized for cell killing from the heat dose alone, the resulting survival curve is relatively flat and virtually superimposable on the curve for cells exposed to drug at 37°C. Again, morphological examination of cells prior to or after receiving the postdrug heat treatment yielded no morphological differences, although heat caused some cells to round up slightly.

The effect of hyperthermia on induction and removal of DNA topoisomerase II cleavable complexes in cells heated (10 min, 45.5°C) after VP-16 treatment (12 μg/ml, 1 h, 37°C) was measured by PFGE and expressed as either FAR or the number of DSBs/100 Mbp (Fig. 6). Heating after drug treatment caused an approximately 50% reduction in the formation of cleavable complexes; however, when cells...
were heated at 45.5°C, they went through a thermal transition where the temperature was ~37°C for ~2 min after the drug was removed. Some repair during this short period at 37°C could account for the slight decrease in the number of initial lesions in the heated samples. Upon incubation at 37°C after heating, the trapped complexes were removed more slowly during a 120-min period than when they were removed in unheated cells.

Comparing 45.5°C before Drug Treatment with 45.5°C after Drug Treatment. To relate these observations for the induction and removal of cleavable complexes to cytotoxicity, drug dose response curves were determined for survival of cells heated prior to or after treatment with various concentrations of VP-16 at 37°C (Fig. 7). As noted in Fig. 5, the drug cytotoxicity for cells heated after drug treatment was about the same as it was for unheated cells. However, when cells were heated for 10 min at 45.5°C prior to drug treatment at various concentrations at 37°C (Fig. 7) or at 12 µg/ml for various time periods at 37°C (Fig. 8), cells were strongly protected from drug cytotoxicity. In contrast, when the induction and removal of cleavable complexes were compared (Fig. 9) between nonheated cells treated with VP-16 (1 h at 12 µg/ml for 37°C) and cells heated prior to drug treatment, only small differences were seen in the induction and removal of complexes. The reduction of about 50% in the initial number of cleavable complexes and their slightly slower rate of removal when the cells were heated before drug treatment certainly should not account for the large amount of protection from cytotoxicity when cells were heated before administration of the drug (Figs. 7 and 8).

Morphological Alterations. An interesting and possibly important observation made was that when cells were examined for morphological changes after treatment with drug, with or without hyperthermia, a large proportion of the cells that were heated at 45.5°C before administration of VP-16 rounded up with significant membrane blebbing (data not shown). However, cells that were treated with VP-16 at 37°C or heated after drug treatment were similar in appearance to nonheated control cells. Thus, one might conclude that the strong protective effect that was observed when heat preceded administration of the drug was due simply to loss of rounded, blebbing nonclonogenic cells (42) during the trypsinization procedure. This was not the case, however, because great care was always taken to ensure that no or very few rounded cells were dislodged and lost when cell samples were prepared for PFGE and survival experiments. For example, equal numbers of cells were plated into 2 replicate flasks to be heated at 45.5°C for 10 min before drug treatment. In both cases, the medium which was poured from the cells and the medium that was used to wash the drug from the cells did not contain any cells as observed both microscopically and by electronic particle data counts. Furthermore, when all the washes and trypsin rinses were combined with the medium into which the cells from one flask were suspended, the final cell count was 1.92 × 10⁶ and essentially the same as 1.88 × 10⁶ obtained when the initial medium and washes were not pooled and saved for the other flask. Therefore, the increase in survival (up to 100-fold, depending on concentration and length of drug treatment) observed when heat preceded drug treatment (Figs. 7 and 8), was not due to loss of rounded nonclonogenic cells because such a loss would.

Fig. 5. Survival of cells treated for 1 h at 37°C with 12 µg/ml VP-16 and then incubated at either 37°C or 45.5°C for various times after the drug treatment. The cells were washed free of drug prior to the incubation period. SEMs (bars) for 2 experiments are shown, with survival values for cells treated with VP-16 only (from one of the experiments) also shown for reference (○). — — —, surviving fraction for cells heated after VP-16, but normalized for heat killing.

Fig. 6. Effect of hyperthermia on the induction and removal of the DNA-topoisomerase II cleavable complexes formed in cells treated with VP-16 and measured as DSBs by PFGE. Cells were treated for 10 min at 45.5°C after the drug treatment or treated with VP-16 only. Cells were treated with VP-16 (12 µg/ml for 1 h at 37°C), the drug was removed, and the cells were washed. Then, cells were heated either at 45.5°C for 10 min (□, ▲) or not (□, △) before transfer into a 37°C bath for various times to allow removal of the cleavable complexes. Data from 2 individual experiments are shown. (A) Removal of cleavable complexes from heated (□, ▲) or nonheated (□, △) cells as measured by the percentage of DNA released from the plug following PFGE. The mean values for unheated and heated controls for the two experiments shown were 3.9 and 4.5%, respectively. (B) Removal of cleavable complexes from heated (□, ▲) or nonheated (□, △) cells as measured by the percentage of initial DSBs remaining after normalization to the initial number of DSBs induced.
human melanoma cells when heat at 45°C was administered immediately prior to VP-16 treatment.

The amount of cytotoxicity that we observed, however, did not correlate with the number of cleavable complexes consisting of VP-16, topoisomerase II, and DNA. Hyperthermia at 42°C during drug treatment or at 45.5°C for 10 min immediately after drug treatment slightly decreased (less than a factor of 2) the number of cleavable complexes (DSBs/100 Mbp) measured immediately after treatment (compare Figs. 2, 4, and 6). However, when heat at 45.5°C for 10 min immediately preceded drug treatment, the number of cleavable complexes was reduced much less than the reduction in cytotoxicity (Figs. 7, 8, and 9) (i.e., less than 2-fold compared with 100-fold for cytotoxicity). With heat, the drug concentration required for isotoxicity (isosurvival) would have to be increased ~8-fold (Fig. 7), compared with only ~1.3-fold for the same number of cleavable complexes (compare Figs. 4 and 9). Also, the large degree of protection from have to consist of as much as 99% of the cells in the flasks; as discussed above, this certainly did not occur. Although most of the cells recovered morphologically within 24 h, we did not attempt to correlate morphological recovery of the rounded cells with the ability to divide and form macroscopic colonies.

DISCUSSION

Our results have shown that mild hyperthermia (42°C) during a 1-h treatment with VP-16 or 10 min at 45.5°C immediately after the 1-h treatment did not enhance drug cytotoxicity but instead resulted in a slight degree of protection as observed by a less than 2-fold increase in survival (Figs. 1, 3, and 7). However, 10 min at 45.5°C immediately before drug treatment caused a large amount of protection as observed by as much as a 100-fold increase in survival (Figs. 7 and 8). These results are consistent with other studies (31, 32) for hyperthermia combined with poisons of topoisomerase II, although the amount of protection reported for m-AMSA (32) was much less than what we have observed for VP-16. Also, Raaphorst et al.4 and Dynlacht and Syzek5 have observed significant protection of human fibroblasts and

Fig. 7. Survival of cells heated (10 min at 45.5°C) prior to (A) or after (B) exposure to various concentrations of VP-16 for 1 h. O, survival of cells treated with VP-16 only. Data are from one experiment, but the much greater protective effect from heat given before the drug than when it is given after the drug is supported by the data presented in Figs. 5 and 8.

Fig. 8. Survival of cells heated at 45.5°C for 10 min prior to treatment with 12 μg/ml VP-16 for various times at 37°C. SEMs (bars) were calculated from two experiments. O, unheated samples; □, heated samples.

4 P. Raaphorst et al., personal communication.
5 J. R. Dynlacht and L. Syzek, unpublished observations.
VP-16 cytotoxicity when heat preceded drug treatment could not be explained by heat increasing the rate of removal of the cleavable complexes as has been reported under certain conditions (14, 32). In fact, the rate of removal may have been slightly less in the heated cells (Fig. 9); but, most importantly, by 180 min after drug removal, when 70–80% of the lesions had been removed, there were 4–5 DSBs/100 Mbp for both heated and unheated cells [compared to values of 2.3 and 1.8 DSBs/100 Mbp for unheated and heated controls, respectively (Fig. 9B)]. Although studies have shown positive correlations between cytotoxicity of topoisomerase II poisons and the number of cleavable complexes (3, 17, 23, 32), our present study and other studies (43) have not found a positive correlation.

In view of the results above, the question naturally arises as to whether the cleavable complexes formed among the topoisomerase II poison, topoisomerase II, and DNA, are the primary lesions responsible for cell lethality. Furthermore, there is evidence that a component of killing by topoisomerase II poisons, especially for high concentrations, has nothing to do with the formation or processing of cleavable complexes (23, 24). Such possibilities include membrane damage (although the rounding and blebbing of cells we observed only when cells were protected from cytotoxicity by heating prior to drug treatment suggest the opposite), formation of toxic oxidation products (43), and other undefined alterations in the cell. Nevertheless, the hypothesis frequently mentioned (3, 17, 23, 25) is that cellular processing of the complexes may result in errors in passing a DNA double helix through the DSBs produced by the VP-16-topoisomerase II-DNA complex, which in turn, could result in lethal chromosomal deletions and exchanges (15, 17, 44–46). Also, DNA repair mechanisms may be involved in processing the potential DSBs, which apparently do not exist in vivo until the chromosomal protein is removed from the DNA by SDS or protease treatment, which causes the complex to detach from the DNA and leave a DSB (2, 3, 17). Therefore, if the complex were completely reversible without any residual effects after removal of the drug, the cleavage of DNA, strand passing, and religation steps should proceed normally as if the drug had not been present (3). Apparently this does not occur because studies show that X-ray-sensitive mutant cells deficient in repair of X-ray DNA breaks also have increased sensitivity to topoisomerase II poisons (25, 44, 47, 48) for cell killing and/or chromosomal aberrations. Thus, these studies suggest that some type of irreparable or misrepaired DNA damage resulting from the cleavable complex is involved in killing by topoisomerase II poisons (1, 3, 14, 25). However, most of the cleavable complexes must be nontoxic upon their removal compared to DSBs induced by X-rays because ~30 DSBs/100 Mbp observed for 12 µg/ml of VP-16 (Fig. 4D) and reported for V-79 cells based on [32P]dUTP calibration (40) causes much less cell killing [survival of ~0.01 (Fig. 3)] than that observed for 60 G y which induces the same number of DSBs (Fig. 4C) but a very low surviving fraction of ~10−20 (calculated for a D0 of 1.5 Gy). Assuming that the cleavable complexes are indeed the primary toxic lesions, the challenge is to identify the cellular/molecular processes at the DNA level that lead to cell death.

Reports in the literature and our observation that heat administered prior to treatment with VP-16 greatly protects from cell lethality without any great effect on either the number of cleavable complexes or their rate of removal may give a clue as to what is occurring at the molecular level: (a) because of the reported heat resistance of both transport of drug into the cell (30) and topoisomerase II enzyme activity (32, 49, 50) and our observation that heat before treatment with VP-16 reduced the number of cleavable complexes less than 2-fold (Fig. 9B), there must have been very little, if any, effect on either transport of VP-16 to the DNA or the activity of topoisomerase II associated with the DNA; (b) the protection from topoisomerase II poisons is most likely not associated with heat-induced delay in cell cycle progression, as has been postulated for protective agents that also delay initiation of DNA synthesis (2, 51) or RNA transcription (5, 43, 51); heat (45.5°C for 10 min) by itself either before or after VP-16 treatment should cause a 12–16 h delay for movement from G1 into S and from G2 into mitosis (52, 53) and thus should have the same effect on cytotoxicity; (c) after cells are heated, nuclear protein appears to aggregate or precipitate in the nucleus (54), with most of the precipitation or aggregation occurring at the nuclear matrix (55, 56); and (d) most of the consensus sequences for binding topoisomerase II are located at the nuclear matrix (57–59) with about 2–3 topoisomerase II molecules for each DNA replication of about 100 kilobase pairs that also is attached to the nuclear matrix (58, 60, 61). Therefore, aggregation of protein at the nuclear matrix may lead to a change in chromatin structure (32, 49). This has been postulated (43) to alter the processing of cleavable complexes in other cases, such as for AT cells (47, 48) and for spermid versus monolayer cultures (43), where changes in sensitivity to topoisomerase II poisons could not be correlated with the number of cleavable complexes (43). If changes in chromatin structure are indeed important in altering the processing of cleavable complexes, our data (showing a lot of protection when heat preceded drug treatment could not be correlated with the number of cleavable complexes (43). If changes in chromatin structure are indeed important in altering the processing of cleavable complexes, our data (showing a lot of protection when heat preceded drug treatment, the nuclear matrix immediately before the drug is administered. For example, if cleavable complexes were especially toxic when they are located at forks in DNA where replication and/or transcription occur, heat-induced inhibition of replication and/or transcription before addition of VP-16 might be expected to greatly reduce cytotoxicity because of an expected reduction in the frequency of such forks in the DNA where cleavable complexes could be located. If this model is correct, transcription (2, 25, 64) as well as replication (2, 25, 45, 64–66) would be important because our VP-16 cytotoxicity data, with survival values as low as 10−3 (Figs. 1, 3, 7, 8) compared with only 30–50% of the cells in S phase (52) indicate that cells are killed in all phases of the cell cycle. Also, although cells treated in S phase appear to be the most sensitive (1, 3, 23, 46, 47, 51, 67), other researchers (2, 5) have concluded that topoisomerase II poisons must be cytotoxic throughout the cell cycle, especially for high drug concentrations. There are other possibilities, in addition to those mentioned above, for mechanisms of both cell killing by topoisomerase II poisons and the protective effect of heating before drug treatment; thus, detailed studies at both the molecular and cellular levels are needed. For example, synchronous cells should be studied to determine how cells treated in G1 or S, with or without heat, are delayed in the cycle followed by either cell lysis, apoptosis, and/or movement into mitosis with the appearance of chromosomal aberrations. Presumably, chromosomal aberrations would be observed (15, 44), but the frequency of cytologically abnormal cells and the frequency of those cells dying without entering mitosis must be related quantitatively to the frequency failing to form macroscopic colonies.

The main point of our present study is that we have confirmed by using pulsed-field gel electrophoresis that mammalian cells treated with hyperthermia, particularly before drug treatment, are protected from drug cytotoxicity without corresponding changes in either the initial number of cleavable complexes or in the number when at least 80% of the complexes have been removed by 3 h after drug treatment. Finally our data (Figs. 1, 3, 5, 7, 8) and those of others (31, 32) suggest that hyperthermia will not enhance the cytotoxicity of VP-16.
and other topoisomerase II poisons used in the treatment of cancer, at least when heat is given before the administration of the drug. Only under the unlikely situation where normal tissue would be selectively heated or the tumor would be selectively cooled would a therapeutic gain be expected when heat is administered before VP-16.

REFERENCES

Hyperthermia Can Reduce Cytotoxicity from Etoposide without a Corresponding Reduction in the Number of Topoisomerase II-DNA Cleavable Complexes


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