ABSTRACT

Differential scanning microcalorimetry of nuclei from cultured cells revealed differences between antitumor drugs in potency and mechanism. Scanning calorimetry of nuclei showed four structural transitions as the temperature was raised from 25 to 120°C. Transitions II (76°C), III (88°C), and IV (105°C), respectively, characterize the denaturation of the nucleosome, the unstacking of bases in nicked DNA after release from the nucleosome and unstacking in the released intact, supercoiled DNA.

Nuclei from human epithelial cells treated with the DNA strand breakers bleomycin and streptonigrin showed an increase in transition III at the expense of transition IV. The effect was dose dependent. At intermediate times of treatment a substantial portion of the chromatin melted between the temperatures of transitions III and IV and this was taken to represent intact supercoiled DNA in which base-pairing had been weakened by loss of some bases.

Treatment of cells with the alkylating agents N-nitroso-N-methylurea and mitomycin C gave results similar to those of the strand-breaker antitumor drugs, except that they were less potent. Irradiation by UV light produced similar effects.

The effects of intercalating drugs were quite distinct from those produced by strand breakers or alkylators. Nuclei from cells incubated with increasing doses of actinomycin D or ethidium bromide gave calorimetric scans that indicated progressive denaturation of the nucleosome with increasing doses of actinomycin D or ethidium bromide. Although McMurray and Van Holde (8) used this physicochemical technique to various human cell types cultured in vitro, three of these four factors might be taken into account. Moreover, the extent of DNA damage induced by antitumor drugs. In practice, the loss of transition IV during drug treatment was correlated with the loss of cellular capacity to divide, regardless of which drugs were used.

INTRODUCTION

A quick assay for the potency of antitumor drugs in human tissues would be useful and differential scanning microcalorimetry of drug-treated human cultures seems a possible basis for such an assay. This calorimetric technique applied to intact nuclei isolated from cultured cells has already proven capable of revealing several features of DNA structure within chromatin (1–3) and so it might assay antitumor drugs whose primary mechanism is DNA damage, observing the extent and nature of DNA damage induced by antitumor drugs. In practice, the potency of a drug depends on its ability to reach target cells, and on its penetration into and transport within a cell as well as possible metabolic modification. By application of scanning calorimetry to various human cell types cultured in vitro, three of these four factors might be taken into account. Mechanisms of action not involving DNA damage would not be measured in such an assay, but there are large categories (4) of antitumor drugs that act primarily on DNA structure without much secondary action. It was in this hope that we tested the promise of this physicochemical technique holds promise as a quick assay for antitumor drugs.

MATERIALS AND METHODS

Cell Culture. Human epithelial cells derived from an aplastic squamous cell carcinoma of the nasal septum (CCL-30, RPMI 2650) were obtained from the American Type Culture Collection, Rockville, MD. The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin G, and 100 units/ml streptomycin. The cells were grown in 150-cm² tissue culture flasks (Corning Glass Works, Corning, NY) at 37°C under a humidified 5% CO₂ atmosphere.

Sterile stock solutions of antitumor drugs (Sigma) in PBS1 pH 7.4 were added to the medium of the cultured cells to the appropriate final concentrations. Cells were incubated in the presence of the drugs for 6–48 h.

Measurement of Replication Rate. The cells were passaged with 4 ml trypsin EDTA (GIBCO). Cellular replication rate was determined as…

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1 The abbreviation used is: PBS, phosphate buffered saline.
viable cell count upon subcultivation per time interval between two successive passages. For cells treated with anticancer drugs, the drug-containing medium was removed and the cells were thoroughly rinsed and transferred into a fresh drug-free medium. Incubation was continued for 24 or 48 h, after which the cells were trypsinized and counted.

Isolation of Nuclei. The culture medium was removed and the layer of cells was washed three times in swelling buffer (0.1 mM hexylene glycol, 1 mM CaCl₂, 0.06 mM PIPES, pH 6.8). The cells were scraped off using a rubber policeman and were resuspended in swelling buffer for 10 min. Nuclei were obtained after disruption of the cells with 25 strokes in a loose fitting Dounce homogenizer and centrifugation for 3 min at 1000 × g. The pellet of nuclei was washed three times in calorimetry buffer (buffer C: 5 mM Tris, 0.2 mM EDTA, 250 mM sucrose, 150 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, pH 7.5). All steps were done at 0–4°C.

Differential Scanning Calorimetry. Samples of nuclei were pelleted by centrifugation for 3 min in an Eppendorf microcentrifuge at 30–40% of full speed. The pellets (5–15 mg moist weight) were transferred to aluminum pans which has been previously siliconized to prevent the accumulation of moisture at the sealing surfaces, and the pans were hermetically sealed.

All calorimetric measurements were done on a DuPont 990 thermal analyzer from 25 to 120°C at a scanning rate of 5°C/min. Total enthalpy was calculated by cutting out peaks from tracings of the scans and weighing them. Base lines were taken between the minimum of the left side of transition II and the minimum on the right side of transition IV or IV(+). After scanning, sample pans were opened and the contents dissolved in 0.1 mM NaOH. The amount of DNA was determined by the absorbance at 260 nm, assuming E°₄⁰ = 280. Differential scanning calorimetry of stock solutions of each of the drugs used in this study revealed that none of them gave a measurable heat absorbance above baseline levels at a temperature range between 25 and 120°C.

UV Irradiation. Cells were cultured in petri dishes. Immediately prior to irradiation, the medium was replaced with 10 ml PBS. The uncovered dishes were placed in a hood (Belco Glass, Inc., NJ) and were irradiated with a germicidal lamp (G30t8, 30 w, General Electric) at a dose rate of 0.02 J/M²/s for 15 to 120 min. Nuclei were isolated immediately after irradiation was completed.

Nonirradiated cells incubated in PBS for the same time periods were used as controls.

RESULTS

The melting profile of nuclei from cultured human tumor epithelial cells was characterized by four structural transitions: (60°C), II (76°C), III (88°C), and IV (105°C). These transitions represent: I, nonchromosomal components; II, the collapse of the nucleosome, III, the melting of relaxed DNA released from the nucleosomal structure; IV, the melting of released superhelical DNA (5). Transition IV dominated the melting profile contributing about two-thirds of the total enthalpy change (Fig. 1, Table 1).

The first type of antitumor drug whose effects on the melting profile were tested was that of DNA strand breakers. Incubation of cells with the antibiotics bleomycin or streptonigrin resulted in rapid loss of transition IV and a concomitant increase in transition III. The response was dose-dependent (Figs. 1 and 2). The kinetics of the bleomycin effect were also studied (Fig. 3) to show that the complete substitution of transition IV by transition III required 45 h when tested at 20 μg/ml. As the enthalpy change of transition IV decreased it was accompanied not only by the change in transition III but also by the transient appearance of DNA melting between 95 and 100°C. We call the structural transition(s) intermediate between III and IV IV(—). The composite curve of IV and IV(—) gives the appearance of a lowering of the melting temperature from 105°C in control nuclei to 99°C after 20 h of incubation with 50 μg/ml bleomycin or 0.1 μg/ml streptonigrin.

The second type of antitumor drug tested was that of alkylating agents. The effects of N-nitroso-N-methyurea on the calorimetric scans were the same as those induced by strand breakers (Fig. 4, Table 1). Complete loss of transition IV after 20 h incubation required >2 mg/ml N-nitroso-N-methyurea. Similar results were also obtained with the alkylating drug mitomycin C (Table 1).

The effects of intercalating drugs on the melting profile of human chromatin were quite distinct as studied using actinomycin D and ethidium bromide. Incubation of cells with actinomycin D resulted in a marked decrease in enthalpy change and melting temperature for transition II and in a dramatic increase in the apparent melting temperature of transition IV from 105 to 115°C after 20 h of treatment with 20 μg/ml actinomycin D (Fig. 5, Table 1). Higher concentrations of actinomycin D were not studied because the thermal analyzer could not scan above 120°C. The kinetics of the actinomycin D effect is shown in Fig. 6.

The effect of ethidium bromide was similar to that of actinomycin D: transition II markedly decreased in both magnitude and melting temperature, whereas transition IV shifted from 105 to 110°C after 20 h of incubation with 10 μg/ml ethidium bromide (Fig. 7). The apparent increase in the melting temperature of transition IV produced by the intercalators is due to increasing amounts of DNA melting at temperatures above 110°C. The melting of such material we refer to as transition IV(+).

Scanning calorimetry was capable of distinguishing among several processes: the conversion of the (intact) superhelical DNA of chromatin to the relaxed form; the destabilization of the superhelical form by alkylators; the destabilization of the nucleosome; the stabilization of the superhelical form by intercalators. Therefore, it was of interest to study the damage induced by UV irradiation. The major result of short term exposure to UV irradiation is the cross-linking of bases in the DNA. As shown in Fig. 8 and Table 1, irradiation of whole cells resulted in rapid loss of transition IV (102–110°C) in the calorimetric profiles of their nuclei with the concomitant appearance of transition IV(—) (95–102°C). As exposures were extended and more excision repair would be expected, increases...
DIFFERENTIAL SCANNING CALORIMETRY OF NUCLEI

Table 1  Enthalpy change (cal/mg/DNA)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>II</th>
<th>III (82–92°C)</th>
<th>IV(–) (92–96°C)</th>
<th>IV (96–112°C)</th>
<th>IV(+) (112–120°C)</th>
<th>Capacity to divide (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.6</td>
<td>1.3</td>
<td>0.9</td>
<td>0.3</td>
<td>6.8</td>
<td>0</td>
</tr>
<tr>
<td>Bleomycin (μg/ml)</td>
<td>20</td>
<td>1.6</td>
<td>1.0</td>
<td>2.1</td>
<td>1.6</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>1.7</td>
<td>1.0</td>
<td>3.7</td>
<td>1.6</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>1.8</td>
<td>1.2</td>
<td>5.6</td>
<td>1.0</td>
<td>0.8</td>
</tr>
<tr>
<td>Streptomycin (μg/ml)</td>
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<td>0.7</td>
<td>1.0</td>
<td>3.9</td>
<td>1.1</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.5</td>
<td>0.4</td>
<td>6.0</td>
<td>0.8</td>
<td>0.9</td>
</tr>
<tr>
<td>Methyl-nitroso urea (mg/ml)</td>
<td>1</td>
<td>1.3</td>
<td>1.3</td>
<td>2.2</td>
<td>1.5</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.6</td>
<td>1.3</td>
<td>4.6</td>
<td>1.7</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.6</td>
<td>1.0</td>
<td>6.6</td>
<td>1.2</td>
<td>0.4</td>
</tr>
<tr>
<td>Mitomycin C (μg/ml)</td>
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<td>0.9</td>
<td>0.8</td>
<td>2.6</td>
<td>0</td>
<td>4.2</td>
</tr>
<tr>
<td>Actinomycin D (μg/ml)</td>
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<td>0.9</td>
<td>1.3</td>
<td>1.3</td>
<td>0</td>
<td>5.9</td>
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<tr>
<td></td>
<td>5</td>
<td>0.8</td>
<td>0.6</td>
<td>1.1</td>
<td>0.3</td>
<td>2.4</td>
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<tr>
<td></td>
<td>10</td>
<td>0.7</td>
<td>0.5</td>
<td>1.2</td>
<td>0</td>
<td>2.2</td>
</tr>
<tr>
<td>Ethidium bromide (μg/ml)f</td>
<td>1</td>
<td>1.3</td>
<td>1.1</td>
<td>1.1</td>
<td>0.3</td>
<td>6.7</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.9</td>
<td>0.2</td>
<td>1.4</td>
<td>0.4</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.5</td>
<td>0</td>
<td>0.7</td>
<td>0.4</td>
<td>3.1</td>
</tr>
<tr>
<td>UV (min)</td>
<td>30</td>
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<td>2.4</td>
<td>1.0</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>2.1</td>
<td>1.0</td>
<td>3.0</td>
<td>1.2</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>1.7</td>
<td>1.1</td>
<td>3.2</td>
<td>1.3</td>
<td>3.5</td>
</tr>
</tbody>
</table>

* After treatment of cells with drugs or UV radiation their capacity to divide within 24 or 48 h of drug-free incubation was measured. The figure given is the cell count after 24 h of drug-free incubation relative to the cell count for control (i.e., untreated) cells. Essentially the same figures were obtained when the counts were made after 48 h of incubation in drug-free medium.

† Repetition of the control 15 times showed a range of ±4% for all transitions except transition I, which varied as much as ±22%.

The enthalpic changes are underestimated (by as much as 40% at the highest drug concentrations) due to light absorption of the drugs at 260 nm. Since all the conclusions in the text are based on ratios of enthalpy changes in any profile, they are unaffected by the underestimation, but the absolute values presented here are not true thermodynamic parameters.

DISCUSSION

The interactions between anticancer drugs and DNA have become an important part of carcinogenesis research. Here we tested differential scanning calorimetry as a quick technique for approximating the DNA-damaging potency of antitumor drugs and distinguishing their mechanisms in cultured human cells. The assay is based on the measurement and characterization of structural transitions in chromatin of intact nuclei as the temperature is raised from room temperature to about 120°C. Under approximately physiological salt conditions, where the isolated nuclei give a melting profile characterized by four major structural transitions. Transition I (60°C) represents the melting of miscellaneous nonchromatin proteins (5). Transition II (76°C) represents the collapse of the nucleosome (5, 8). If the DNA released by the collapse of the nucleosome is relaxed it melts as transition III (88°C) but if it is intact (and supercoiled) it melts at 105°C (5, 7). Thus, in principle, scanning calorimetry ought to reveal DNA damage in terms of nucleosomal structure, base pairing, and intact supercoiled loops. The present study demonstrated that these three potentials can be realized in practice, and that antitumor drugs can be classified accordingly. The first class of antitumor drugs studied were DNA strand breakers. Both bleomycin and streptonigrin are in this class, which is characterized by an apparent destabilization of the supercoiled DNA to lower melting forms and the conversion of intact supercoiled DNA to the nicked, relaxed form. This was evidenced as a transient lowering of the melting temperature of transition IV to generate IV(–) as well as the replacement of transitions IV and IV(–) by transition III. The lowering of the temperature of transition IV most likely represents enhancement of base unstacking (9) and the removal of base moieties (10, 11). Bleomycin attacks DNA to produce both single strand and double strand breaks (4, 12) while streptonigrin produces mainly single strand breaks (4, 13). Such differences were not revealed by scanning calorimetry, since the observed changes in transitions II, III, and IV were similar in the calorimetric profiles of nuclei treated with streptonigrin and bleomycin. Scanning calorimetry obviously does provide a quick and very sensitive assay, however, for strand breaks in general.

The second class of DNA-damaging antitumor drugs studied
DIFFERENTIAL SCANNING CALORIMETRY OF NUCLEI

Fig. 2. Differential scanning calorimetry of nuclei obtained from cultured human tumor cells after treatment with streptonigrin for 20 h. Heat absorption curves as a function of temperature are shown for nuclei isolated in buffer C. DNA amounts in the samples were: control, 0.37 mg; streptonigrin (0.1 µg/ml), 0.33 mg; streptonigrin (0.5 µg/ml), 0.54 mg.

Fig. 3. Differential scanning calorimetry of nuclei obtained from cultured human tumor cells after treatment with bleomycin for 20-45 h. Heat absorption curves are shown for nuclei isolated in buffer C. DNA amounts in the samples were: control, 0.50 mg; 20 h, 0.70 mg; 45 h, 0.34 mg.

were alkylators, methyl nitrosourea, and mitomycin. Methyl nitrosourea forms covalent bonds to nucleophilic centers in biological materials, especially proteins and nucleic acids (4, 14). Yang and Woolley (15) studied the effect of methyl nitrosourea on the organization of pancreatic chromatin fragments, as reflected by their melting behavior at low salt concentrations. They found that methyl nitrosourea treatment destabilized the chromatin, markedly reducing its melting temperature. Our data for physiological salt concentrations do not show a change in the melting of relaxed DNA (transition III) but we did observe a destabilization of the intact superhelical form in that transition IV shifted to lower apparent melting temperatures IV(−) before the relaxation occurred that shifted it to transition III. The difference in results points to the differences in experimental approaches. Although the means of administration of the drug differed, it probably did not matter; Yang and Woolley injected large doses into animals over 8 days, while we used defined drug concentrations directly on the tested cells and for periods of 20 h or less. A more important difference was that in solubilizing chromatin, Yang and Woolley fragmented it, destroying the structure that gives rise to transition IV and so they could not have detected changes in the supercoiled loops of DNA, which depend on the intactness of the DNA. A second important difference was that in the low salt concentrations used by Yang and Woolley proteins profoundly affect DNA melting while in the physiological range DNA melting is indifferent to protein (16). Since calorimetry exhibited a normal melting temperature for nicked, relaxed chromatin in physiological salt conditions, the destabilization seen by Yang and Woolley in low salt must have reflected protein modification rather than DNA damage. The calorimetry approach, then, is more specific for DNA and can reveal both strand breaks and interference with the normal, intact, supercoiled form.

The pattern that characterizes alkylating antitumor drugs is a destabilization (lower melting temperature) of transition IV followed by its conversion to transition III. That is, transition IV(−) appears to be a kinetic intermediate between IV and III. This is the same pattern that characterized strand-breaking antitumor drugs, as might have been expected since there are many reports that the in vivo mechanism of action of the alkylators involves substantial levels of strand breaking. UV also seemed to induce transition IV(−) as an intermediate between IV and III. The primary modification of DNA by UV radiation is the formation of intrastrand pyrimidine dimers (17) which produce partial unstacking of bases in the DNA double strand, and this seems a reasonable explanation of the decrease in melting temperature of transition IV. By analogy, methyl nitrosourea and mitomycin might lower the temperature of
transition IV by the cross-linking of DNA which they are known to produce (4). Mitomycin has been directly observed to induce conformational changes in DNA (18) and the destabilization of intact, supercoiled DNA observed in the present work would be consistent with those results. The conversion of transitions IV and IV(−) to transition III is clearly an expression of the strand breakage known to be induced by these alkylators and UV exposure. In any case, the biologically relevant effect includes both processes, strand breaking and conformational change, as was the case for DNA strand-breaking antitumor drugs. Thus, the DNA damage induced by both strand breaking and alkylating antitumor drugs is characterized by a decrease in transition IV, which is replaced by transition IV(−), which is in turn replaced by transition III.

A third class of antitumor drugs induces quite different changes in the calorimetric profiles. The intercalators, ethidium bromide and actinomycin D, cause transition II to lower its temperature and transition IV to increase its temperature. The binding of ethidium bromide to nucleosomal core particles was studied by McMurray and Van Holde (8), who demonstrated that this binding results in a dissociation of the nucleosomal core particle. It was concluded that the stiffening of the DNA helix hinders the DNA in its binding around the histone octamer. McMurray and Van Holde pointed out that their results might not be applicable to intact chromatin, since the latter has extensive lengths of DNA rather than the relatively unstable, short lengths (145 base pairs) of DNA possessed by core particles. This reservation is pertinent because it has been reported (19) that ethidium bromide intercalates preferentially in the linker DNA between nucleosomes when nuclei are exposed to the drug. Nevertheless, our data show that the dissociation of core particles by ethidium bromide in fact does have its counterpart in whole chromatin, since transition II was decreased in temperature and enthalpy by the drug. Either the preference of ethidium bromide for linker DNA is not absolute, or the binding in the linker affects the nucleosomal DNA indirectly. This destabilization of the nucleosome is a characteristic of ethidium bromide, daunorubicin, and actinomycin D and presumably of other intercalators as well. In addition to destabilizing the nucleosome, the intercalators seem to stabilize the superhelical DNA, causing it to melt at 110°C (or higher) rather than at 105°C. The ability of intercalators to alter the superhelicity of DNA is well established and presumably underlies the change in transition IV to transition IV(+).
The foregoing discussion addressed the ability of differential scanning microcalorimetry as a quick method for revealing the nature of DNA damage induced in human cells by antitumor drugs. Now we turn to a consideration of the quantitative aspects of calorimetry as an assay for biological potency. The quantification of the profiles is compromised to a modest extent by uncertainty in drawing baselines for integration of the peaks in the profile. When peaks are widely separated there is no problem, but when peaks overlap substantially, as was frequently the case between transitions III and IV(−), between IV(−) and IV, and between IV and IV(+), precise quantification is not possible. The problem is aggravated because, like most null-balance instruments, the differential scanning calorimeter is exquisitely sensitive to subtle factors just in the baseline. The enthalpic change calculated from each peak must be considered as only approximate. These approximations, as shown in Table 1 do demonstrate a reasonable correlation between the potency of a drug toward DNA damage, and its capacity to divide. In order to develop a precise numerical relationship between dose, DNA damage, and the capacity to divide, a large number of assays would be required for each of several drugs. It is not obvious at this stage whether such relationships would be linear or logarithmic or more complicated, but the data showing dependence of the DNA cleavage and loss of dividing capacity on the amount of exposure to the drugs are sufficient to show that quantitative relationships could be established. A scanning calorimetric assay for antitumor drugs would possess several advantages. The use of cell cultures would overcome variations in response due to routes of drug administration, and using human cells would avoid questions that arise about the suitability of animal models. Various cell lines are available so that allowances could be made for metabolic differences between cells. Indeed, it is possible that the assay could be adapted to pieces (10–15 mg) of whole tissues from individual patients to judge the variation between individuals and the drug of choice for any particular patient.

REFERENCES

1. Touchette, N. A., and Cole, R. D. Differential scanning calorimetry of nuclei obtained from UV-irradiated human tumor cells. Curves for heat absorption as a function of temperature are shown for nuclei isolated in buffer C. DNA amounts in the samples were: control, 0.70 mg; UV 30', 0.55 mg; UV 60', 0.63 mg; UV 120', 0.55 mg.

Fig. 8. Differential scanning calorimetry of nuclei isolated from UV-irradiated human tumor cells. Curves for heat absorption as a function of temperature are shown for nuclei isolated in buffer C. DNA amounts in the samples were: control, 0.70 mg; UV 30', 0.55 mg; UV 60', 0.63 mg; UV 120', 0.55 mg.
Differential Scanning Calorimetry of Nuclei as a Test for the Effects of Anticancer Drugs on Human Chromatin

Miriam Almagor and R. David Cole


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