DNA STRAND SCISSION AND ITS REPAIR FOLLOWING EXPOSURE OF CELLS TO INHIBITORS OF OXIDATIVE PHOSPHORYLATION

John Hilton and Michael D. Walker
Section of Neuro-oncology, Baltimore Cancer Research Center, NCI
Baltimore, Maryland 21211

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Summary. Mouse L1210 leukemia and HeLa cells exposed to 2,4-dinitrophenol, oligomycin and rotenone under conditions which led to depletion of ATP pools exhibit DNA damage expressed as irreversible DNA strand separation in alkali. Removal of the agents allows both the repletion of ATP pools and repair of DNA damage.

Introduction. The nature of compounds which cause DNA damage generally have the property to modify the structure of DNA and to render it chemically unstable or sensitive to endonucleolytic attack. Many such compounds have been found also to be carcinogenic or mutagenic. However, in the course of investigations into DNA damage by nitrosourea anti-tumor agents (1), we have observed that 2,4-DNP which is looked upon conventionally as an inhibitor of oxidative phosphorylation leads to DNA damage resembling that caused by monofunctional alkylating agents and ionizing radiation. The compounds chosen for the present study, 2,4-DNP, oligomycin and rotenone, although having quite different modes of action (2,3,4) have the common property of being inhibitors of mitochondrial ATP production.

Methods and Materials. Mouse leukemia L1210 cells were maintained in RPMI 1630 medium and HeLa cells were grown as monolayers in Basal Medium (Eagle) in an atmosphere of 5% CO_2. Both media were supplemented with 10% fetal calf serum and antibiotics (penicillin, 110 units/ml; streptomycin, 100 µg/ml). Glucose-free media were prepared from analytical grade reagents and were supplemented with 10% dialysed fetal calf serum. The addition of the appropriate amount of glucose to these media supported normal cell growth for at least two generations. Cells, labelled in complete media with [^{14}C]thymidine (0.1 µCi/ml, 2 x 10^{-6} M) for 2-3 generations, were washed free of residual isotope and incubated in isotope-free medium for 3 hr. before use. Dinitrophenol was added to cultures as an aqueous buffered solution (pH 7.4). Oligomycin and rotenone were dissolved in acetone such that the final concentration of acetone within the cultures was 0.5%. Following the addition of these agents, the cells were washed twice at 4° in the medium in which they had been exposed earlier and then either incubated in fresh medium at 37° for DNA repair or suspended in saline-EDTA (0.075 M NaCl, 0.024 M EDTA pH 7.4) at 4°. HeLa cells were harvested following exposure to 0.25% trypsin at 4° for 15 min. Cells were irradiated with a ^{60}Co source at 0° at a dose rate of 100 rad/min.

The extent of DNA strand separation in alkali was determined as described by Rydberg (5). Briefly, 5 x 10^4 cells are injected into 1 ml of lysis medium (0.03 N NaOH,

Abbreviations: 2,4-DNP, 2,4-dinitrophenol; RPMI 1630, Roswell Park Memorial Institute Medium 1630; BME, Basal Medium (Eagle); SDS, sodium dodecyl sulfate.

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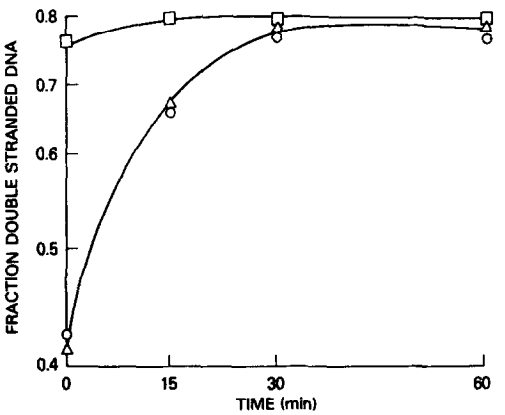

Figure 1. L1210 cells in glucose-free 1630 medium were exposed to 1 mM 2,4-DNP (O), 200 rad gamma irradiation (△) or were untreated (□). The washed cells were transferred to complete 1630 medium and samples removed during the 60 min repair period were analysed for the extent of repair of damage present at time zero.

0.9 M NaCl, 0.01 M Na_2HPO_4, pH 12) at room temperature. After 30 min. the solution is rapidly neutralized by the addition of HCl, briefly sonicated and made 0.4% in SDS. Double and single stranded DNA is separated by chromatography on hydroxylapatite (5). ATP was determined in neutralized perchloric acid cell extracts by the luciferase assay (6).

Results. In the remainder of this report, the term "DNA damage" refers to a change in the nature of cellular DNA which causes it to become more liable to irreversible strand separation in alkali; the inference being that the degree of complete strand separation is related to the extent of strand scission.

Our initial observation was that L1210 cells exposed to 1 mM dinitrophenol for 60 minutes exhibited a degree of DNA damage equivalent to approximately 200 rad gamma irradiation. Since a characteristic of cells exposed to ionising radiation is their ability to rapidly repair such damage (7), cells exposed to dinitrophenol were similarly examined for their repair potential. Fig. 1 describes the time course of repair of DNA damage at 37° in cells exposed to 200 rad irradiation or 1 mM dinitrophenol for 1 hour. Clearly, the time course and extent of repair of damage caused by exposure to either agent is identical. The fraction of DNA remaining double stranded after neutralization of the alkaline lysate reaches the value in control cells within about 30 minutes. Exposure of HeLa cells in BME to 1 mM dinitrophenol for 60 minutes at 37° was found

TABLE 1

Effect of Dinitrophenol on ATP Levels and DNA Damage

Treatment	Cell	Medium	ATP nmoles/10^6 cells	Fraction DNA double-stranded
None	L1210	1630	4.8	0.79
None	L1210	glucose-free 1630	4.6	0.77
1 mM DNP	L1210	1630	0.1	0.43
1 mM DNP	L1210	glucose-free 1630	0.1	0.44
None	HeLa	BME	11.1	0.82
None	HeLa	glucose-free BME	10.6	0.79
1 mM DNP	HeLa	BME	10.8	0.81
1 mM DNP	HeLa	glucose-free BME	1.0	0.62

to have no effect on strand separation of HeLa DNA in alkali. Since HeLa cells depend largely on glycolysis for their energy production, the consequence of pre-incubation in glucose-free medium was examined. Table 1 describes the effect of exposure of L1210 and HeLa cells to 1 mM dinitrophenol in normal, glucose containing media and glucose-free media both in terms of ATP levels and DNA damage. Whereas L1210 cells suffer an almost complete loss of ATP and extensive DNA damage in either normal or glucose-free media, HeLa cells are resistant to 1 mM dinitrophenol in normal medium, but sensitive as measured by reduced ATP levels with DNA damage in glucose-free medium.

We have also determined that other inhibitors of mitochondrial ATP formation show qualitatively similar effects to dinitrophenol. Exposure of L1210 cells for 60 minutes at 37° to oligomycin (1 μg/ml) or rotenone (10^{-7} M) in glucose-free media results in extensive DNA damage. After exposure to either inhibitor, the DNA damage is repairable when cells are washed and returned to glucose-containing medium at 37°. Table 2 shows the results of exposure of L1210 cells to 10^{-7} M rotenone in glucose-free medium followed by repair in either glucose-free medium or complete medium. Exposure to rotenone (10^{-7} M) causes both a dramatic reduction in ATP levels and

TABLE 2

Repair of DNA Damage in L1210 Cells Exposed to Rotenone

L1210 cells in glucose-free RPMI 1630 were treated for 60 min. at 37°, then washed twice in glucose-free medium. For repair, cells were resuspended in either glucose-free or complete medium and incubated a further 60 min. at 37°.

Treatment	Repair Medium	ATP nmole/10^6 cells	Fraction DNA double-stranded
None	---	5.20	0.78
0.5% acetone	---	5.10	0.79
10^{-7}M rotenone	---	0.07	0.45
10^{-7}M rotenone	glucose-free 1630	0.10	0.35
10^{-7}M rotenone	complete 1630	3.20	0.76

extensive DNA damage. Further incubation of washed cells in glucose-free medium does not alter significantly either condition. In the presence of complete glucose-containing medium however, ATP levels increase 30-fold and the fraction of double stranded DNA returns to the control level.

Discussion. Strand scission in DNA is invariably the consequence of a modification of its structure. Such modification may in itself lead to strand scission through the induction of chemical instability (8) or present a point of attack for a repair endonuclease. Our observations on the production of DNA damage by exposure of cells to inhibitors of oxidative phosphorylation are not easily accommodated by the above generalization.

Although we cannot presently rule out the possibility that these inhibitors, of diverse chemical structure, have some common property which enables them to act directly on cellular DNA, such an explanation appears unlikely. Our results more strongly support the thesis that their effect on the integrity of cellular DNA is the result of their common ability to reduce cellular ATP levels.

It is important to distinguish between DNA degradation resulting from cell death and DNA damage produced at levels of exposure to agents which allow the consideration of a specific course of events in cells which exhibit criteria of viability. In the present study these criteria include the cells ability to restore depleted ATP levels and an ability to repair single strand breaks in DNA.

The refractoriness of ATP content and DNA stability in HeLa cells in glucose-containing medium to exposure to dinitrophenol and the sensitivity of both parameters in glucose-free medium indicates a causal relationship between cellular ATP levels and DNA damage. Similarly, in L1210 cells exposed to rotenone low cellular ATP levels and the appearance of DNA damage may be contrasted with DNA repair and an increase in ATP levels when the washed cells are further incubated in complete medium. However, if glucose-free medium was provided during the repair period, there was neither DNA repair nor a significant increase in cellular ATP content.

The question of how decreased cellular ATP levels result in DNA damage remains conjectural. Some mechanisms, however, can be eliminated. Since, for example, the cultures are capable of DNA repair and restoration of ATP levels, an explanation based on cell death and ensuing autolysis is unlikely. There have been several reports which suggest that cells carry out a process resembling DNA repair which may be involved in the maintenance of cell integrity. If such a process existed, the reduction of ATP levels, expected to slow down repair but not affect base excision, would result in the accumulation of single-strand breaks in DNA. Gautschi et al (9) however, after carefully repeating earlier work, demonstrated that the hypothetical repair process was very probably an artifact of the preparation of the DNA samples and therefore concluded that the integrity of DNA is not maintained by a balance of excision and repair. More recently, Smith and Hanawalt (10) have reached a similar conclusion.

Cellular endonucleases, perhaps those normally involved in DNA repair, appear the most likely candidates for producing single strand breaks in DNA of ATP depleted cells. Investigations to attempt a demonstration of either an increased nucleolytic activity or an increased susceptibility of DNA to endonuclease attack are in progress.

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