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Morphological Transformation, DNA Strand Separation, and Antinucleoside Immunoreactivity following Exposure of Cells to Intercalating Drugs

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SUMMARY

The intercalating drugs quinacrine and proflavine induced increases in single-stranded DNA detected in the nuclei of mouse BALB/c 3T3 1–13 cells. The denatured DNA was detected by fluorescein-labeled antinucleoside antibodies, which bind to single-stranded but not double-stranded DNA. Exposure of cells to the potent mutagen proflavine increased the fraction of immunoreactive nuclei from 0.65 to 0.8. With the weaker mutagen quinacrine, higher concentrations were needed to induce increases in immunoreactivity. Both intercalating drugs rapidly induced morphological transformation in mouse 3T3 cells. Treatment with proflavine resulted in higher transformation frequencies than were found with quinacrine. Significant increases in cell transformation frequency were obsereved at the concentrations which induced high levels of immunoreactivity. These results suggest that DNA strand separation is itself, or at least accompanies, an early step in cell transformation by intercalating drugs.

INTRODUCTION

Covalent attachment to DNA and other macromolecules is a common feature of the action of many chemical mutagens and carcinogens. However, covalent attachment is not a necessary step in mutagenesis with intercalating drugs such as hycanthone (1). This concept led us to obtain more information on the cellular effects of other intercalating mutagens which do not covalently attach to DNA. The importance of DNA's unwinding as an early event in mutagenesis is of special interest because some intercalating drugs are widely used clinically.

Intercalation by acridines alters DNA's physical properties; the interaction was correlated by Lerman (2) with acridine mutagenesis. Recently, Drinkwater *et al.* (3) noted excellent correlation between the potency of frameshift mutagens which do attach covalently to DNA and their ability to unwind purified viral DNA *in vitro*. We found (1, 4, 5) that hycanthone, a potent mutagen (6, 7), is not covalently attached to DNA or other major macromolecular fractions in HeLa cells. Poland and Glover (8) found that the carcinogen 2,3,7,8-tetrachlorodibenzo-*p*-dioxin was not bound covalently *in vivo* to rat liver macromolecules; DNA binding was detected in only 1 molecule per DNA equivalent of 35 cells. Schupbach (9) showed that the intercalating agent chloroquine is mutagenic and that it appears to act by distorting the DNA helix, thus shifting the reading frame. Mefloquine, a nonintercalating analogue (10), is not mutagenic. It appears that alteration of DNA conformation is an early step in carcinogenesis and mutagenesis which need not be accompanied by covalent attachment.

We used the technique of antinucleoside immunofluorescent staining to detect the unwinding of DNA in nuclei of fixed cells. Antinucleoside antibodies are base-specific and react with single-stranded but not double-stranded nucleic acids (11, 12). Nuclei of G_1 phase cells are normally unreactive, whereas S-phase nuclei, containing sites of unwound DNA at the replication forks, are reactive (13, 14). Nuclear ANIM[4] correlates well with S-phase determinations by [^{3}H]thymidine autoradiography in individual cells (15). The induction of DNA unwinding by X-rays, alkylating agents, and other mutagens was detected in HeLa nuclei (1, 5, 14) and in human peripheral blood lymphocytes (16) by the ANIM technique.

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[4] The abbreviations used are: ANIM, antinucleoside immunoreactivity; PBS, phosphate-buffered saline (140 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 1.5 mM KH_2PO_4).

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As expected from the immunocytological studies, increased quantities of single-stranded DNA could be recovered from X-irradiated or methylmethanesulfonate-treated Chinese hamster ovary cells, using benzoylated naphthoylated DEAE-cellulose chromatography (17).

The nonmutagenic protein synthesis inhibitor cycloheximide also reversibly induces ANIM, although it does not induce DNA repair synthesis or strand breaks. We have shown that this is probably due to depletion of a class of rapidly turning-over chromatin proteins, tightly bound to DNA, which appear to make single-stranded regions unavailable to the antibodies (18).

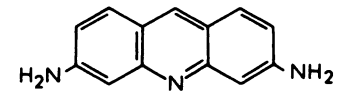
We have compared two structurally similar intercalating agents, the acridine derivatives proflavine and quinacrine (Fig. 1), for their ability to induce morphological transformation and DNA strand separation in mouse 3T3 cells. Quinacrine (Atabrine) was a widely used antimalarial.

MATERIALS AND METHODS

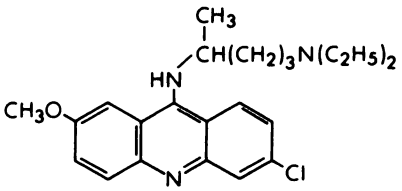
BALB/c 3T3 1–13 (Sivak) mouse cells (19) were grown in Eagle's minimal essential medium supplemented with 10% fetal bovine serum in 75-cm^2 plastic flasks; the population doubling time in these cultures was 15.5 hr. Cells were exposed to the various treatments either directly in the flasks (1–2 × 10^6 cells/T75 flask) or in subcultures in 60-mm or 100-mm Petri dishes (1–2 × 10^5 cells/dish).

In transformation assays, cells were treated with the agents for 1 or 3 hr. Cells were then removed with trypsin, and 1.0 × 10^4 cells were inoculated into each 60-mm Petri dish. The cells were incubated in a moist CO_2 incubator at 37° for 4 weeks and the medium was changed twice weekly. Colonies of transformed, piled-up cells were then scored according to the criteria of Reznikoff *et al.* (20). Rapidly growing, tightly clustered cell colonies (Type 1) were ignored; only cells growing in random, piled-up, criss-cross patterns (Types 2 and 3) were scored as positive.

Antinucleoside immunofluorescence assays. Fluorescein-labeled rabbit antibodies against bovine albumin-nucleoside conjugates were prepared as described by Erlanger and Beiser (11). Details of the antinucleoside immunofluorescence staining have been given previously (14).



PROFLAVINE



QUINACRINE

FIG. 1. *Chemical structures of the acridine derivatives proflavine and quinacrine*

Examination of cells for antinucleoside immunoreactivity was easier when the cells were well spread. However, equivalent immunoreactivity determination results were obtained when cells were treated with drugs in subconfluent monolayers, rinsed three times in fresh medium and once with PBS, and trypsinized; the cell suspensions were then smeared on glass slides, air-dried, fixed, and stained.

Plating efficiency was determined by inoculating 100–300 drug-treated single cells into 100-mm Petri dishes. Colonies were counted after 7 days of incubation.

Determination of macromolecular synthesis. Radioactive precursors were obtained from New England Nuclear Corporation (Boston, Mass.). [^{3}H]Thymidine (1 μCi/ml, 55 μCi/mmole), [^{3}H]uridine (1 μCi/ml), or a ^{3}H-labeled amino acid mixture (5 μCi/ml) was added to the medium in 60-mm dishes containing 1–2 × 10^5 cells, and the labeling proceeded for 1 hr. The medium was removed, the cell monolayer was gently rinsed twice with cold PBS, and the cells were lysed with 3 ml of saline containing 0.33% sodium dodecyl sulfate. The lysate was collected and mixed with an equal volume of 10% trichloroacetic acid. Samples containing ^{3}H-labeled amino acid were heated to 90° for 1 hr. They were then filtered through Millipore HA filters, dried, and counted by liquid scintillation.

Drugs. Proflavine hemisulfate and quinacrine dihydrochloride were obtained from Sigma Chemical Company (St. Louis, Mo.). All solutions and media containing these agents were protected from strong (especially fluorescent) light.

RESULTS

Induction of ANIM in mouse 3T3 cells by intercalating agents. Antinucleoside immunoreactivity examinations made on unsynchronized 3T3 cells from logarithmically growing cultures showed about 60% immunopositive cells, in good agreement with the high proportion of S-phase cells we have found in the cultures, as verified by [^{3}H]thymidine autoradiography.

For both 1-hr and 3-hr exposures to a 1 μM concentration of the potent mutagen proflavine, a significant increase in ANIM was obtained; with the weaker mutagen quinacrine, a similar increase was not observed until a concentration of 5–10 μM was used (Fig. 2). With these unsynchronized mouse cells, a large fraction of the population is in S-phase and therefore ANIM induction was observed against the relatively high background of 0.65. Nevertheless, the mean values and standard errors shown in Fig. 2 clearly show that 1 μM proflavine induced high ANIM (the differences between the experimental and control populations are <18 times the standard errors of the differences). In similar experiments with HeLa cells (data not shown), 1-hr exposure to 1 μM proflavine increased the proportion of immunoreactive cell nuclei from 0.32 ± 0.02 SE to 0.70 ± 0.06 SE. For quinacrine, no increase in ANIM in HeLa cells could be detected using 1 μM, but with increasing concentrations up to 10 μM quinacrine, the immunoreactive fraction progressively increased to 0.80 ± 0.04 SE.

Inhibition of macromolecular synthesis by proflavine and quinacrine. Data of Table 1 suggest that protein synthesis inhibition was not responsible for ANIM in-

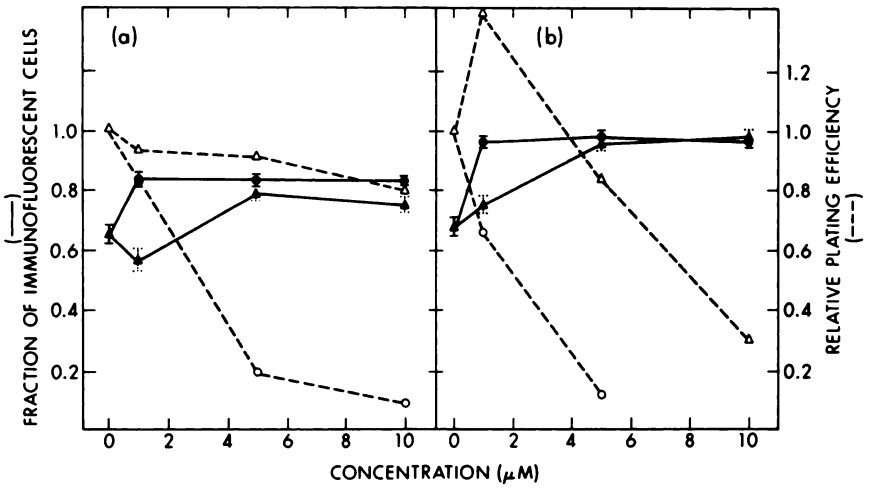


FIG. 2. *ANIM induction and cell killing*

a, A 1-hr exposure to the agents; b, a 3-hr exposure. *Solid symbols* indicate immunofluorescence; *open symbols* indicate relative plating efficiencies. Standard errors were obtained from at least three separate experiments. For relative plating efficiencies, standard errors were ±5% of the means, and are omitted. For ANIM, the standard error =

$$\pm \sqrt{(\%\ \text{positive}) \times (\%\ \text{negative}) \div (\text{no. of cells scored})}$$

●, ○, Proflavine; ▲, △, quinacrine. The absolute plating efficiency of control cells was 5.85% ± 0.3%. Although absolute plating efficiencies for this cell line were normally about 30%, they were lower in experiments such as this one, which included extensive washing and manipulations.

In a separate experiment an absolute cell cloning efficiency of 31% was found for untreated cells. A 3-hr exposure to 1 μM, 5 μM, and 10 μM drug increased the percentage of immunoreactive cells from the background level of 67% to 75%, 95%, and 97% with quinacrine and to 96%, 98%, and 96% with proflavine, using the concentrations shown.

duction by these agents. Although both agents inhibit protein synthesis, a high level of inhibition (≥50%) was not obtained with 1 μM proflavine or 5 μM quinacrine, concentrations at which ANIM was significantly increased (Fig. 2). Inhibition of RNA or DNA synthesis is not likely to be a factor in ANIM induction, since we have shown (5) that the RNA synthesis inhibitor lucanthone, and the DNA synthesis inhibitors cytosine arabinoside and hydroxyurea do not induce ANIM in HeLa cells. In mouse cells, 1 μM proflavine induced high ANIM with minimal inhibition of RNA or DNA synthesis. We cannot exclude the possibility that the ANIM induction by 5–10 μM quinacrine might be due in part to inhibition of macromolecular synthesis, leading to exposure of single-stranded DNA regions by a second mechanism, i.e., by elimination of a class of DNA-binding proteins (18).

Morphological transformation by proflavine and quinacrine. In the Ames test, proflavine is a more potent mutagen than quinacrine (21–23). In animal cells, mutation and the morphological transformation of animal cells in culture are not equivalent, but they do seem to be related phenomena. Little information on morphological transformation by proflavine and quinacrine was found in the literature. Therefore, we tested the ability of these agents to induce morphological transformation in the mouse 3T3 cell system. Figure 3a shows that quinacrine

TABLE 1

Inhibition of macromolecular synthesis in mouse 3T3 cells

Agent	1-hr exposure[a]			3-hr exposure[b]		
	DNA	RNA	Protein	DNA	RNA	Protein
			% inhibition[c]			
Proflavine						
1 μM	4.6 ± 0.1	22.0 ± 0.9	17.0 ± 0.8	15.0 ± 0.4	12.7 ± 0.4	2.4 ± 0.1
5 μM	35.4 ± 0.7	42.9 ± 0.8	46.8 ± 2.7	55.3 ± 1.0	61.1 ± 1.4	41.0 ± 1.5
10 μM	64.0 ± 2.2	57.0 ± 1.3	86.5 ± 6.9	82.6 ± 2.0	84.2 ± 1.7	95.5 ± 6.5
Qulnacrine						
1 μM	8.6 ± 0.2	5.3 ± 0.1	12.7 ± 3.1	18.6 ± 0.4	7.6 ± 0.2	28.8 ± 1.4
5 μM	42.5 ± 1.4	44.0 ± 1.4	27.7 ± 1.6	69.9 ± 2.6	75.3 ± 2.8	48.3 ± 2.6
10 μM	67.3 ± 1.8	68.5 ± 2.2	87.9 ± 5.6	88.1 ± 2.5	95.3 ± 4.2	80.9 ± 5.4
Cycloheximide, 50 μg/ml			99.9 ± 23.3			99.9 ± 5.9

[a] Radioactive precursors were present during the 1-hr exposure to the agents.

[b] Radioactive precursors were present during the final hour of exposure.

[c] Numbers represent percentage inhibition of radioactive incorporation (± standard error) in treated groups compared with controls. Control group incorporation was: [^{3}H]thymidine, 48,876 cpm; [^{3}H]uridine, 6,262 cpm; [^{3}H]amino acids, 288 cpm (incorporation was low with amino acids since the growth medium contained abundant unlabeled amino acids). See Materials and Methods.

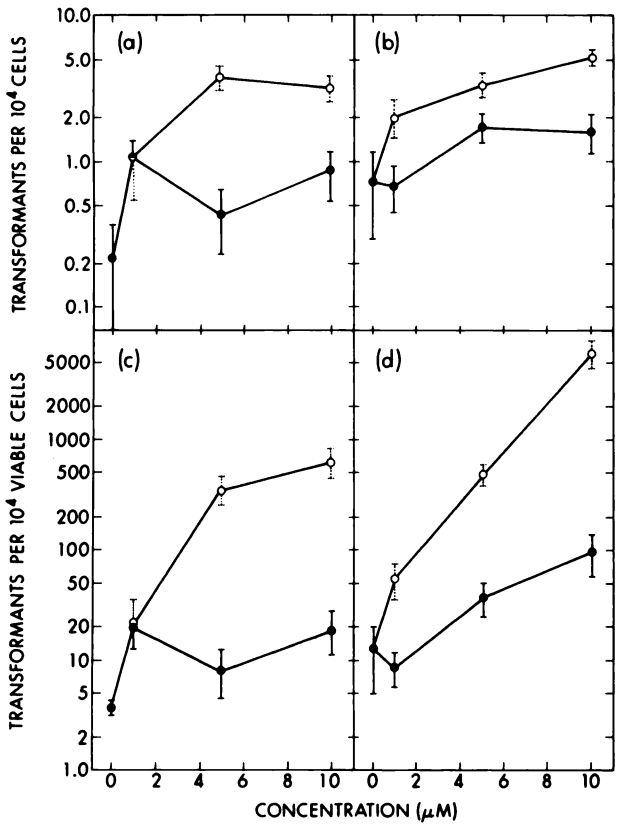


FIG. 3. *Morphological transformation of mouse 3T3 cells by intercalating agents*

a, A 1-hr exposure to the agents; b, a 3-hr exposure; c, a 1-hr exposure, data adjusted for cell killing; d, a 3-hr exposure, data adjusted. ●, Quinacrine; ○, proflavine.

The total number of morphologically transformed foci found with proflavine or quinacrine treatment was significantly higher than control values. This was determined (24) by using the formula

$$\frac{[n - n_{exp}] - 1/2}{\sqrt{n_{exp}}}$$

where n is the number of foci found in the assay dishes and n_{exp} is the expected number of foci, based on control values. There were 7–10 dishes for each experimental point shown. The distribution of foci among the dishes containing drug-treated cells appeared to be random. Sivak and Tu (19) recommend that determinations of foci per plate may avoid uncertainties inherent in assays which can be influenced by drug cytotoxicity.

We determined that the frequency of transformed foci was significantly higher than control values ($p \leq 0.01$) for all values shown except for cells exposed for 1 hr to 5 μM quinacrine and for 3 hr to 1 μM quinacrine. In the *lower panels* we express morphological transformants as a fraction of the number of colony forming cells. This was estimated from the data of the *upper panels*, corrected for the absolute plating efficiency and then corrected for the relative loss of plating efficiency due to drug cytotoxicity. Drug cytotoxicity data were taken from Fig. 2. For example, the 160-fold difference between the uncorrected number of transformants per 10^4 cells plated after a 3-hr exposure to 5 μM proflavine (b) and the corrected value panel (d) is accounted for by the 16-fold correction for the absolute plating efficiency and the 10-fold correction for drug toxicity, determined from inspection of Fig. 2b.

induced morphological transformation; proflavine appeared more potent. With 3-hr exposure the diffference between the two agents was smaller, but proflavine was still more potent (Fig. 3b). In Fig. 3a and b the number of transformants per 10^4 cells plated is given. When nonviable cells were ignored, i.e., correcting the data for cell killing by these agents, the transformation frequency per 10^4 viable cells was more than 1 decade higher for proflavine than for quinacrine, at both 1-hr (Fig. 3c) and 3-hr (Fig. 3d) exposures. Drug cytotoxicity effects are shown as the relative plating efficiency on the right ordinate of Fig. 2, which can be used to estimate the influence of drug cytotoxicity in Fig. 3 since all of these determinations were made in a single experiment.

High apparent transformation frequencies for proflavine (Fig. 3d) are likely a reflection of drug cytotoxicity; the transformants represent a large fraction of the surviving cells.

Studies by Sivak and Tu (19) and others indicate that a high level of drug toxicity may increase the apparent transformation frequency because of the lower initial cell density in the assay plates; it is well known that high cell densities suppress transformation (19). Transformation frequencies with both drugs were significantly higher than the spontaneous values in both experiments.

DISCUSSION

Among the effects of mutagens and carcinogens are covalent attachment to DNA, cell cycle arrest (25), DNA repair synthesis induction (26), and inhibition of RNA maturation (27). Denaturation and unwinding of DNA strands seems to be another of the effects of these agents and of ionizing radiation. The crucial events remain unknown, but the present study confirms our previous suggestion that DNA strand separation, as measured by ANIM, is an important early step in mutagenesis and transformation (1). These disturbances can be induced

TABLE 2

Morphologic transformation of BALB/c 3T3 cells

In each drug treatment group, 2×10^6 cells growing in T-75 plastic flasks were treated for 3 hr. The cells were rinsed, trypsinized, and diluted; 200 cells were plated in 100-mm Petri dishes and for 7 days were incubated for colony formation. For the transformation assay, 10^4 cells were plated in 60-mm Petri dishes (9 dishes per group). Transformed colonies were counted 4 weeks later. Cells were plated immediately after exposure to 280-kV X-rays. The transformation frequency was calculated as: transformed colonies (Type 2 or 3)/(cloning efficiency × cells per dish × no of dishes).

In another experiment, in which quinacrine was not tested, control cultures showed a morphological transformation frequency of 0.45×10^{-4}; with 300 rads, 21×10^{-4}; and with 10 μM proflavine, 8.1×10^{-4}, for a 3-hr exposure. Cloning efficiencies were 42%, 20%, and 16%, respectively.

Treatment	Cloning efficiency[a]	Transformation frequency[a, b]
	%	× 10^4
None	34 ± 2.4	0.65 ± 0.27
300 rads	19 ± 1.8	10.2 ± 1.1
Proflavine		
1 μM	40 ± 2.6	2.52 ± 0.53
10 μM	10 ± 1.3	44.6 ± 2.2
Quinacrine, 10 μM	11 ± 1.3	7.1 ± 0.94

[a] Values are means ± standard error of the mean.

[b] Transformation frequencies shown for all of the treatment groups are significantly higher than the control value at a 95% level of significance or greater.

by commonly used intercalating drugs such as quinacrine.

Intercalating drugs such as the thiaxanthenones (1, 4, 5) and the acridine derivatives studied here are useful model compounds since they are not lethal to the cells at concentrations which induce ANIM and induction is readily reversible upon removal of the agents. With these compounds the more potent mutagens are more potent inducers of morphological transformation and of ANIM.

The potent mutagens hycanthone and proflavine appear to act by intercalating and unwinding DNA strands locally, as indicated by immunoreactivity. The weaker mutagens, lucanthone and quinacrine, intercalate but cause less disturbance in the DNA double-helical structure, resulting in less mutagenesis and transformation. The analogue chloroquine, which is not mutagenic at low concentrations, is an extremely poor inducer of ANIM. We reported previously that a number of other very weakly mutagenic thiaxanthenones were also weak inducers of ANIM (1).

We failed to detect covalent attachment of hycanthone to cell components (1) and similar results have recently been reported for other systems (8). There is no evidence that the acridine derivatives studied here bind covalently. Despite their failure too bind covalently, those intercalating agents which induce high levels of ANIM are potent mutagens in bacteria and inducers of transformation in animal cells. Differences in the mode of intercalation with DNA by the agents we studied may provide insight into their different activities as mutagens and drugs.

Differences in crowding, contact, and nutritional features under conditions of drug cytotoxicity could lead to discrepancies—making the transformation index less than ideal—as discussed in detail by Sivak and Tu (19), who observed this effect. We observed morphological transformation by both agents in experiments with high as well as low absolute plating efficiencies for control cells and under differing levels of cell cytotoxicity induced by the drugs (Fig. 3; Table 2).

Some mutagenic agents, such as X-rays, may act in a complicated multistep manner in which cell density can strongly influence the determination of transformation rate (28). Despite these uncertainties, our results clearly show that quinacrine and proflavine induce morphological transformation of 3T3 cells, accompanying the other disturbances described in this report.

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