Reversal of Malignant Transformation by Tumor DNA*

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Abstract. Walker-256 carcinosarcoma cells grown in tissue culture medium containing inhibitory DNA prepared from the tumor are shown to have an unaltered growth *in vitro*, but a diminished capacity to produce malignant tumors on injection into the rat. Coincident with this change in virulence is the induction of tRNA methylase inhibitors in these malignant cells. Within days after the DNA is removed from the growth medium, the tRNA methylase inhibitors disappear and the oncogenicity reappears.

The oncogenicity of neoplastic cells prepared from the Walker-256 carcinosarcoma was strikingly reduced when the cells were incubated with DNA extracted from the same tumor.¹ This was demonstrated by injection of the DNA-treated cells into rats. Relatively low concentrations of DNA (100 μ g/ml) and short incubation times (60–90 min) were required to produce this effect. Surprisingly, the molecular weight of the inhibitory DNA could also be relatively small (50,000). Heat denaturation prevented this inhibition, while treatment of the deoxyribonucleoprotein with proteolytic enzyme augmented the effect. Similarly prepared DNAs from bacteria or from various organs of other mammalian species were without comparable activity.

Subsequent removal of the nucleoprotein and fractionation of the inhibitory DNA by the Ledoux² procedure yielded several classes, with molecular weights ranging from 50,000 to more than 5×10^6 , all of which were inhibitory to the tumor cell transplantability. Treatment of the smaller fraction with DNase I (16 hr, 0.5 mg/ml, 38°C) destroyed its inhibitory capacity. However, identical treatment of the higher molecular weight fractions increased the number of inhibitory molecules present.

When Walker-256 (W-256) DNA was injected into rats 24 hr prior to the injection of tumor cells, the resulting tumor-cell proliferation was not retarded. This was also the case whenever DNA was injected at a different site but at the time as the W-256 cells. From these results it appeared likely that the growth inhibition was due to a direct effect of the DNA on the tumor cell rather than on the host; consequently, we decided to examine the effect of DNA on W-256 cells in tissue culture. We were particularly concerned with any effects on tumor cell growth *in vitro*, tumor-cell tRNA methylase activity, and tumor-cell oncogenicity *in vivo*.

Methods. DNA preparation: Rats were killed under ether anesthesia, 10 days after subcutaneous implantation of W-256 tumor. Whole neoplasms were removed as aseptically as possible and immediately frozen on dry ice. They were stored at -20°C until processing. The first alcohol precipitate obtained by the Kay, Simmons, and Dounce³ procedure was stored in 95% ethanol at -20°C . The deoxyribonucleo-protein was dissolved in sterile, buffered (pH 7.5) 0.2 M NaCl-0.02 M sodium phosphate-pronase (1 mg/ml)-penicillin G (0.1 mg/ml) and incubated at 37°C with constant stirring for 2 hr. The solution was brought to 1 M NaCl by the addition of the solid salt, then centrifuged at 0°C for 30 min at $37,000 \times g$. The supernate was put on a 5×50 -cm column of Biogel A, 200-400 mesh, 0.5 M, in a (4°C) cold box, and eluted with autoclaved 1 M NaCl-0.02 M Na_2HPO_4 (pH 8)-penicillin G (0.1 mg/ml). 15-ml fractions were collected aseptically. Tubes with $A_{260}-A_{320} > 0.5$ were pooled and concentrated in a Diaflo pressure dialysis system with a UM-10 membrane. Dialysis was continued for three more days at 4°C in bags that had been boiled, treated with EDTA, and washed with distilled water; five changes (1-liter each) of 0.01 M sodium phosphate buffer (pH 7.4) with added penicillin G (0.1 mg/ml) were used.

UV absorbance was measured at 230, 260, 280, and 320 nm.⁴ The $A_{260} - A_{320}$ was usually 15-20 units (0.75-1.00 mg/ml). The A_{230}/A_{260} and A_{280}/A_{260} ratios were between 0.50 and 0.55. The duplex nature of the DNA was determined by its partition coefficient in the aqueous polyethyleneglycol-dextran two-phase system of Albertsson.⁵ Protein was determined by the method of Lowry *et al.*⁶ DNA solutions were stored at 4°C until used and were mixed directly with McCoy's medium⁷ just before application to growing W-256 cells.

Primary tissue cultures of W-256 cells: The W-256 neoplasm was excised as aseptically as possible and placed in cold saline A.⁸ Single-cell suspensions in McCoy's medium were prepared by trypsinization as described by Marcus *et al.*⁸ and Eagle.⁹ Cells were plated in Falcon plastic tissue-culture flasks, $1-2 \times 10^6$ cells/ 75 cm^2 and incubated at 37°C in 5% CO_2 -95% air with 100% relative humidity. McCoy's medium, enriched with 15% fetal calf serum, streptomycin (100 $\mu\text{g}/\text{ml}$), and penicillin G (200 $\mu\text{g}/\text{ml}$), was used routinely and changed every 2 days. Cell lines have been maintained by transferring every 5-7 days.

Oncogenicity: To determine the ability of W-256 cells to produce a malignant tumor in rats, cells were removed from the tissue-culture flasks by trypsin treatment. After the cell count and trypan blue staining were completed, 10^5 unstained cells in 0.5 ml of Hank's¹⁰ balanced salt solution were injected into the right flanks of ten anesthetized female Sprague-Dawley rats. Animals were examined every 2 days for the onset of, and death due to, tumor.

Preparation and assay of tRNA methylase: Isolation. Tumor cells were removed from the culture flasks with 0.2% crystalline trypsin and pelleted by centrifugation at 4°C for 3 min at $1085 \times g$. The cell pellet was washed twice with 0.25 M sucrose-0.01 M MgCl_2 -0.01 M Tris (pH 8.0), then homogenized by hand in a Ten Broeck homogenizer for 3 min. The homogenate was centrifuged at $105,000 \times g$ for 60 min at 0°C . The $105,000 \times g$ supernatant was used as the source of crude tRNA methylase. tRNA methylase was separated from methylase inhibitors by the acid precipitation method of Kerr.¹¹

Assay. The reaction mixture, in a total volume of 0.4 ml, contained 25 μmol of Tris, pH 9; 160 nmol of dithiothreitol; 50 μg of *Escherichia coli* (K-12) submethylated tRNA; 6.0 nmol of *S*-adenosyl-[^{14}C -methyl]methionine; 75 μmol of ammonium acetate; and enzyme in buffer. On occasion, the radioactive *S*-adenosylmethionine was supplemented with nonradioactive *S*-adenosylmethionine and ammonium acetate was omitted. The reaction mixture was incubated at 37°C for 45 min. The reaction was stopped by the addition of 0.2 ml of neutral 4.0 M hydroxylamine. After an additional 20 min at 37°C , the reaction tubes were placed on ice. 100- μl aliquots were applied in duplicate to Whatman 3MM filter-paper discs, hot-air dried, and washed by the procedure of Manns and Novelli.¹² After the cold trichloroacetic acid washes, one set of the duplicate discs was

washed in hot (90°C) 5% trichloroacetic acid while the other set was washed in cold 5% trichloroacetic acid; both sets were then washed with cold trichloroacetic acid, ethanol, and ether. The filter pads were dried and placed in vials with a toluene scintillation fluid for counting. Controls without added *E. coli* submethylated tRNA and blanks without enzyme were also assayed.

Experimental procedure: Pooled W-256 cells were suspended from as many line flasks of similar transfer number as were necessary to give $1-3 \times 10^6$ cells for each large flask, and $0.4-0.7 \times 10^6$ cells for each small flask. Only cells derived from a single suspension were used in any given experiment, to ensure a common origin for all cells. Growth rates were determined either by sacrificing duplicate small flasks at intervals for protein determinations¹³ or by performing daily cell counts on large flasks. As cell division appeared to enter the log phase of growth, usually 3-4 days after seeding, the large and small flasks were divided equally into two groups and additions were made to an enriched, appropriately concentrated, McCoy's medium just before the flasks were changed. DNA derived from W-256 tumors, dissolved in 0.01 M sodium phosphate buffer (pH 7.4), was added to the experimental group while the control flasks received an equal volume of 0.01 M sodium phosphate buffer. Both groups were maintained on added penicillin G (400 $\mu\text{g}/\text{ml}$) without streptomycin. After this treatment was completed, the cells were again removed with 0.15% trypsin solution and resuspended in Hank's buffered salt solution, counted, and stained with trypan blue for animal injection and tRNA methylase studies.

In the experiment reported here, 0.6×10^6 cells were plated on each of 8 small flasks, and 2.4×10^6 cells on each of 12 large flasks. Fig. 1 shows the growth rates measured

FIG. 1. Growth of W-256 cells in cultures. Walker-256 cells were grown in McCoy's medium η , plus 15% fetal calf serum and penicillin G (200 $\mu\text{g}/\text{ml}$). The experimental vessels were supplemented with W-256 DNA final concentration (120 $\mu\text{g}/\text{ml}$) in 0.01 M sodium phosphate (pH 7.4), and the control vessels with a similar amount of phosphate, at 46 hr.

by total protein¹³ before the flasks became overgrown on the fifth day. DNA was added 46 hr after plating to a final concentration of 120 $\mu\text{g}/\text{ml}$.

After 48 hr of treatment, a sufficient number of cells were removed from each group to inject 10^5 unstained cells into each of ten rats and to assay tRNA methylase activity. In addition, some of the DNA-treated cells were subcultured to standard medium for periods of 3 and 8 days, at which times rats were again injected and tRNA methylase activity was determined.

The medium in the remaining original flasks was changed to the special one containing either 120 $\mu\text{g}/\text{ml}$ of DNA or phosphate buffer, and cells were allowed to continue growing for an additional 24 hr, so that they had 72 hr of treatment in all. Again, aliquots were taken for animal injections and tRNA methylase determinations. All the flasks were overgrown and contained many floating cells unstained with trypan blue; the monolayer was therefore washed several times before trypsin application to be sure that only the firmly attached cells were used for animal injection and tRNA methylase activity. To further assure ourselves that the tumor cells were healthy, we determined the 6-day

growth curve for 0.3×10^6 cells plated on small flasks from both groups, and found it to be normal. The 72 hr, DNA-treated cells were also plated in several large flasks and allowed to grow in regular medium for another 9 days, when they were injected into rats and tested for methylase activity.

The respective media in the final flasks were changed as above and treatment was continued for a total of 96 hr. After this length of time (6 days since the original plating) all flasks were extensively overgrown. In both groups there was a greater number of tumor cells dispersed in the medium than were attached to the flasks. 10^5 unstained, newly divided, freely-floating cells were injected into animals and subcultures were made from each group.

Results. Animal studies: For the cells treated with DNA for both 48 and 72 hr, there was not only a considerable lag in the onset of the neoplasms, but there was a significant reduction in the number of animals that acquired and succumbed to the neoplasm in the 90-day interval of observation (Fig. 2, *top*). Not a single animal developed a tumor in the group receiving the small young viable cells treated with DNA for 96 hr (Fig. 2, *bottom*, VI), whereas 90% of

FIG. 2. Effect of inhibiting DNA on oncogenicity of W-256 cells. Animals were injected subcutaneously with 10^5 W-256 cells in Hanks' solution after several intervals of exposure of the cells to W-256 DNA at $120 \mu\text{g/ml}$ (medium as in Fig. 1). *Group I*, 48-hr control cells; *Group II*, cells grown 48 hr in DNA medium; *Group III*, 72-hr control cells; *Group IV*, cells grown 72 hr in DNA medium; *Group V*, 96-hr control cells; *Group VI*, cells grown in DNA medium 96 hr; *Group VII*, control cells for *Group VI*; *Group VIII*, cells grown in DNA medium 48 hr followed by 72 hr in control medium.

the control group receiving the same type of cells developed and died of the neoplasm (Fig. 2, *bottom*, V). 3 days after the tumor cells had been removed from the medium containing DNA and allowed to grow on the standard medium, their ability to produce a tumor was practically the same as that of the control cells (Fig. 2, *bottom*, VII and VIII). 8 days after treatment, even the cells treated with DNA for 96 hr had unimpaired virulence.

TABLE 1. *tRNA methylase activity of control and DNA-treated W-256 cells.*

Enzyme source	Protein (μ g)	tRNA methylase (specific activity)*
48-hr cells	52	365
" "	104	298
" " plus DNA	62	179
" " "	103	36
72-hr cells	50	632
" "	100	622
" " plus DNA	51	55
" " "	102	39
8-day cells after DNA treatment	55	392
	110	333

* Picomoles of ^{14}C incorporated into tRNA per mg protein in 45 min.

tRNA methylase activity: The tRNA methylase activity of the tissue culture cells was determined after 48 and 75 hr of treatment, and again after the DNA-treated cells had been transferred back to the regular medium for 8 days. The results are recorded in Table 1. After 48- and 72-hr exposures to DNA, there was a marked decrease in the specific activity of the tRNA methylase extracted from the cells, compared with that in buffer-treated controls. 8 days after the return of the cells to regular medium after 48-96 hours' exposure to DNA, the tRNA methylase activity returned to the control or untreated values.

Freezing and storage of crude cell extracts resulted in loss of activity; therefore, only fresh extracts were used in all experiments.

In this particular experiment, the $105,000 \times g$ supernates (crude enzyme source) were prepared from control W-256 cells and from cells treated for 24 hr with DNA. The supernates were dialyzed against two changes of buffer, 400-ml total, for 3 hr. Neutralized pH 5 supernates and precipitates were also prepared from the undialyzed $105,000 \times g$ supernates. The neutralized supernate of the DNA-treated cells was also added to the dialyzed crude enzyme from the untreated cells. The enzyme activity was measured with and without added ammonium acetate in each case. The results are shown in Table 2.

Ammonium acetate added to the reaction mixtures produced a more than 5-fold increase in the methylase activity of crude dialyzed preparations from DNA-treated cells compared with a slight decrease in activity from the control

TABLE 2. *Induction of tRNA methylase inhibitor by DNA treatment and effect of ammonium acetate.*

Enzyme source	tRNA methylase activity*—	
	— NH_4OAc	+ NH_4OAc
1. Control cells, crude enzyme	58	46
2. DNA-treated cells, crude enzyme	17	79
3. Control cells, pH 5 precipitate	100	300
4. DNA-treated cells, pH 5 precipitate	137	316
5. 1 + 4, neutralized	32	110

Assayed as outlined in the text, except that 52 nmol of *S*-adenosyl-[methyl- ^{14}C]methionine was used.

* Picomoles of ^{14}C transferred per mg protein in 45 min.

cells. However, ammonium acetate stimulated the pH 5 precipitate of both groups. The combination of neutralized pH 5 supernate from DNA-treated cells with dialyzed crude enzyme of control cells resulted in a marked reduction in the methylase activity of the control enzyme when the reaction was run without added ammonium acetate. With added ammonium acetate, the opposite effect was observed.

Discussion. Continuous exposure of W-256 tissue culture cells to DNA prepared from the tumor does not impair the growth rate of these cells in tissue culture measured at 48 and 72 hr. However, these DNA-treated cells had a diminished ability to produce malignant tumors when injected into the strain of rats from which the neoplasm originated. This effect may be demonstrated if the tissue culture cells are incubated with DNA for 90 min in the absence of protein, but the effect is also evident in growing neoplastic cells *in vitro* within 24 hr when the medium contains not only protein but all the other necessary constituents for unaltered growth. The impaired oncogenicity was most apparent in cultures that were allowed to overgrow, and when the injected cells were limited to the newly formed, small floater cells that did not stain with trypan blue.

Coincident with the decrease in the oncogenicity is a decrease in tRNA methylase activity and the appearance of a tRNA methylase inhibitor. The presence of a tRNA methylase inhibitor is *suggested* by the decrease in enzyme specific activity of the tRNA methylases extracted from the DNA-treated cells (Table 1 and Fig. 3). Its presence is clearly *demonstrated* by the addition of the

FIG. 3. Effect of DNA treatment on tRNA methylase activity of W-256 cells.

neutralized pH 5 supernate of the DNA-treated cells to the dialyzed enzymes prepared from the control cells when ammonium acetate is left out of the reaction mixture.^{11,19}

When the W-256 tissue culture cells are returned to a medium that does not contain inhibitory DNA, their ability to produce malignant tumors in animals and their tRNA methylase activity return to their pretreatment levels. The inhibitor apparently disappears rapidly after the inducer is removed. Whenever DNA preparations added to the medium turned out (occasionally and inexplicably) to be noninhibitory, the W-256 cells displayed an accelerated growth

in vitro and *in vivo* and a corresponding slight increase in tRNA methylase activity.

The malignant transformation of normal cells either *in vivo* or *in vitro* by viruses^{14,15}, chemical carcinogens¹⁶, or changes in the tissue culture medium¹⁷ is associated with a rise in tRNA methylase activity. This also is evident in spontaneous malignancies of humans and experimental animals.¹⁸ In neoplasia, the elevated tRNA methylase activity may be a reflection of a basic biochemical lesion—the failure to elaborate tRNA methylase inhibitors. By inducing such methylase inhibitors in malignant cells we have simultaneously reversed the biochemical lesion and the malignant state.

Abbreviation: W-256, Walker-256 carcinosarcoma.

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