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Actions of *cis*-Diamminedichloroplatinum on Cell Surface Nucleic Acids in Cancer Cells as Determined by Cell Electrophoresis Techniques[1]

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ABSTRACT

Whole-cell electrophoresis determinations, using a null technique to measure a cloud of cells, were made on a variety of tumor cell suspensions in order to examine the charged groups on the cell surfaces. We report here evidence, derived from these measurements, which indicates the presence of nucleic acids on the surfaces of the tumor cells. Cells of the ascitic and solid forms of the Sarcoma 180 mouse tumor, grown in ICR mice, showed a decrease of 10 to 20% in electrophoretic mobility when incubated with nucleases. One variant, S-180, exhibited sensitivity to RNase only; another variant was sensitive to both RNase and DNase, while different tumor lines were shown to be sensitive to DNase only. Treatment of tumor-bearing animals with a therapeutic dose of cisplatin resulted in a loss of tumor cell mobility identical to that produced by the nuclease incubations of the control cells. Incubation of the cisplatin-treated tumor cells with nucleases produced no change in mobility. The isoelectric points of these cells were determined and are consistent with the loss of a group with a low pK value, such as the phosphates of RNA and DNA, in both cisplatin-treated cells and nuclease-incubated cells. Using the S-180 tumor sensitive to both RNase and DNase, the rates of the two enzymes were additive, but the mobility decreased to the same level regardless of whether the enzymes were used alone or together. This suggests that both enzymes act on the same site. RNase and DNase immobilized on agarose beads were capable of lowering the mobility of the cells upon incubation, confirming the surface location of these nucleic acid residues. S-180 tumor cells were also placed in a tissue culture medium at 37° for up to 22 hr and were treated *in vitro* with cisplatin and other metabolic inhibitors. Nucleic acid or protein synthesis inhibitors produced a loss of the cell surface nucleic acids. In another *in vitro* experiment, the nucleic acids were first removed by nucleases, and, when the cells were incubated at 37°, the nucleic acids reappeared. Disappearance with the inhibitors or reappearance after digestion exhibited a half-time of about 2 hr. Surface nucleic acids were detected by this electrophoresis technique on all of several types of tumor cells but not on any normal cells examined.

INTRODUCTION

RNA has been found on the surface of cells by several investigators (4-6, 8, 10, 16-18). Weiss *et al.* (15-17) and Bennett *et al.* (2) have shown that this surface RNA is present on fast-growing cells, that it probably exists in patches, and that it is important in Ca^{2+} binding on the membrane. They and others have shown RNA on tumor cells, fetal liver cells, and stimulated lymphocytes using whole-cell electrophoresis (2, 5, 16-18), membrane preparations (4, 6), and ^{32}P release (10).

DNA has also been discovered on cell surfaces, particularly lymphocytes and tumor cells, using membrane isolation and extraction techniques (9), autoradiography (12), surface staining (1), and anti-DNA antibodies (13).

These nucleic acids have been partially characterized according to size and synthesis rate (6, 11, 12), and both the RNA and DNA are believed to represent subpopulations of the total cellular nucleic acids.

In this study, we examine the Sarcoma 180 tumor, and we show that in one strain of this tumor there is a surface group sensitive to RNase and/or DNase. Additional experiments lead us to conclude that this group is probably some type of surface RNA:DNA complex. Antitumor drugs, particularly cisplatin, lead to the loss of this surface nucleic acid, and this may play a role in tumor regression. From the results of these tests, we conclude that these nucleic acids originate within the tumor cell and are linked to cellular metabolism. We also show that some other cell types, not examined previously, show surface nucleic acids. In our studies thus far, we find these nucleic acids on tumorigenic cells only.

Our studies of cell surface nucleic acids are based upon whole-cell electrophoresis techniques. The electrophoretic mobility is a measure of the average surface charge of a cell, and it can be used in conjunction with selective membrane effectors to determine the types of groups present on the cell periphery. Presumptive nucleic acid moieties located on the surface could be detectable, because they contribute to the negative charge of the cell by virtue of the negatively charged phosphate backbone of these molecules. The types of effectors we have used to probe for the existence of surface nucleic acids include: alterations in pH of the media in which the cells are measured; incubation with enzymes which remove the nucleic acids; addition of molecules which bind to nucleic acids; and finally, incubation with nucleic acid synthesis inhibitors and antitumor drugs. In particular, we show evidence that the antitumor drug cisplatin acts in such a way that it causes the long-term disappearance of cell surface nucleic acids in mouse tumor cells.

MATERIALS AND METHODS

Cells. Two variants of the mouse Sarcoma 180 tumor were used in this laboratory over different periods. The first, which is hereafter called S-180, was serially transferred in ICR mice for seven years. After a colony-wide infection led to the loss of this tumor line, a new S-180 line, hereafter called S-180J, was obtained and was also grown in ICR mice.

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Tumor cells were injected i.p. (2×10^6 cells), and at later times, the cells were removed from sacrificed animals by aspiration from the peritoneal cavity. The cells were washed by centrifugation in 0.145 M sodium chloride after removal from the animals and were then stored on ice until used in incubation experiments or until measured for their electrophoretic mobility.

The P388 and P815 cells were grown i.p. in DBA/2 mice. AKR spleen and thymus cells were obtained from preleukemic (normal appearing) and leukemic (sick appearing) AKR mice. The L1210 and V79 cells were grown in tissue culture.

The liver, spleen, thymus, fetal cells, and solid Sarcoma 180 tumors were removed from animals and bathed in a sodium citrate:EDTA:NaCl solution (0.027 M sodium citrate: 0.27 M EDTA: 0.08 M NaCl). For all tissues except the solid tumor, the animals were perfused through the heart with sodium citrate:EDTA:NaCl solution before removal of the organ. The cells were then forced through a 40 mesh stainless steel screen, and the large pieces were allowed to settle out. The supernatant was run through a 26-gauge needle, and the resulting suspension was cleaned by centrifugation.

Buffers. The buffer used for all electrophoresis measurements was a citric acid:phosphate buffer with a pH range from 2.2 to 8.0. For pH 7.4, concentrations (M) are: NaCl, 0.137; KCl, 0.004; Na_2HPO_4, 0.011; citric acid, 0.006. The Na_2HPO_4:citric acid ratio was adjusted to obtain the other pH values.

All enzyme incubations were performed in modified Hanks' solution (NaCl, 0.137 M; KCl, 0.005 M; $MgSO_4$, 0.0016 M; $CaCl$, 0.00081 M; glucose, 0.011 M; NaH_2PO_4, 0.001 M; and Na_2HPO_4, 0.0045 M). The bicarbonate was removed to eliminate the need for a CO_2 atmosphere. Since this buffer was used only for short-term incubations this is an acceptable modification.

The tissue culture media for the *in vitro* incubations after drug treatment were either Medium 199 with Hanks' base plus 10% fetal calf serum or NCTC 135 with Earle's balanced salt plus L-glutamine plus 10% fetal calf serum.

Enzymes. Several sources of RNase and DNase were used early in this study, but since they all acted similarly, the following enzymes were used regularly. RNase A (Sigma Chemical Co., St. Louis, Mo.; type XI-A) was used at a concentration of 0.2 mg/ml for incubation with cells. DNase I was Sigma type I and was used at a concentration of 0.1 mg/ml. No protease contamination was detected at the levels necessary for a measurable effect upon the cellular electrophoretic mobility. Immobilized DNase was obtained from Worthington Biochemical Corp., Freehold, N. J., and immobilized RNase was obtained from Miles Laboratories, Inc., Elkhart, Ind. *Eco*RI and *BamI* were also obtained from Miles Laboratories, Inc. The sources for the other enzymes were as follows: trypsin, ICN Nutritional Biochemicals, Cleveland, Ohio; neuraminidase, Sigma type V; protease, Calbiochem-Behring Corp., La Jolla, Calif.; hyaluronidase, Sigma type IV; immobilized DNase, Worthington Biochemical Corp.; and immobilized RNAse, Miles Laboratories, Inc.

For enzyme treatment of the cells, the enzymes were dissolved in Hanks' solution, and the cells were added. Incubation was at 37° for the times shown in the text, usually between 20 and 40 min.

Electrophoresis Measurement. The electrophoretic mobility measurements were made with an apparatus constructed in this laboratory and modified from the design of Goetz and Penniman (7). A rectangular chamber was used with the orientation as shown in Chart 1. The chamber was obtained from the Arthur H. Thomas Co., Philadelphia, Pa., and was sealed within a glass water jacket to allow temperature regulation. The temperature was held at 18 ± 0.1°, using a Haake Inc. (Saddle Brook, N. J.) pumping water bath. The viewing plane of the chamber was oriented laterally so that the cells could sediment and still remain in focus. A magnification of × 200 was used, and the focal point chosen was the closest stationary plane within the chamber (3). A modified Nikon, Inc. (Garden City, N. J.) Model MS inverted microscope was oriented horizontally and fitted with a sliding mirror and a television camera to display the image of the chamber on a television monitor. The image of the cells, which was projected by the microscope objective onto a grid in the eyepiece, was moved laterally by a motor-driven sliding mirror. This lateral movement was used to counterbalance the motion of the cells due to the electric field inside the chamber. When the cells appeared stationary at the eyepiece, the mirror motion was equal and opposite to the velocity of the cells. Since the mirror was moved by a precision servomotor-tachometer system, an accurate voltage representation for the average cell velocity was obtained. When using this apparatus, the movement of a whole cloud of cells was nulled, rather than only single cells. This gave a more precise representation of the average mobility of the cells with fewer measurements required. Fifteen left and right determinations (nullings) were taken for each mobility measurement. Each determination lasted for 5 to 10 sec, and then the current and mirror movement were reversed. Approximately 15 to 50 cells were in the field of view for each determination. Silver:silver chloride electrodes in saturated KCl were used to provide current to the chamber. Approximately 15 cm of tubing, filled with the electrophoresis buffer, separated the electrodes from the chamber. The field strength within the chamber was determined by measuring the electric current through the chamber and the conductivity of the suspending buffer. The electrophoretic mobility was then the velocity divided by the field strength and is given here as μm/sec/V/cm.

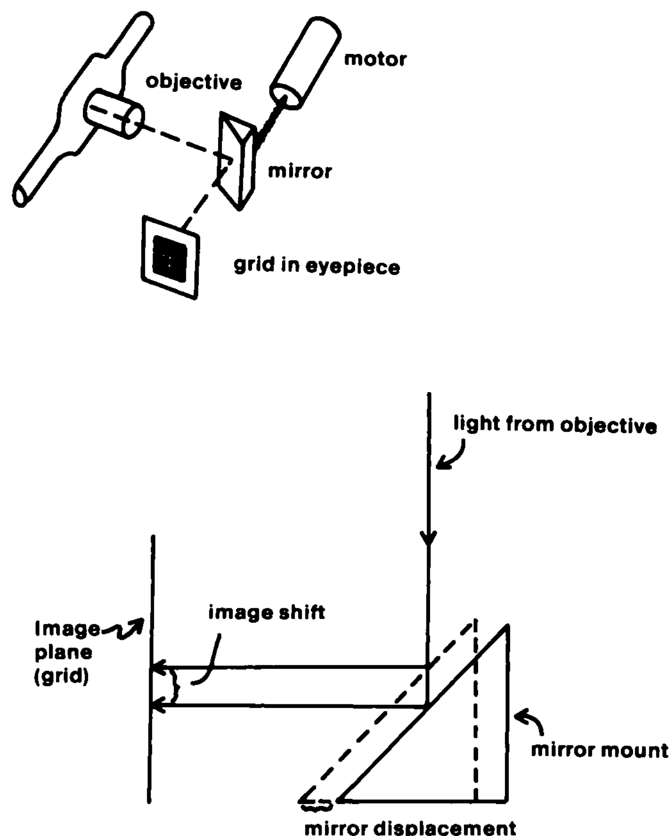

Chart 1. Diagram of light path from electrophoresis chamber to ocular grid.

RESULTS

Electrophoretic Mobilities as a Function of the Tumor Growth Time

In Chart 2, the electrophoretic mobilities of the Sarcoma 180 tumor are plotted as a function of time after i.p. injection of the tumor into the mice. One set of animals received the tumor on Day 0, the other set received the tumor on Day 0 and cisplatin (7 mg/kg) injected i.p. on Day 1. Chart 2 shows the mobilities

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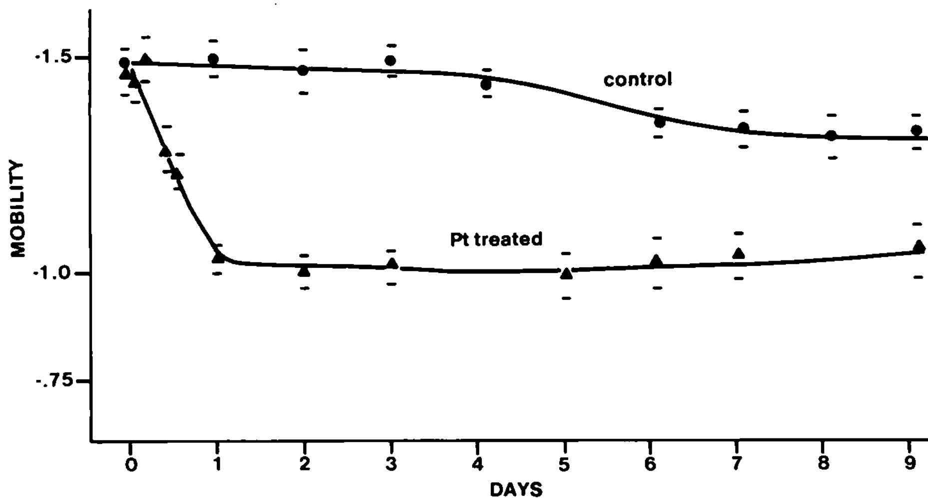

Chart 2. Electrophoretic mobility of S-180 tumor cells with and without cisplatin treatment as a function of tumor growth time. *Bars*, S.E.

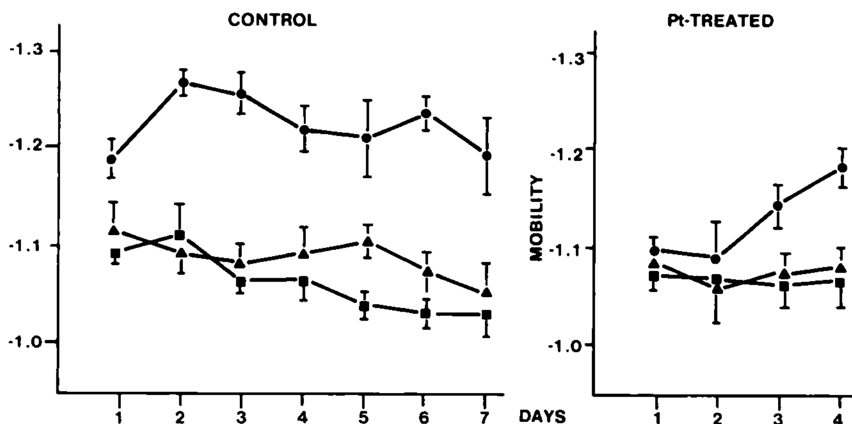

Chart 3. Mobility as a function of tumor growth time for control and cisplatin-treated cells. ■, RNase incubation; ▲, DNase incubation; and ●, incubation controls. *Bars*, S.E.

through the subsequent 9 days of tumor growth. After the ninth day, the peritoneal cavities of the controls became very large and bloody, while in the platinum-treated animals, the peritoneal cavities contained very few cells.

For the first 4 hr after cisplatin injection, there was no change in mobility, suggesting that cisplatin did not lower the mobility by simply binding to the membranes. There was a striking reduction of the mobility of the treated cells which began about 7 hr after treatment.

Light microscopy examinations of peritoneal cell samples by Dr. Steven Stockham (veterinary pathologist at Michigan State University) revealed a large relative concentration of macrophages and neutrophils infiltrating the tumors after cisplatin treatment. This led to ambiguities in measuring tumor cell mobilities beyond 2 to 3 days after cisplatin injection due to the unreliability of visually determining which cells were neoplastic. Verification of the *in vivo* action of cisplatin upon the neoplastic cells was obtained by allowing the tumor to grow 3 days in the mice before treatment with cisplatin. After this period of growth, the peritoneal cells were almost exclusively neoplastic. These data are presented in the following section using the tumor, S-180J.

Electrophoretic Mobilities as a Function of Tumor Age and Enzyme Treatment

S-180J. In Chart 3, the mobilities as a function of time and enzyme treatment are shown for the S-180J, a variant strain of the Sarcoma 180 tumor. The mobilities for 7 days of tumor growth are shown along with the results of RNase and DNase incubation of samples from each day. The mobilities of tumor cells from animals treated with cisplatin 3 days after tumor injection are also shown together with the results of RNase and DNase incubation. The *abscissa* of the cisplatin treatment graph represents days after cisplatin injection. The untreated tumor cells showed high mobility and were sensitive to DNase and RNase. Cisplatin treatment of animals resulted in a dramatic reduction of tumor cell mobility, where greater than 90% of the cells present were tumor cells. Cisplatin treatment at Day 3 rarely resulted in tumor cures but led only to an increased life span. The resurgence of tumor growth was seen as a reappearance of nuclease-susceptible groups on the platinum-treated tumors.

Enzyme Incubations. Incubations of the S-180 and S-180J with several enzymes are documented in Table 1. Both control and platinum-treated cells of both tumor types are listed after incubation separately with each of the enzymes, neuraminidase, trypsin or Pronase, hyaluronidase, DNase, and RNase. In the S-180, RNase was the only enzyme which, after incubation, mimicked the effect of platinum treatment in the controls and had no effect on the platinum-treated cells. In the S-180J, both RNase and DNase mimicked the effect of platinum treatment on cell mobility. Both proteases and neuraminidase lowered the mobilities of the controls and treated cells, and each enzyme lowered the mobilities of the controls and the treated

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Table 1
Effects of enzyme incubations on mobility of S-180 tumor cells

	Electrophoretic mobility (μm/sec/V/cm)					
	Control	Protease[a] (10 μg/ml)	Neuraminidase (1 unit/ml)	RNase (0.2 mg/ml)	DNase (0.1 mg/ml)	Hyaluronidase (1000 National Formulary units/ml)
S-180	1.32 ± 0.011[b]	0.97 ± 0.018[c]	0.96 ± 0.86[c]	1.06 ± 0.016[c]	1.36 ± 0.04	1.30 ± 0.03
S-180 + cisplatin[d]	1.08 ± 0.015	0.93 ± 0.025[c]	0.85 ± 0.52[c]	1.05 ± 0.024	1.06 ± 0.05	
S-180J	1.19 ± 0.015	0.93 ± 0.03	0.92 ± 0.016[c]	1.03 ± 0.05[c]	1.02 ± 0.05[c]	1.25 ± 0.03
S-180J + cisplatin	1.06 ± 0.07	0.94 ± 0.02	0.91 ± 0.014[c]	1.05 ± 0.06	1.05 ± 0.06	

[a] The protease used for the S-180 was Pronase, and that for the S-180J was trypsin.
[b] Mean ± S.E.
[c] Significantly different from respective controls at a significance level of 0.01 or better.
[d] The cells listed as "+ cisplatin" are tumors from animals that have been treated with 7 mg cisplatin per kg.

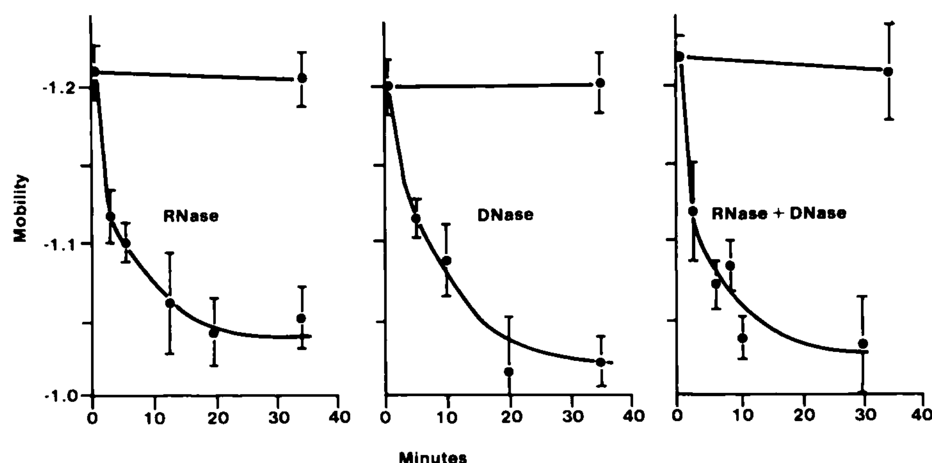

Chart 4. Change in mobility of the S-180J as a function of the time of exposure to RNase and DNase. *Top curve* in each Chart is the incubation control. *Bars*, S.E.

cells to a common level. RNase, but not DNase, decreased the surface charge of the S-180 cells, but both were active against the S-180J.

Enzyme Kinetics. Time courses for DNase, RNase, and RNase plus DNase are shown in Chart 4, *a* to *c*. The kinetics of RNase, DNase, and RNase plus DNase is approximately first-order curves with rate constants of 0.065, 0.074, and 0.161 min^{-1}, respectively. The rate constant for RNase plus DNase is approximately equal to the sum of each of the separate rate constants.

The level to which the mobility drops was about equal for both RNase and DNase and RNase plus DNase. From this and the additivity of their rates, it is probable that they are both acting on the same site.

During the course of RNase digestion, samples were removed from the 37° incubation at the indicated times and were stored on ice until all samples were taken. At this point, the RNAse was washed from all samples by repeated centrifugation. If the positively charged RNase molecules were simply binding to the surfaces of cells instead of exhibiting enzymatic activity, then this procedure would have detected it, because all time points were exposed to RNase for the same length of time. At ice temperatures, the coulombic interaction between a positively charged RNase molecule and a negatively charged surface molecule would be favored due to the low thermal energy present (assuming that no conformational changes of the membrane rule out the interaction). The early time points should have shown the same drop in mobility as the later time points, which they did not. Therefore, only the enzymatic activity was the cause of the reduced mobility. This result is further verified by the results obtained with immobilized RNase (described below) which cannot attach to the membrane permanently.

pH Profiles of the S-180 and S-180J. When the cells are measured in buffers of varying pH, a profile is obtained which usually shows an isoelectric point where the cells have no net charge. This point is approximated by interpolating between negative and positive mobilities. In Chart 5, the pH profiles for control and platinum-treated S-180 cells are shown. The isoelectric points are 3.4 and 3.9, respectively. This shift to higher pH in the platinum-treated cells could be due to the loss of a group with a pK lower than 3.4.

The loss of a group with a pK of 1 to 2 is consistent with the shift in Chart 5. Phosphate groups have pKs in this range, and they are located in membrane phospholipids and in the backbone of polynucleic acids. The pK of the phospholipid phosphates is between 1.0 and 2.0, and the pK of RNA is around 1.0.

The major phospholipids of mammalian cell membranes are zwitterions with the positive group the most exterior group. This eliminates the possibility that one of these groups was responsible for the effect seen in Chart 5. Loss of nucleic acids, however, could be responsible for the effect.

In Chart 6, the pH profile of the S-180J tumor is given. The *curve* of *solid circles* is the untreated control, the *open circles* are the platinum-treated cells, and the *squares* and *triangles*

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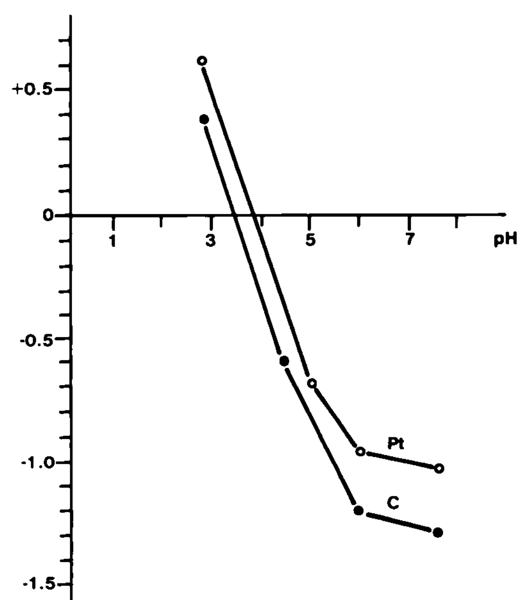

Chart 5. Plot of mobility *versus* pH for control *(C)* and cisplatin-treated *(Pt)* S-180J cells.

represent cells incubated with RNase or DNase, respectively. Only the control is significantly different from the other 5 curves. This is consistent with the hypothesis that nucleic acids are responsible for the low pK moiety.

Incubation with Agarose Bead-immobilized DNase and RNase. Immobilized enzymes were used in order to prevent the RNase and DNase from entering the cell and thereby causing indirect membrane changes. In Table 2, the results of an incubation with agarose-bound DNase and RNase are shown. After the incubation of the cells with the bound enzymes, Ficoll was added to a final concentration of 15%. When the mixture of cells and beads was centrifuged in this solution for 5 min at 750 rpm, only the agarose beads sedimented. The cells in the supernatant were then washed free of the Ficoll. Mobilities were determined but not shown for Ficoll wash alone and plain Sepharose beads with Ficoll wash. There was no effect due to these procedures.

The effect of the bound enzymes was to reduce the mobility in the same manner as the free enzymes. The treatment with the bound enzymes was followed with incubations in free enzymes. This produces no further change, indicating that the enzyme effect was truly a surface effect.

Platinum:Thymine Blue Attachment to the Cell Membrane. Platinum:thymine blue was shown to bind strongly to nucleic acid (14). This binding was believed to be mediated by the positive charge of the platinum complexes. These complexes contain a mixture of positively charged oligomer chains of various molecular weights and are not crystallizable, but their specificity, at low concentrations, for nucleic acids has been demonstrated by electron microscopy (1). We used low concentrations of platinum:thymine blue in an attempt to specifically lower the mobility of cells by neutralizing the negative charge of surface nucleic acids.

Platinum:thymine blue was incubated with S-180J cells at a concentration of 10 μg/ml for 30 min at 37°. Table 3 shows the effect of the platinum:thymine blue on the mobilities of control and platinum-treated cells, as well as cells incubated first with RNase or DNase and then with the platinum:thymine blue. The platinum:thymine blue lowers the mobility of the controls but has no effect on the treated or enzyme-incubated cells.

Incubation with Anti-DNA Antiserum. Rabbit anti-DNA antiserum (a generous gift of Dr. Edward Golub of Purdue University) was made by injecting calf thymus DNA plus bovine serum albumin into rabbits. The titer of this antiserum is low due to the low antigenicity of nucleic acids.

S-180J control and cisplatin-treated cells were incubated with anti-DNA antiserum, and the mobility results are shown in Table 4. These results show that there is a small reduction in control cells at the dilutions shown but not in the platinum-

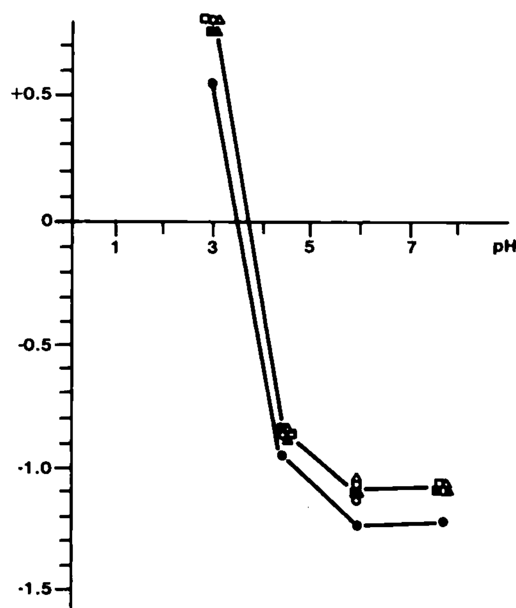

Chart 6. Plot of mobility *versus* pH for the S-180J. ●, ■, ●, control cells; ○, □, △, cisplatin-treated cells. □, ■, RNase incubated; △, ▲, DNase incubated; and ○, ●, incubation controls.

Table 2

Mobility effects due to incubation of cells with immobilized RNase and Dnase for 45 min at 37°

	Mobilities (μm/sec/V/cm)	Concentration
S-180J control	−1.143 ± 0.011[a]	
S-180J + free RNase	−1.048 ± 0.013	0.1 mg/ml
S-180J + free DNase	−1.046 ± 0.013	0.2 mg/ml
S-180J + agarose-bound RNase	−1.080 ± 0.023	6 units/ml (Miles)
S-180J + agarose + free RNase	−1.060 ± 0.032	0.1 mg/ml
S-180J + agarose-bound DNase	−1.070 ± 0.020	400 units/ml (Worthington)
S-180J + agarose + free DNase	−1.060 ± 0.022	0.2 mg/ml

[a] Mean ± S.D.

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Table 3

Effects of binding of platinum:thymine blue on mobilities of S-180J cells

Platinum:thymine blue was incubated with the cells for 10 min at 37° at a concentration of 10 mg/ml.

	Mobilities (μm/sec/V/cm) with following enzyme incubations		
	Control	RNase	DNase
S-180J control	−1.21 ± 0.02[a]	−1.08 ± 0.04	−1.08 ± 0.015
Platinum:thymine blue added	−1.11 ± 0.02	−1.06 ± 0.02	−1.05 ± 0.02
S-180J cisplatin treated	−1.08 ± 0.03	−1.07 ± 0.02	−1.08 ± 0.02
Platinum:thymine blue added	−1.06 ± 0.04	−1.11 ± 0.01	−1.04 ± 0.01

[a] Mean ± S.D.

Table 4

Mobility changes due to the incubation of cells with anti-DNA antisera

When treated with a 1:2 dilution of normal rabbit sera, the mobility of S-180J cells was −1.22 ± 0.014. Antisera were incubated at 23° for 30 min, and then, the cells were washed once by centrifugation.

	Mobilities (μm/sec/V/cm)			
		Dilutions of anti-DNA antisera		
	Control	1:10	1:15	1:2
S-180J	−1.23 ± 0.003[a]	−1.18 ± 0.010[b]	−1.17 ± 0.003[b]	−1.16 ± 0.04[b]
Cisplatin treated	−1.09 ± 0.03		−1.08 ± 0.02	

[a] Mean ± S.D.
[b] Significantly different from control at the 0.01 level.

Table 5

Effects of restriction endonuclease EcoRI and S_1 nuclease on cell mobility

The cisplatin-treated cells on Day 1 show a curious phenomenon which was seen occasionally. The overall mobility does not drop, but the sensitivity to enzymes disappears. By Day 2, the full effect of the cisplatin seems to have occurred.

	Mobilities (μm/sec/V/cm)						
		*Eco*RI activity				S_1 nuclease activity	
	Control	20,000 units	10,000 units	5,000 units	Control	330,000 units/ml	125,000 units/ml
S-180J					−1.22 ± 0.02	−1.23 ± 0.02	−1.24 ± 0.01
+ 10 mM $MgSO_4$	−1.20 ± 0.03[a]	−1.03 ± 0.03[b]	−1.08 ± 0.01[b]	−1.16 ± 0.03			
+ 10 mM EDTA	−1.23 ± 0.02	−1.21 ± 0.03	−1.20 ± 0.01	−1.21 ± 0.04			
S-180J cisplatin treated (Day 1)							
+ 10 mM $MgSO_4$	−1.15 ± 0.02	−1.15 ± 0.02	−1.18 ± 0.02	−1.19 ± 0.01			
+ 10 mM EDTA	−1.17 ± 0.01	−1.17 ± 0.02	−1.15 ± 0.02	−1.14 ± 0.03			
S-180J cisplatin treated (Day 2)							
+ 10 mM $MgSO_4$	−1.05 ± 0.02	−1.03 ± 0.02	−1.05 ± 0.02	−1.09 ± 0.03			

[a] Mean ± S.D.
[b] Significantly different from control at the 0.01 level.

treated cells. Incubation with normal rabbit serum at the lowest dilution also had no effect.

Incubation with the Restriction Enzymes *Eco*RI and *Bam*I. In the S-180J, the sensitivity to RNase is accompanied by a sensitivity to DNase. The possibility of surface DNA was further examined by the use of the specific endonuclease restriction enzyme *Eco*RI. This enzyme recognizes the following double-strand sequence

and cleaves the double-stranded DNA at the slash marks.

In Table 5, *Eco*RI is shown to be active at high enzyme concentrations in control cells but not in platinum-treated cells, and if EDTA is added to remove the activation ion Mg^{2+}, the effect disappears.

Also shown in Table 5 is the effect of Nuclease SI which is specific for single-stranded RNA or DNA. Even at very large concentrations of the enzyme, no effect is seen. This supports the results of *Eco*RI experiments in indicating that the surface nucleic acid is double stranded.

In Table 6, the effect of restriction enzyme *Bam*I on cell mobility is shown. An effect is noticeable at lower enzyme concentrations than is seen in *Eco*RI. Both of these enzymes are used at extremely high concentrations, however, and probably damage the nuclei of the cells.

***In Vitro* Tumor Cells Incubated with Several Antimetabolic Drugs.** *In vitro* studies were undertaken to rule out the possibility that the interaction between tumor and host was responsible for the loss in mobility due to cisplatin and to offer a less complicated system for testing the effects of other drugs. In these experiments, cells were removed from a tumored animal and incubated with cisplatin or other compounds in Hanks' buffer and then placed into tissue culture media for up to 22 hr

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Table 6
Effects on cell mobility of incubation with restriction endonuclease BamI

	Mobilities (μm/sec/V/cm)			
	Control	2000 units/ml	500 units/ml	100 units/ml
Untreated				
+ Magnesium	−1.18 ± 0.02[a]	−1.00 ± 0.01	−1.09 ± 0.01	−1.18 ± 0.02
+ EDTA	−1.19 ± 0.03	−1.12 ± 0.03	−1.21 ± 0.02	−1.23 ± 0.04
Cisplatin treated				
+ Magnesium	−1.07 ± 0.02	−1.09 ± 0.04	−1.05 ± 0.02	−1.05 ± 0.02
+ EDTA	−1.054 ± 0.04	−1.09 ± 0.02	−1.07 ± 0.01	−1.06 ± 0.03

[a] Mean ± S.D.

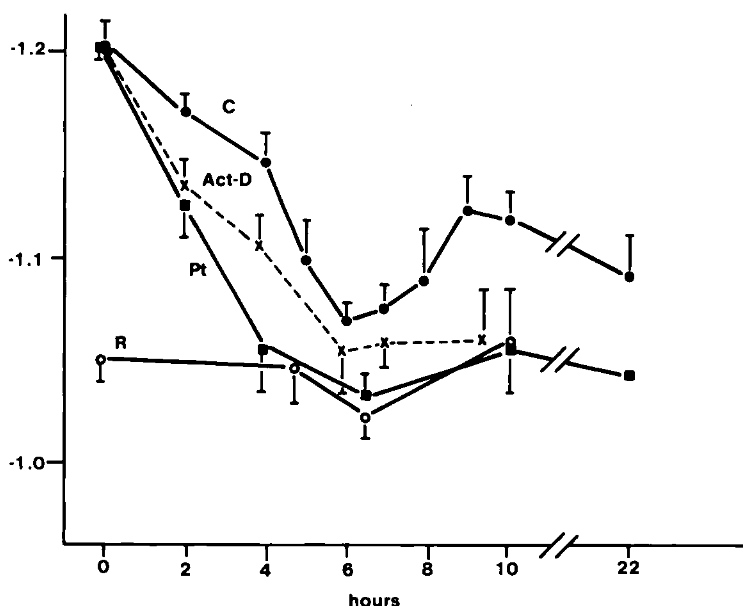

Chart 7. Plot of mobility *versus* time in *in vitro* incubations. Cells were removed from animals and preincubated with cisplatin *(Pt)* or with actinomycin D *(Act-D)* for 40 min before time zero. Time shown is time in normal tissue culture media. *R*, RNase incubation of samples taken during the course of the *in vitro* incubations. *C*, control.

at 37°. The culture medium was changed at regular intervals of 1 and 2 hr. Samples were removed using a sterile technique, and the mobilities were measured.

The curves in Chart 7 show a continual decline and a characteristic dip between 4 and 8 hr. The effect of cisplatin is to increase the rate of decline leading to an even lower value with only a slight dip. The RNase treatment of controls *(open circles)* is a curve similar to that of cisplatin beginning at about 4 hr.

An RNA synthesis inhibitor, actinomycin D, is also shown as the *dashed line* in this Chart. It too causes a reduction of the mobility to a level similar to RNase and platinum. Its effect is slower, but this may be dose dependent.

Other inhibitors were also tested to further substantiate the evidence for nucleic acids on the cell surface and their possible origin inside the cell.

In Chart 8*c*, the effects of mercaptopurine and methotrexate are shown. They produce an effect similar to cisplatin by lowering the mobility to that produced by RNase. Both of these drugs are DNA synthesis inhibitors and are used in antitumor therapy. The effects of puromycin, a protein synthesis inhibitor, are shown in Chart 8*d*. It lowers the mobility much further than the other drugs but in the process also removes the RNase-susceptible group. At the 5-hr mark, the *bar* under the *triangle* indicates the mobility of that 5-hr sample after an RNase incubation: there is no change. We had seen in other work that the loss of protein (such as with protease treatment) causes the loss of the nuclease-susceptible group.

From these results, we conclude that puromycin causes more membrane changes than just the loss of nucleic acids.

The link of the presence of nuclease-susceptible groups to cellular metabolism is shown in Chart 8, *a* and *b*. In these experiments, cells were first treated with RNase or DNase and then placed in tissue culture media. Within 4 hr, the mobility increased, and the curves joined the curve of the control cells. It appeared that these groups are renewed on the surface and that the cell may be continually producing this material and perhaps even exuding it into the medium.

Examination of Other Types. Several other cell types were examined for their sensitivity to DNase and RNase, and the results are given in Table 7. In this small number of cell types tested, only tumorigenic cells were sensitive to these enzymes. In general, we noticed that only cells of lymphocyte origin were sensitive to DNase.

DISCUSSION

The method of whole-cell electrophoresis is an indirect method of measuring cell surface changes, but it was chosen because of its sensitivity to small charge changes on the cell surface. A gain or loss of 10^6 to 10^7 charges is easily detectable by this method.

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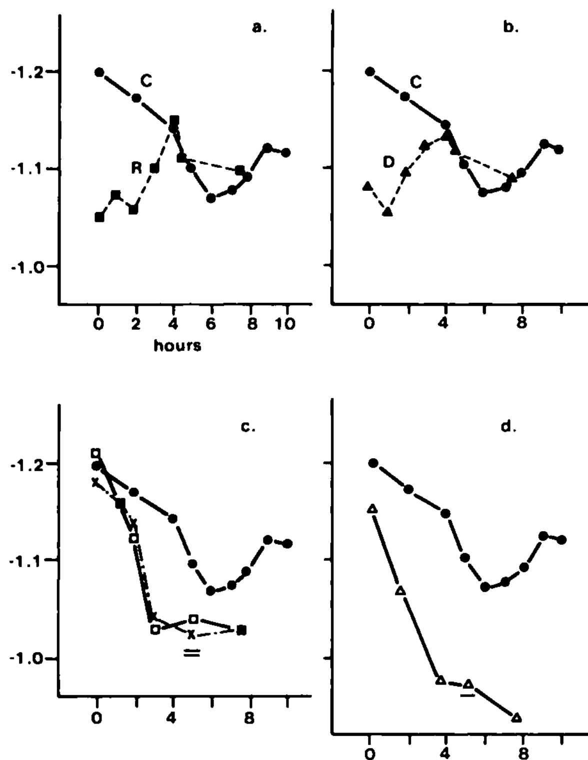

Chart 8. Effects of various enzyme and drug preincubations on the recovery of mobilities of S-180J cells. The mobilities of control cells (nonincubated) as a function of time in tissue culture are repeated in *a* and *b* (*C*). *a*, recovery of mobilities with time in tissue culture after preincubation with RNase (■); *b*, as in *a* for DNase preincubation (▲); *c*, effects of preincubations with 6-mercaptopurine (0.5 mg/ml) (□) and methotrexate (0.5 mg/ml) (×); *d*, effects of preincubation with puromycin (1.0 mg/ml) (△).

Table 7
Summary of nuclease effects on cell mobilities of several cell types

	RNase	DNase
S-180	+[a]	–
S-180 solid	+	–
S-180J	+	+
S-180J solid	+	+
P815 ascitic	+	+
P388 ascitic	–	+
AKR (leukemic)		
Spleen	–	–
Thymus	–	+[b]
AKR (preleukemic)		
Spleen	–	–
Thymus	–	–
Fetal cells	–	–
Liver	–	–
V-79 (tissue culture)	–	–
L1210 (tissue culture)	–	+
L1210 (virus infected)	+[b]	+[b]
L1210 (cisplatin resistant)	+	+[b]

[a] +, significant effect of the enzymes at a level of 0.01; –, no significant effect of the enzyme treatment.
[b] Significance at the 0.05 level.

The type of measurements made by the apparatus used for this study yields an average mobility for a group of cells. If there are 2 populations of different mobility, they might not be detected as being separate. In order to assure that the populations observed in this study were unimodal, a distribution of cell mobilities was determined using a stopwatch to time individual cells. Both the control and platinum-treated cells showed unimodal distributions of mobility. In the cisplatin-treated tumors, however, it was necessary to avoid measuring the mobility of host cells that infiltrate the peritoneal fluid. These were mainly neutrophils and macrophages, and their average mobilities were greater than the cisplatin-treated S-180 cells.

A possible criticism of these findings is that the nucleic acid detected on the cell periphery is simply exogenous debris from broken cells. We cannot refute this completely, but we have incubated cells with debris from mechanically disrupted cells and then measured the mobility. Cells which had their surface nucleic acid removed by RNase or DNase showed no change in mobility with subsequent incubation with cell debris. If it is cell debris, it is not a random type of debris.

We have observed that trypsin, Pronase, and neuraminidase can remove the nuclease susceptibility along with the protein and sialic acid for which these enzymes are specific. This suggests to us that the nucleic acids exist on the periphery of the cell, perhaps anchored by interactions with proteins and sugar moieties.

The 2 strains of the Sarcoma 180 showed different responses to RNase and DNase. S-180J responded to both, while S-180 responded only to RNase. With these data and the results for the other cell types shown in Table 7, we have documented susceptibilities to RNase alone, DNase alone, and both RNase and DNase. The existence of these 3 classes of cells provides evidence that there is no common impurity, such as a protease, present in the RNase and DNase preparations which could result in the lowered mobility.

There is the possibility that some cross-contamination of nucleases was present in the commercial RNase and DNase used. The importance of this is minimal for 2 reasons. The first is the presence of the 3 classes of cells, as stated above, which exhibit 3 mutually exclusive susceptibilities to the 2 enzymes. The second is that we have removed magnesium ions from incubation mixtures, and only the DNase susceptibilities were eliminated, RNase continuing to lower the mobilities in those cells sensitive to RNase.

The enzyme kinetics results demonstrated that RNase, DNase, or RNase plus DNase lowered the electrophoretic mobility to a common level. We have followed RNase-treated cells with DNase incubations and vice versa (data not shown) and have found no further reduction in mobility. This is consistent with an RNA:DNA complex loosely bound to the cell surface. When either RNA or DNA is digested, the binding ability of the complex decreases enough to allow it to separate from the cell.

The relatively high concentrations of RNase and DNase needed to digest the surface nucleic acids may indicate that these molecules have a low sensitivity to nucleases. This could be due to double-stranded RNA or RNA:DNA hybrids resulting in a lowered sensitivity which might be advantageous if these nucleic acids were being exuded by the cell for a purpose. The need for high RNase and DNase levels could also be partially explained by the presence of more surface nucleic acids than can be detected by electrophoretic measurements. This would lead to a need for a higher concentration of nucleases to observe the desired effect. The 2-dimensional surface of the cell membrane also contributes to the need for higher levels of nucleases by reducing the effective concentration of the bound nucleic acids compared to the freely diffusing nuclease molecules in solution.

We have used 3 separate surface effectors to add further

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evidence for cell surface nucleic acids. Each of these effectors is specific for nucleic acids, but in each case, the conclusions drawn must be tempered by the inherent drawbacks of each experiment. The platinum:thymine blue binds strongly to nucleic acids but probably also binds electrostatically to other strong anions. Therefore, we have kept the concentration low in hopes of avoiding nonspecific binding. The antisera to DNA did not show high activity but did show significant reduction in mobility over control sera. We attribute the reduction in mobility to the binding of other antibodies in the sera which may have different specificities. The restriction enzymes showed significant reduction in mobility. These enzymes are very specific for DNA. However, they were used in very high concentrations, and the effect of the levels of these enzymes on whole cells is unknown. Taken together, however, we believe that the data of these experiments imply further support for cell surface nucleic acids.

The short-term *in vitro* cell incubations offered an ideal system to study the effect of several compounds on the tumor cells without the interaction of the whole organism of the mouse. Although the cells were not in a steady-state condition, they were also not altered to the degree necessary for long-term tissue culture growth.

Immediately after removal from the animal, the cells began to lose surface charge even when undergoing no treatment. The dip in mobility between 4 and 8 hr was always seen and was probably due to the cells adjusting to the external environment. After 22 hr of *in vitro* incubation, the mobility had reached a stable level of about $-1.1\ \mu m/sec/V/cm$. When cells were incubated with RNase at different points during their time *in vitro,* they always responded to the enzyme. The level of mobility reached after RNase incubation was relatively constant regardless of how long the cells had been incubated *in vitro*. Since the control cells appeared to lose surface charge with time spent *in vitro,* and since RNase treatment lowered the mobility to a common level, this may indicate that the control cells adjusted to the *in vitro* environment by losing surface nucleic acids.

The preincubation of the cells *in vitro* with cisplatin, actinomycin D, methotrexate, and mercaptopurine resulted in lowered mobility to a level equal to the level of RNase-treated cells. This is consistent with the nucleic acid synthesis inhibitory effects of these compounds. Puromycin and cycloheximide (data not shown), protein synthesis inhibitors, reduced the mobility level much further than the nucleic acid synthesis inhibitors. This indicates that a different surface group is being effected and agrees with the results of protease treatment shown in Table 1.

In those cells pretreated with RNase or DNase, the control levels of mobility were reestablished after 4 hr of *in vitro* incubation. This period of 4 hr is in agreement with the time period for the loss of the surface charge in those cells treated with inhibitors and suggests that the surface nucleic acid has a half-life on the cell surface of approximately 2 hr.

The antitumor drug, cisplatin, and other platinum derivatives have been the subject of intensive research in this laboratory, and it was the possible interaction of cisplatin with nucleic acids that suggested this work. There is substantial evidence that cisplatin interacts with nucleic acids, particularly by binding to purines, and that this binding interferes with such processes as DNA synthesis. In an electron microscopy study, we had observed the binding of platinum:pyrimidine blue to the surfaces of cells, leading us to propose the presence of cell surface DNA (1). We therefore considered the possibility that a part of the antitumor action of cisplatin was associated with its interaction with or effect on a surface nucleic acid molecule.

The results of this study show that the S-180 tumor cells lose surface charge when the animal bearing the tumor is given a therapeutic dose of cisplatin, or when the tumor cells are exposed to cisplatin and other antitumor drugs *in vitro*. The lost charge can be mimicked by incubation of the S-180 with RNase and the S-180J with RNase or DNase. The *in vitro* studies indicate that the surface nucleic acid is linked to the metabolism of the cell and, particularly, to the synthesis of nucleic acid. This suggests to us that cisplatin, which is known to interact with nucleic acids and inhibit new DNA synthesis, causes the loss of surface nucleic acid by interfering with its normal synthesis in the cell.

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