SURFACE-ASSOCIATED DNA IN ASCITES SARCOMA-180: AN AUTORADIOGRAPHIC STUDY

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ABSTRACT

SURFACE-ASSOCIATED DNA IN ASCITES SARCOMA-180:
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By

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Platinum-pyrimidine complexes (platinum-thymine or uracil) show binding to the cell surface membrane of many tumorigenic cells such as ascites sarcoma-180, and workers in this field have presented experimental evidence which indicates that these complexes bind to DNA at the surface membrane.

Ascites sarcoma-180 cells, labelled by intraperitoneal injection of ^{3}H-thymidine into Swiss Webster mice, were coated with NTB 2 or Ilford L4 emulsion, and the resulting autoradiograms were developed in Microdol X after an exposure of 3 or 5 weeks. Histograms (silver grain number versus location) were then prepared. Although the light microscope results are invalid due to heavy beta particle scatter, the electron microscope results confirm the presence of cell surface-associated DNA.

The lack of a strong correlation between cell surface silver grains and platinum binding suggests that the surface-associated DNA has an infrequent replication time

or that the platinum compound (platinum-thymine) is not specific for DNA. The exact source and function of this membrane-associated DNA remain unknown.

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INTRODUCTION

Recent evidence has shown that nucleic acids are associated with the cell surface membrane in many kinds of cells (2,12,14,18). Lerner et al. (18), using various biochemical techniques, have shown that there is a species of DNA associated with the plasma membrane of diploid human lymphocytes. Aggarwal et al. (2), using a more direct method, have demonstrated the presence of nucleic acids on the surface membrane of many tumorigenic cells with the use of certain platinum-pyrimidine complexes (platinum-thymine or uracil). These complexes, which are now being used as potent antitumor agents (10), have been shown to react rapidly in dilute concentrations (2.5 mM) with DNA in solution to cause its precipitation into deeply stained blue fibers. A somewhat slower reaction rate has been observed for RNA, and the end result is the same: the precipitation of RNA into deep blue fibers. Proteins react with these complexes at least 100 times slower than DNA (2).

Because of their electron density and selective nucleic acid binding, the complexes have been shown to be very effective stains for electron microscopy (1,28). In intact cells these complexes stain nucleic acid

containing structures like chromatin, ribosomes, and the nucleolus. Using these complexes as stains, prominent electron-dense patches have been demonstrated at the plasma membrane surface of tumorigenic cells. Such patches have been shown to be removed if pretreated with DNase I or neuraminidase, at least in the case of ascites sarcoma-180 cells. This result, along with the fact that the platinum-pyrimidine complexes are selective for nucleic acids, has suggested that there is cell surface-associated DNA, and that it is bound to the cell surface membrane through neuraminic acid (2).

In an attempt to further strengthen the above finding, it was decided to use the technique of autoradiography at both the light and electron microscope level with ^{3}H-thymidine as the label. Earlier studies with diploid human lymphocytes using ^{3}H-thymidine have demonstrated the association of DNA with the plasma membrane (12,18). These studies were based mainly on membrane fractions, and it was difficult to rule out the possibility of nuclear or mitochondrial contamination. In the present study involving autoradiography, intact ascites sarcoma-180 cells were used. Thus, contamination problems due to membrane fractionation and isolation were eliminated. Ascites sarcoma-180 tumor cells have been used as the model system for the study of surface-associated nucleic acids in the past (2), and therefore this system was used for the present autoradiographic study also.

Furthermore, these cells are easy to maintain in the peritoneal cavity of mice.

It was hoped that through the use of autoradiographic techniques and the ascites sarcoma-180 model system, developed silver grains would be observed superimposed over the electron-dense platinum patches at the cell surface membrane. The following pages contain the results of this study.

MATERIALS AND METHODS

Female Swiss Webster mice, 4-5 weeks old, which were given intraperitoneal injections of 4×10^6 ascites sarcoma-180 tumor cells, were kindly donated by Dr. Barnett Rosenberg, Department of Biophysics, Michigan State University. The cells were allowed to proliferate within their respective hosts for 5 days (24). On day 5, 10 mice were given 3 separate intraperitoneal injections of tritiated thymidine spaced at 4 hour intervals, each consisting of 7.0 μCi of tritiated thymidine/0.5 ml sterile 0.85% NaCl (19). The specific activity of the tritium sample was 364 mCi/mmol (Schwarz/Mann Radiochemicals, Orangebury, N.Y.).

The first group of mice was sacrificed 2 hours after the last injection of the label. Mice from a second group were sacrificed at periods of 24, 48, 72, and 96 hours after the last injection of the label. A third group of mice with the ascites tumor did not receive any ^{3}H-thymidine and thus served as the control group. The body cavity of each mouse was exposed and physiological saline (0.85% NaCl) was pipetted into the peritoneal cavity. The saline solution along with the suspended ascites sarcoma-180 cells was then withdrawn from each

mouse and put into centrifuge tubes. Cell samples (labelled and unlabelled) were washed twice with saline solution (0.85% NaCl) in order to extract the red blood cells. Small pieces (1 mm^3) of intestine, kidney, and liver were also surgically removed from the animals of the first and third groups. All of these tissues, along with the ascites sarcoma-180 cell samples, were separately fixed at 4°C for 1 hour in a 2% glutaraldehyde solution in 0.05 M Sorenson's phosphate buffer at pH 7.4.

After the fixation procedure, all of the tissue samples were washed in the previously mentioned buffer for 5 minutes. Each of the ascites tissue samples and the intestine tissue samples from the first and third groups of animals were then divided into approximately 2 equal portions. One portion from each of these samples was stained in a 1% solution of platinum-thymine blue 70 (Tousimis Research Corporation, Rockville, Md.) for 1 hour at 4°C before going through the acetone dehydration series.

The different tissue samples were separately dehydrated in the following acetone series: 50% acetone (5 min), 70% acetone (5 min), 90% acetone (5 min), and 3 separate changes of dry acetone (15 min each). The tissue samples were left overnight in separate vials containing a 1:1 mixture of dry acetone and Araldite resin. The Araldite resin consisted of 27 parts Araldite 502, 23 parts dodecenyl succinic anhydride, and 1 part

dimethyl amine methyl phenol (Ladd Research Industries, Inc., Burlington, Vt.). The tissues were then transferred into separate vials containing pure Araldite resin and left overnight to insure proper infiltration. Next, the tissues were placed into BEEM polyethylene capsules which were then filled with Araldite resin and vacuum infiltrated for 1 hour. The capsules were placed into a 45°C oven for 24 hours, and then into a 60°C oven for an additional 24 hours. Autoradiograms were prepared from sections of tissues obtained from all 3 groups of animals, but quantitative grain counts were made on blocks from mice belonging to the first and third groups only.

Light Microscope Autoradiography

Thick sections (0.5 μm) were cut using an LKB Ultrotome III Ultramicrotome from each of the different tissue blocks. Five to ten sections from each tissue block were placed on a drop of double distilled water approximately 3 cm from the end of each glass slide. These slides were then placed onto a hot plate to evaporate the water and to properly affix the sections to the slide. The slides were placed into Coplin jars along with enough Harris' hematoxylin to cover the sections (25). The Coplin jars were then placed into a 60°C oven for 4 hours, after which the slides were rinsed in double distilled water to remove excess stain.

The entire emulsion applying procedure occurred in a darkroom which was illuminated with a safelight equipped

with a 25-watt bulb and a Kodak Wratten Series 2 filter (dark red). The safelight was located 4-5 feet above the working area.

Kodak NTB 2 emulsion was placed into a 43°C water bath for 35 minutes in order to thoroughly melt it. The slides, with thick sections affixed, were first dipped into double distilled water (43°C) and then, after the slides were freed of excess water, they were dipped into the melted emulsion for 1 second and withdrawn (8). The slides were placed in an upright position to dry in total darkness for 30-45 minutes. Once dry the slides were placed into black plastic slide boxes (10 slides/box) along with approximately 10 grams of drierite contained in a Kimwipe. The boxes were then taped shut with black plastic tape, placed into Kodak light-tight boxes, and put into the refrigerator (3°C) for a 3 week exposure period.

After 3 weeks the slide boxes were removed from the refrigerator and brought into a darkroom which was illuminated in a manner similar to that used during the emulsion coating procedure. Five slides at a time were placed into a staining dish slide carrier and processed through consecutive staining dishes which contained the following reagents:

Reagent*	Processing Time	Solution Temperature
Microdol X	3.0 min	23°C
2x distilled water	0.5 min	23°C
Kodak Rapid Fixer	2.0 min	23°C
2x distilled water	7.0-10.0 min	23°C
2x distilled water	7.0-10.0 min	23°C
2x distilled water	7.0-10.0 min	23°C

*The Kodak Rapid Fixer and the Microdol X were prepared immediately before use from double distilled water and the Microdol X was filtered. In addition, fresh solutions were used after processing every 25 slides.

After processing, the slides were allowed to dry and then were stored in slide boxes.

The labelled and unlabelled thick sections were viewed under the apo 100 oil immersion lens of a Zeiss Photomicroscope II. Many random pictures were taken of the labelled and unlabelled ascites cells at a magnification of 400x. The film was developed and the negatives were printed on 8" x 10" sheets of Kodachrome RC photographic paper. The final magnification of the labelled ascites cells was 4,360x. The silver grain distribution above the other labelled and unlabelled tissue was noted.

All labelled ascites cells which were completely visible on the light autoradiographs were used to compile data, except for those cells in which the nuclear or surface membrane was not visible, and those which did not contain any silver grains. Measurements were made from the center of each developed silver grain. The grains were measured from the nuclear envelope into the cytoplasm

in units of 0.5 μm and from the cell surface membrane both into the cytoplasm and into the intercellular space, again in units of 0.5 μm. All photopositive grains which were within 1.0 μm (2 average grain diameters) of the nuclear membrane were considered to have resulted from the tritiated thymidine incorporated into the DNA of the nucleus.

If a silver grain was located farther than 1.0 μm from the nuclear envelope, it was measured as a certain distance from the closest source; that is, either the nucleus or cell surface membrane. Grains which were located in the intercellular space were considered to have resulted from the closest cell surface membrane, provided that they were within a reasonable distance of that membrane (1.0 μm).

Area measurements were also made. The nuclear and plasma membrane outlines of 8 nearly circular cells were transferred to tracing paper from the light level autoradiographs. A line was then drawn around each nucleus at a distance of 4.0 mm (1.0 mm $\simeq$ 2,500 Å in actual cell distance), thus establishing a buffer area surrounding the nucleus. Lines were drawn both 1.0 mm inside of and outside of each plasma membrane, thus making a 2.0 mm band (band 3), which included the plasma membrane of each cell. Four additional bands (2.0 mm each) were then drawn: 2 inside of and 2 outside of band 3 (band 1 innermost--band 5 outermost). The lines defining bands 1

through 5 were approximated as circles, and the area of each band was calculated. Any intrusion of nuclear buffer area was subtracted from each band area. The ratio of these band areas was then established, and the grain number, corrected for area, was calculated for each band.

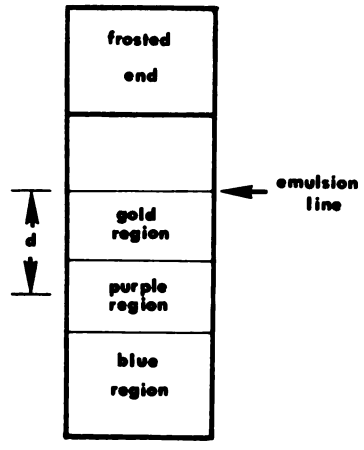
Electron Microscope Autoradiography

Pale gold sections (1,000 Å) were obtained from the same labelled and unlabelled blocks from which thick sections were taken. The sections were cut using an LKB Ultrotome III Ultramicrotome and then each section was transferred with a hair loop to a drop of double distilled water on a microscope slide, previously coated with a 0.4% solution of parlodion in isoamyl acetate. One to five sections were arranged along a line parallel to and approximately 3.5 cm from the frosted end of the slide. The sections were stained with a saturated solution of uranyl acetate for 1.5 hours and then carbon coated (50 Å) using a Hitachi HUS-4 vacuum evaporator.

The entire emulsion coating procedure was performed in a darkroom which was illuminated with a safelight equipped with a 25-watt bulb and a Kodak Wratten Series 1 filter (dark red) (9). The safelight was located 4-5 feet above the working area, and it was adjusted so as to allow only an indirect illumination of this area.

Four grams of Ilford L4 emulsion were weighed into a clean glass beaker along with 15 ml of double distilled

water. The beaker was placed into a 44°C water bath and the contents were mixed with occasional gentle stirring. After 20 minutes the mixture was cooled to room temperature in an ice bath. A parlodion-carbon coated test slide was then evenly coated with the emulsion using a disposable eyedropper. Most of the slide surface was coated short of the frosted end, and then the slide was placed in a vertical position and left to dry for 10 minutes. When dry the slide was examined under fluorescent light to determine the position of the purple colored region (monolayer of silver bromide crystals) with reference to the emulsion line (see diagram).



Distance "d", extending from the middle of the purple region to the emulsion line, was taken as a guide to coat subsequent slides with sections. In such cases the slides were coated with the emulsion from a distance "d" above the sections to the end of the slide. This procedure insured that a monolayer of emulsion was applied over the sections. After coating, the slides were left in a

vertical position to dry for 10-15 minutes, at which time they were prepared for a 5 week exposure period in a manner identical to that of the NTB 2 coated slides.

After 5 weeks the slides were developed (9). The slide boxes were removed from the refrigerator (3°C) and brought into the darkroom. Illumination of the working area was provided by the same safelight filter which was used for the coating procedure. Five slides at a time were removed from the slide boxes and placed into a staining dish slide carrier which was then processed through the following reagents:

Reagent*	Processing Time	Solution Temperature
Microdol X	2.5 min	23°C
2x distilled water	0.5 min	23°C
Kodak Rapid Fixer	2.0 min	23°C
2x distilled water	2.0 min	23°C
2x distilled water	2.0 min	23°C
2x distilled water	2.0 min	23°C

*The Kodak Rapid Fixer and the Microdol X were prepared immediately before use from double distilled water and the Microdol X was filtered. In addition, fresh solutions were used after processing every 25 slides.

The developed slides were allowed to dry in such a way as to protect them from dust contamination.

A razor blade was used to cut a relatively large circle around the sections. Hydrofluoric acid (5%) was then applied with a disposable plastic eyedropper in order to free the circular piece of film from the glass slide. The slide was slowly lowered into double distilled water

so as to cause the parlodion-emulsion film containing the sections to float free on the surface of the water. Copper grids (200 mesh) were placed on the film directly above the sections. Another slide, with masking tape attached to it to provide a rough surface, was brought directly above the grids and turned over while scooping the grids out of the water. By using this technique, the copper grids were now positioned underneath the sections. The slides were placed into a 45°C oven until dry, and then the grids were easily picked up with a forceps and stored in grid boxes.

The sections were viewed under a Hitachi HU-11E electron microscope operated at 75 kV. Random pictures were taken of the labelled ascites cells at a magnification of 6,000x. The negatives were printed on 8" x 10" sheets of Kodachrome RC photographic paper with a final magnification of 15,100x. Data were collected on a total of 139 labelled ascites cells. Unlabelled ascites cells, as well as labelled and unlabelled kidney, liver, and intestine cells, were observed under the electron microscope, and the silver grain distribution was recorded.

The electron microscope autoradiographs were analyzed in a manner similar to that of the light level autoradiographs except that the location of the silver grains was measured in units of 650 Å and grains within 5,200 Å of the nucleus were considered as having been caused by nuclear label.

Area measurements were obtained from 15 out of the 139 cells which were the basis for the electron microscope autoradiography results. The nuclear and plasma membrane outlines of the 15 cells were traced onto paper. A line was drawn around each nucleus at a distance of 8 mm (1.0 mm ≃ 650 Å in actual cell distance) so that a nuclear buffer area existed around each nucleus. Lines were then drawn at a distance of 1.5 mm both inside of and outside of each plasma membrane, thus establishing band 3, which included the plasma membrane of each cell. Bands 1 and 2 (3.0 mm each) were drawn on the inside of band 3, and bands 4 and 5 (3.0 mm each) were drawn on the outside of band 3. The bands were then cut out, making sure to exclude any nuclear buffer area which intruded into the bands. The bands were weighed in groups, the ratio of band areas was established, and the grain number, corrected for area differences, was calculated for each of the 5 bands.

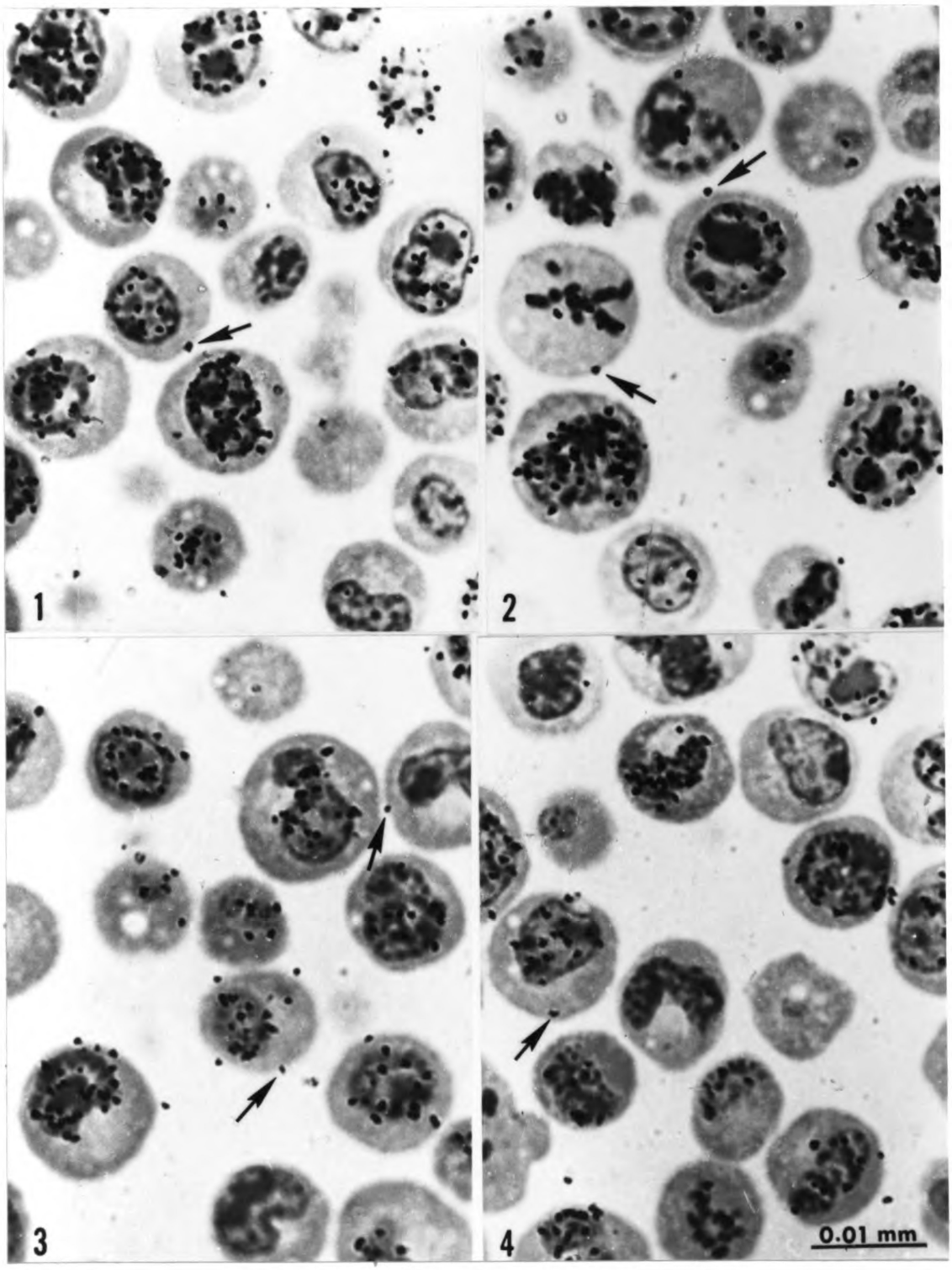
RESULTS

Examination of random autoradiograms from random thick sections of ascites sarcoma-180 cells show a localization of the silver grains mostly over the nucleus. Some grains also are found in the cytoplasmic area while others are present on the plasma membrane (Figures 1 through 4). Examination of random light microscope autoradiographs from such preparations led to the accumulation of data which were then condensed into a histogram (Figure 5). The histogram is based on 154 random cells showing a combined total of 2,822 silver grains. This number includes 1,750 nuclear grains which are not included in Figure 5. Most of the grains are localized within 1.0 μm of the nuclear membrane while fewer grains are found within the cytoplasm. The number of grains increases as one approaches the plasma membrane, where one can find a distinct peak. Again the number of grains decreases as the distance increases away from the plasma membrane into the intercellular space.

In order to estimate the background contribution to the data in Figure 5, the unlabelled ascites cells were examined for both silver grain number and distribution. Since both the labelled and unlabelled ascites cells were

Figures 1 through 4. Light microscope autoradiographs.

Light microscope autoradiographs of random sections of ascites sarcoma-180 cells labelled with tritiated thymidine (≃ 0.8 μCi/gm mouse body weight), fixed in glutaraldehyde (2%), and embedded in Araldite. Sections (0.5 μm) were stained with Harris' hematoxylin, coated with NTB 2 emulsion, and developed for 3 minutes in Microdol X after a 3 week exposure period. Note the cell surface membrane-associated silver grains (arrows). The marker in the lower right-hand corner of Figure 4 also pertains to the magnification of Figures 1, 2 and 3.



Figures 1-4

Figure 5. Histogram of the uncorrected light microscope results.

Histogram of the light microscope autoradiography results (uncorrected for band area differences) showing the distribution of silver grains superimposed over the ascites sarcoma-180 cells. The histogram is based on 154 cells which show a combined total of 2,822 silver grains, 1,750 of which are nuclear grains and are not included in this figure. Note that the silver grain number drops off steadily with increasing distance from the nuclear membrane. Note also the silver grain peak at the cell surface membrane.

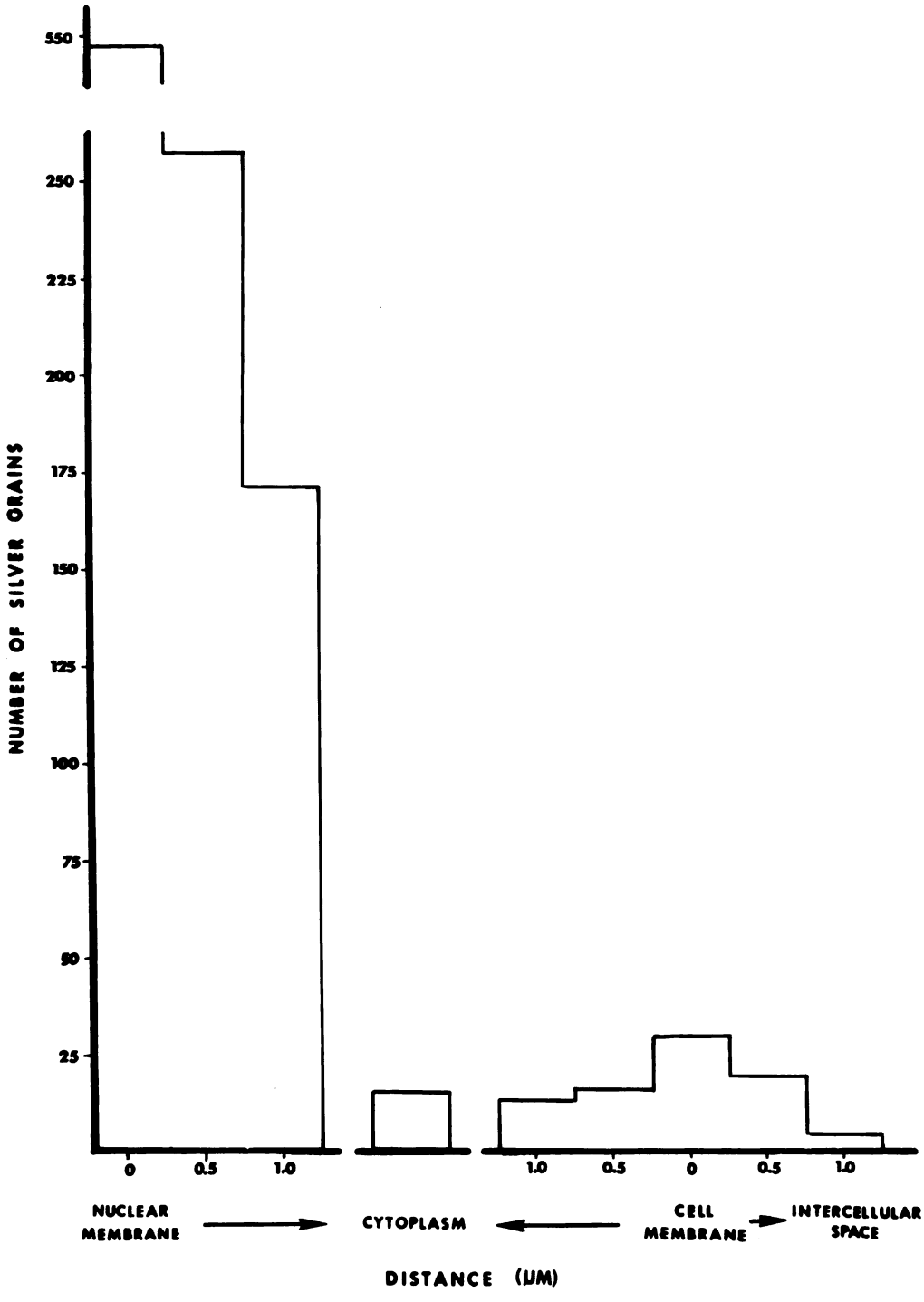

550
250
225
200
175
150
125
100
75
50
25
NUMBER OF SILVER GRAINS
0
0.5
1.0
1.0
0.5
0
0.5
1.0
NUCLEAR MEMBRANE
CYTOPLASM
CELL MEMBRANE
INTERCELLULAR SPACE
DISTANCE (µM)

Figure 5

handled in an identical manner, the silver grain content of the unlabelled tissue was used as an indication of the background grain number and distribution in the labelled tissue. Out of 242 unlabelled cells which were observed, only 17 randomly distributed cells were found to contain silver grains. Among these 17 cells 19 background grains were observed, including 5 over the nuclei, 10 in the cytoplasmic area, and 4 over the cell surface membrane. Since 19 background grains were observed among 242 unlabelled cells (1 background grain/13 cells), it may be interpreted that Figure 5, which was based on 154 cells, includes approximately 12 grains attributable to sources other than the label (^{3}H-thymidine). The surface membrane-associated silver grain numbers for the light microscope autoradiography, both uncorrected and corrected for band area differences, are given in Table 1.

Table 1. Cell surface membrane-associated silver grain numbers, both uncorrected and corrected for band area differences--light microscope autoradiography results

Band	1	2	3	4	5
Uncorrected silver grain numbers	13	16	29	19	4
Corrected silver grain numbers	13	8	10	5	1
Ratio of the band areas (area of band X/area of band 1)	1.0	1.9	2.8	3.5	4.0

For greater clarity and resolution, the above studies were repeated using electron microscope autoradiographic techniques. The distribution of silver grains was found to be mostly over the nucleus. Some grains were also found localized over the plasma membrane and in the cytoplasmic area (Figures 6 through 10), with few (approximately 24 grains) over the mitochondria (Figure 10).

Grain counts were made from random electron microscope autoradiographs and plotted into a histogram for meaningful interpretation (Figure 11). The histogram was structured from the results of 139 random ascites cells. A total of 3,299 silver grains were counted, of which 2,655 were found within the nuclear areas of the ascites cells, and thus were not included in Figure 11. As was observed in the light autoradiography results, the number of silver grains decreases as one moves away from the nuclear membrane, but then increases as one approaches the plasma membrane where one can find a peak. The number of grains then decreases as the distance increases away from the plasma membrane into the intercellular space.

Twenty-nine out of 330 unlabelled ascites cells were found to contain photopositive grains. Seventeen grains were found in nuclear regions, 20 grains were found superimposed over cytoplasmic areas, and no grains were located over the cell surface membranes. Thirty-seven background grains among 330 unlabelled cells represents approximately 1 grain/9 cells. Therefore, approximately 15 background

Figures 6 through 10. Electron microscope autoradiographs.

Electron microscope autoradiographs of ascites sarcoma-180 cells labelled with tritiated thymidine (≃ 0.8 μCi/gm mouse body weight), fixed in glutaraldehyde (2%), and embedded in Araldite. Thin sections (pale gold) were stained with a saturated solution of uranyl acetate and coated with Ilford L4 emulsion. Development in Microdol X for 2.5 minutes was followed by a 5 week exposure period. Note the cell surface membrane-associated silver grains.

Figure 6. Electron microscope autoradiograph.

Note that not all cells contain surface membrane-associated silver grains. Also note the lack of mitochondrial silver grains and the surface binding of platinum-thymine blue.

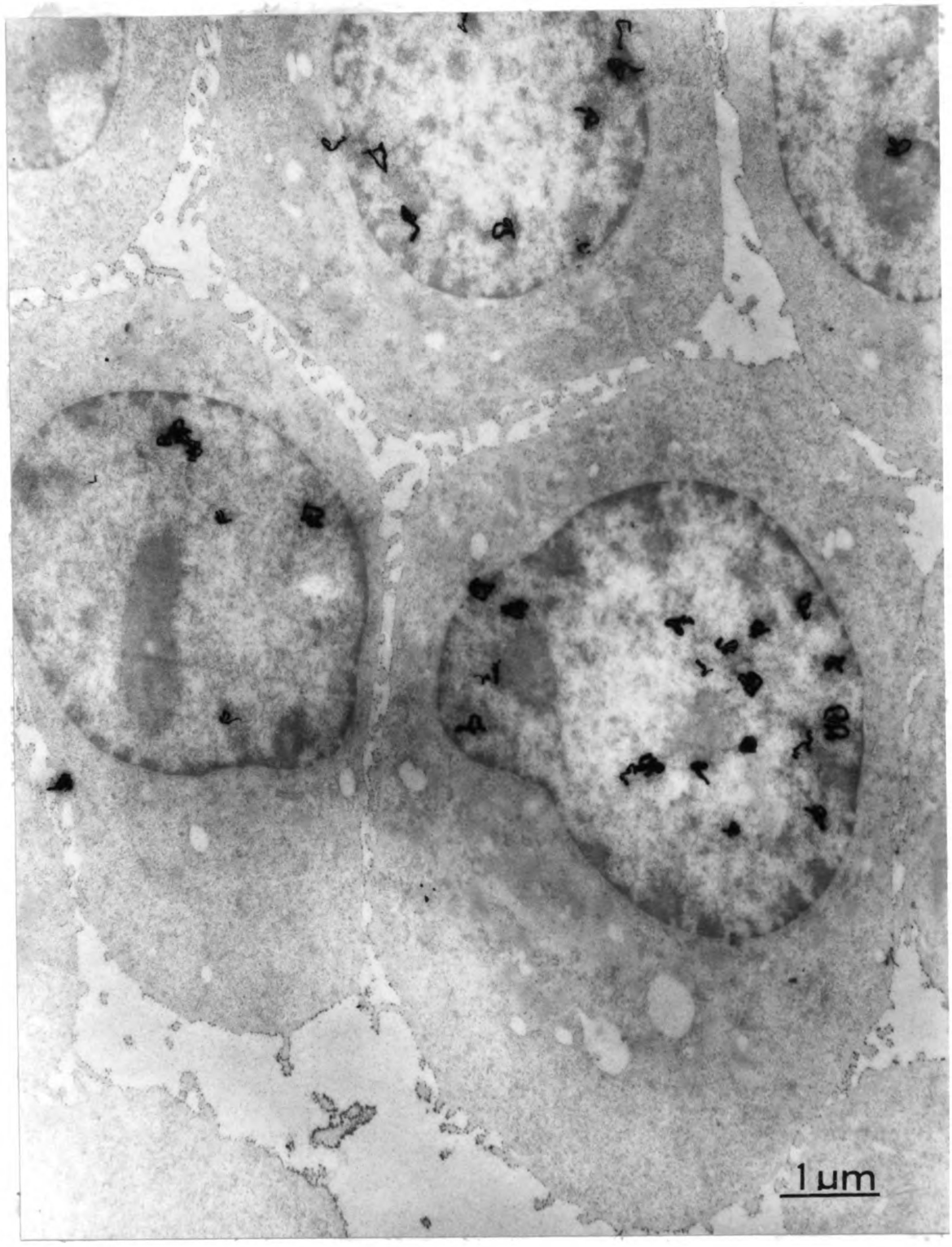


Figure 6

Figure 7. Electron microscope autoradiograph.

Note the presence of silver grains at positions somewhat removed from the nucleus (beta particle scatter). Many mitochondria are also visible; however, they do not show any silver grains.

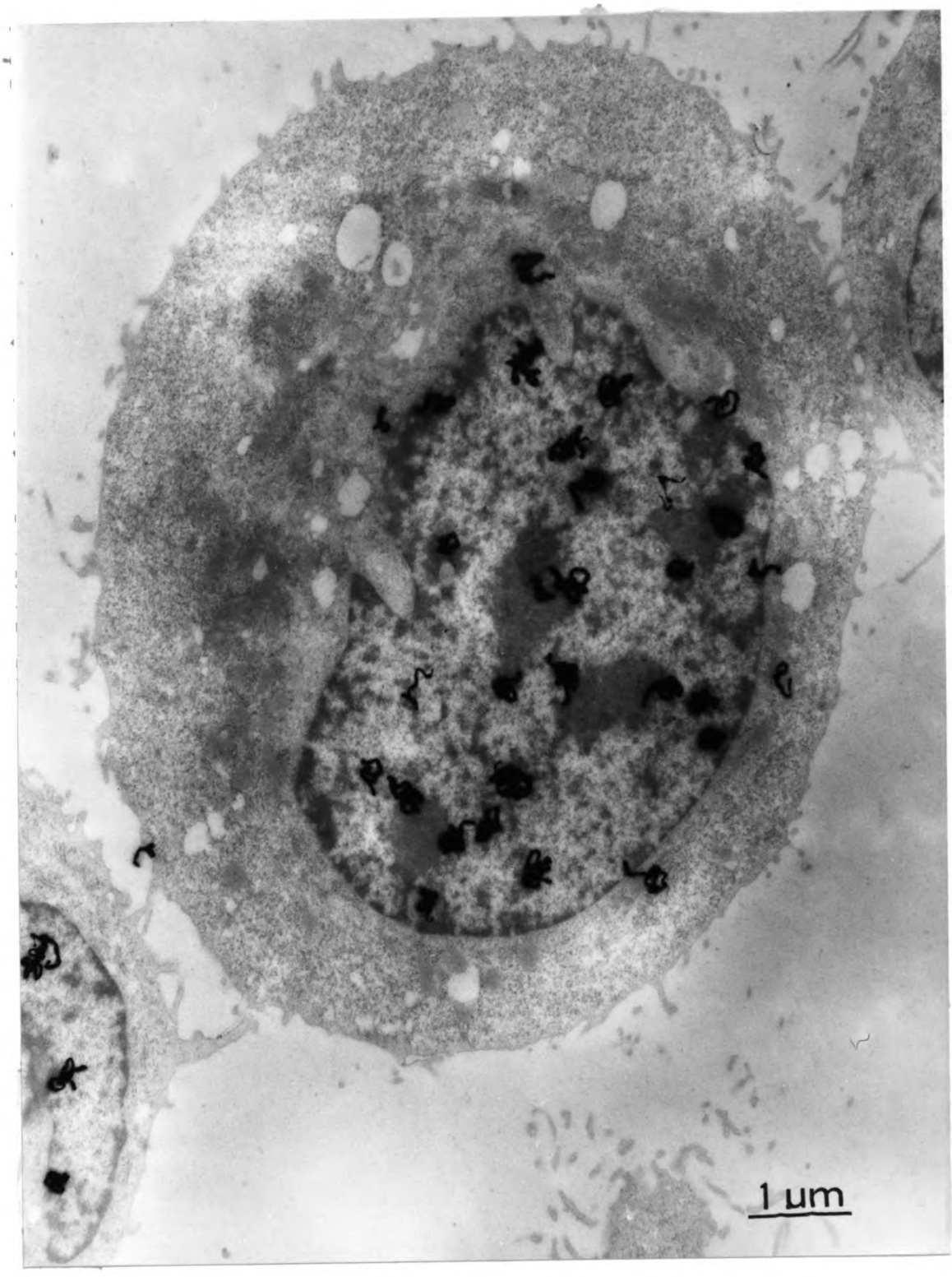


Figure 7

Figure 8. Electron microscope autoradiograph.

Note the general confinement of the silver grains to the nucleus except for the surface. The mitochondria do not show any grain development.

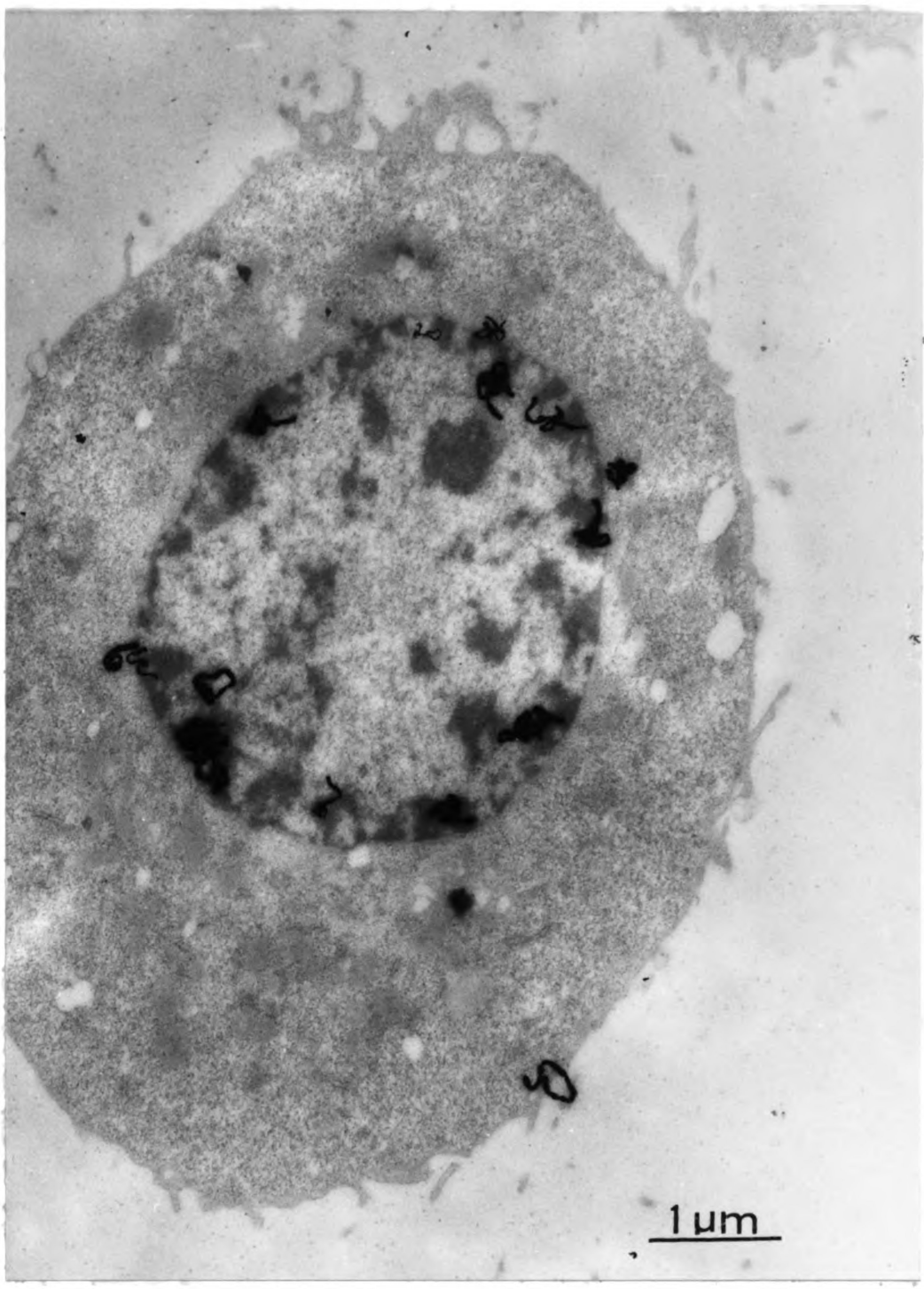


Figure 8

Figure 9. Electron microscope autoradiograph.

Note the platinum surface binding, the general confinement of the silver grains to the nucleus, and the lack of mitochondrial silver grains.

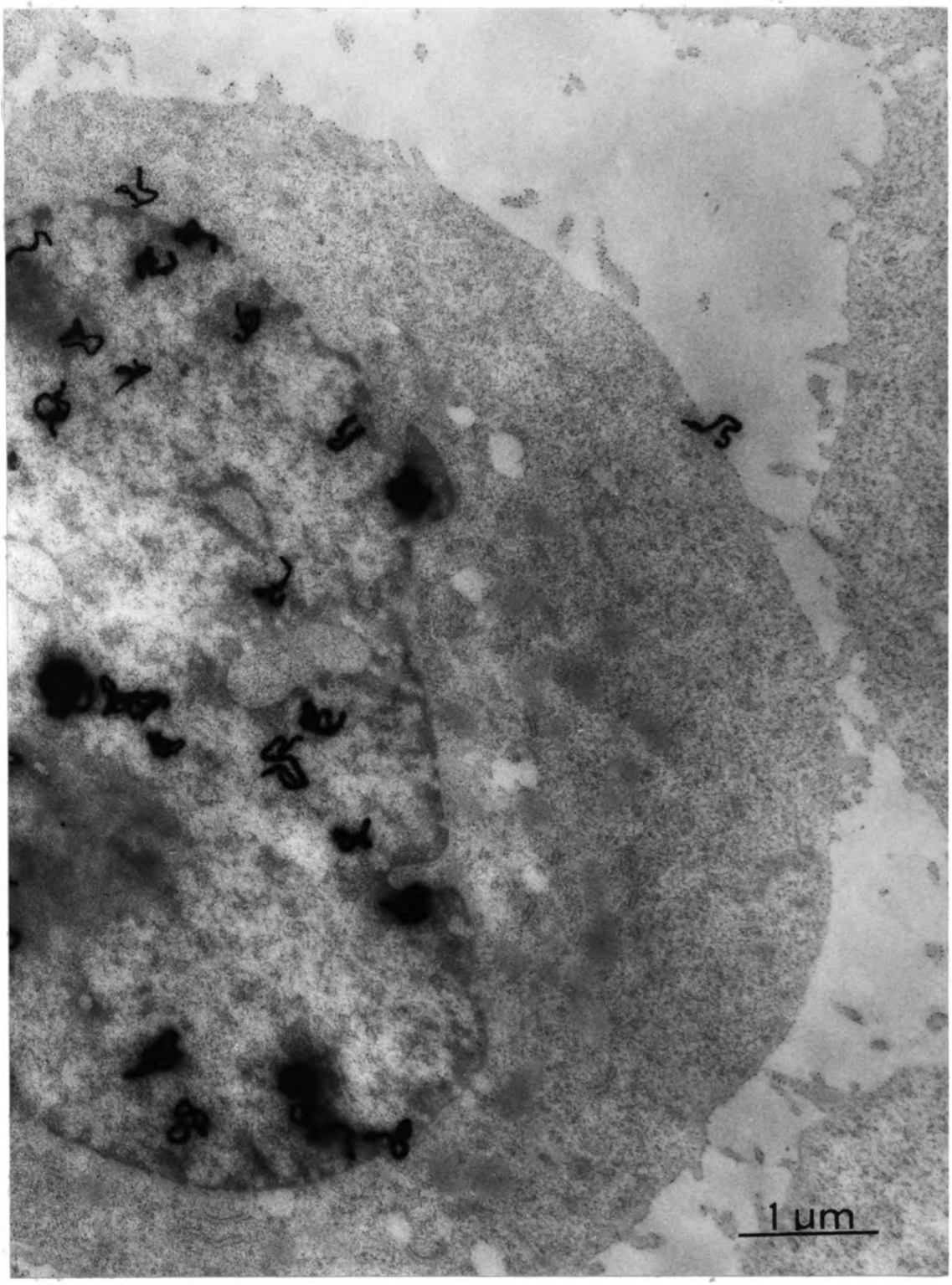


Figure 9

Figure 10. Electron microscope autoradiograph.

Note the prominent platinum-thymine surface binding and the cytoplasmic silver grain located above a mitochondrion.

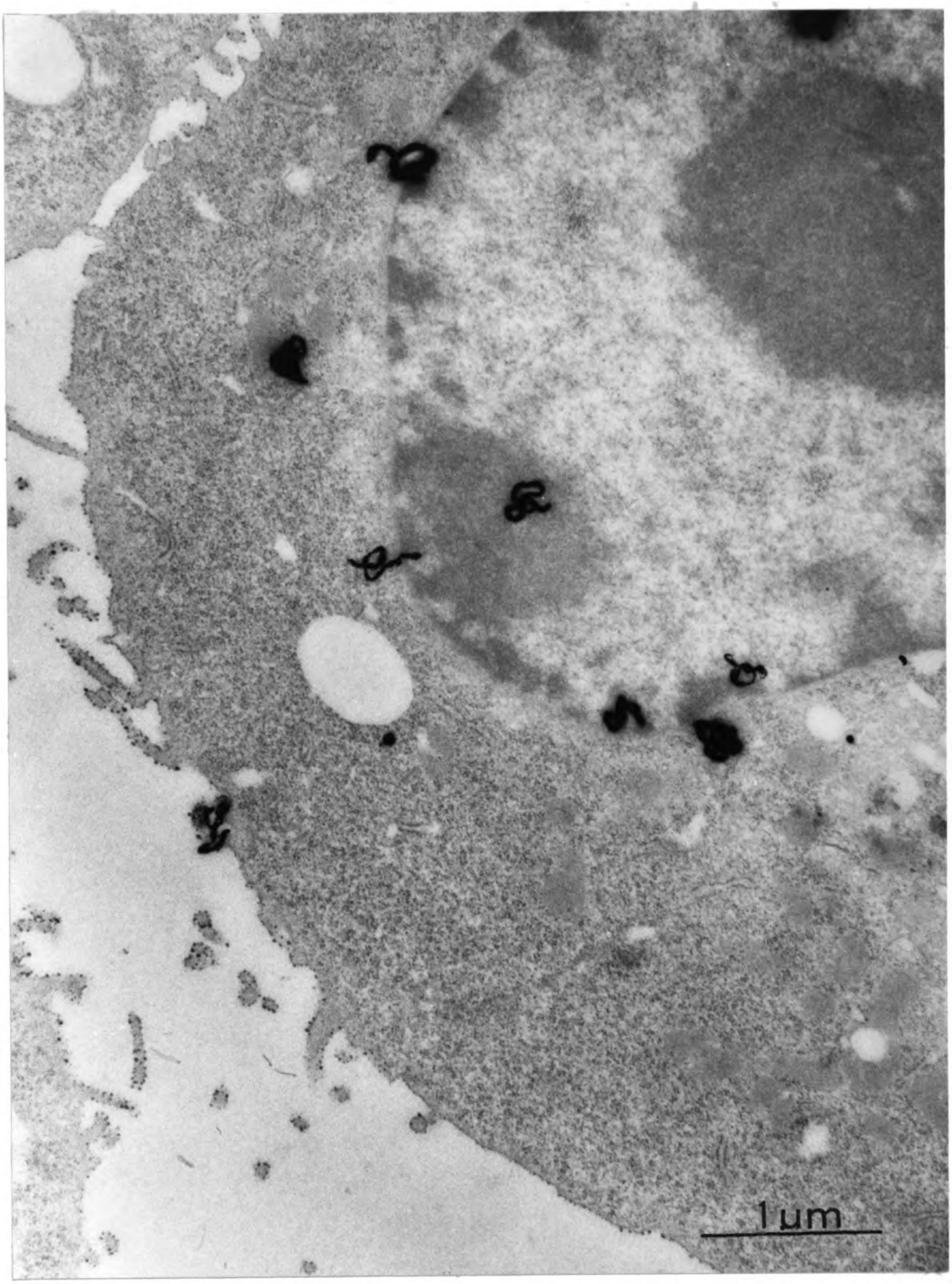


Figure 10

Figure 11. Histogram of the uncorrected electron microscope results.

Histogram of the electron microscope autoradiography results (uncorrected for band area differences) showing the distribution of silver grains superimposed over the ascites sarcoma-180 cells. The histogram is based on 139 random cells which contain a combined total of 3,299 silver grains, 2,655 of which are over the nuclear areas and are not included in this figure. Note that the silver grain number drops off with increasing distance from the nuclear membrane but peaks again at the cell surface membrane.

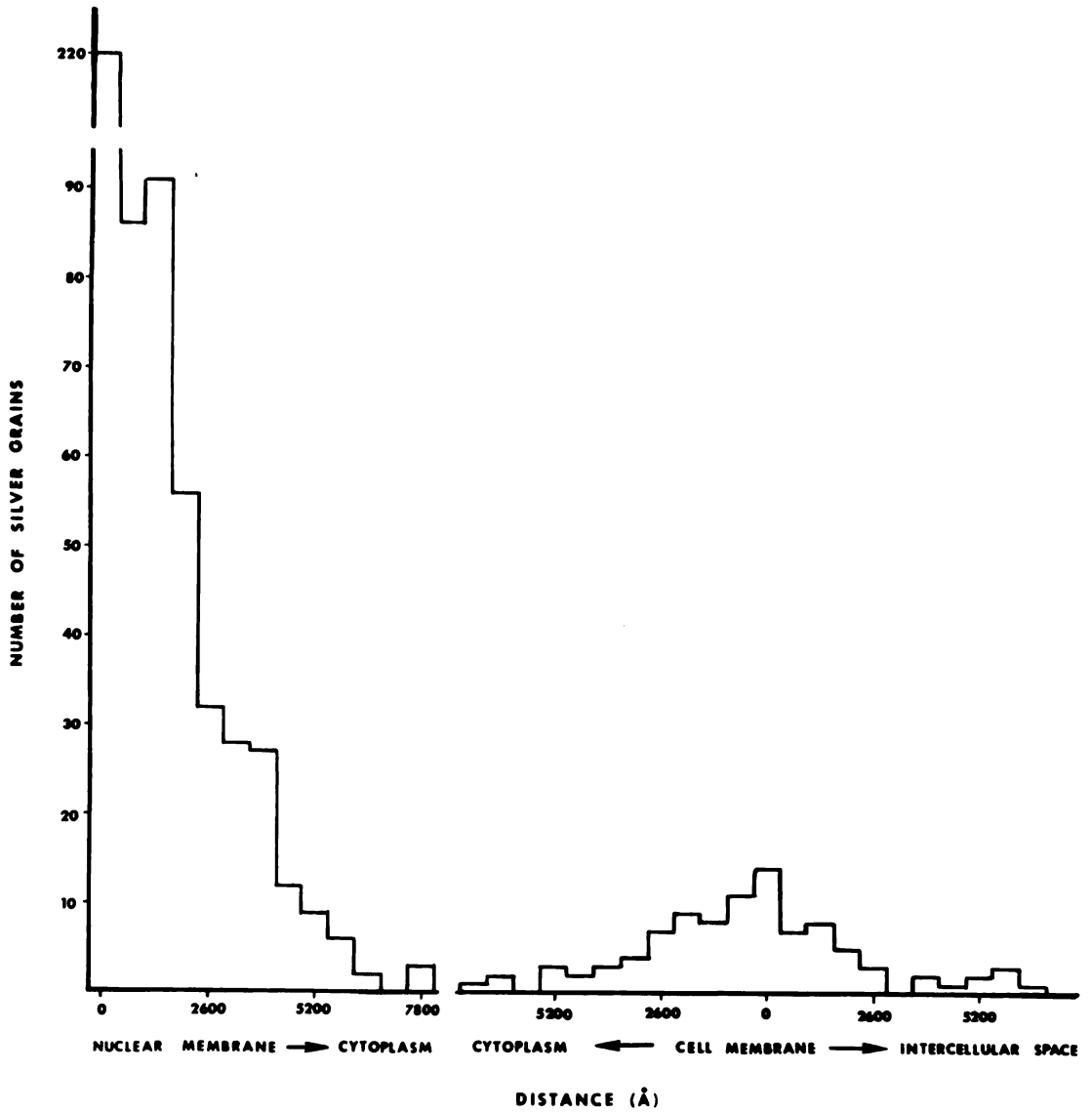

220
90
80
70
60
50
40
30
20
10
NUMBER OF SILVER GRAINS
0
2600
5200
7800
5200
2600
0
2600
5200
NUCLEAR MEMBRANE → CYTOPLASM
CYTOPLASM ← CELL MEMBRANE → INTERCELLULAR SPACE
DISTANCE (Å)

Figure 11

grains are included in the data represented by Figure 11. The surface membrane-associated silver grain numbers for the electron microscope autoradiography, both uncorrected and corrected for band area differences, are given in Table 2.

Table 2. Cell surface membrane-associated silver grain numbers, both uncorrected and corrected for band area differences--electron microscope autoradiography results

Band	1	2	3	4	5
Uncorrected silver grain numbers	9	24	32	16	3
Corrected silver grain numbers	9	21	25	12	2
Ratio of the band areas (area of band X/area of band 1)	1.0	1.15	1.28	1.36	1.5

Few silver grains (approximately 24) were observed directly above the mitochondria in the electron microscope autoradiographs of the ascites cells (Figure 10). Many cases were observed in which whole fields of mitochondria did not show any silver grains (Figures 6 through 9).

Examination of the labelled kidney, liver, and intestine tissue at both the light and electron microscope level revealed silver grain numbers which were not meaningfully greater than those of the unlabelled kidney, liver, and intestine cells. The vast majority of these supposedly labelled cells did not show any silver grains. Since the

labelled tissue did not contain many grains above the expected background number, it was impossible to collect any meaningful data from these nontumorigenic control tissues.

The cell surface binding of the ascites cells by the platinum-thymine compound took a different form from that which had been observed earlier (2); that is, mostly in the form of dense patches. The present form consisted of numerous fine platinum granules which were observed everywhere along the cell surface membrane (Figures 6, 9 and 10). Neither the dense platinum patches nor the platinum granules were found on the cell surface membrane of the intestine cells which were observed.

It should be pointed out that although no actual grain counts were made from the autoradiograms prepared from the second group of animals, surface membrane-associated silver grains were observed in connection with the ascites cells obtained from these animals. It should also be pointed out that there were few mitochondrial silver grains observed, and the platinum surface binding took the same form as that observed in ascites cells taken from the first group of animals.

DISCUSSION

During the present investigation autoradiographic evidence for the association of DNA with the plasma membrane of ascites sarcoma-180 cells has been demonstrated by making use of the specificity of ^{3}H-thymidine for DNA. Evidence of cell surface membrane-associated DNA is by no means new or unique since other researchers have demonstrated its presence in connection with many kinds of cells (2,12,14,18). For example, Kakefuda et al. (14) have demonstrated the presence of DNA in the region of the plasma membrane in 3T3/BALB and chicken embryo cells, and Lerner et al. (18) have demonstrated the association of DNA with the plasma membrane of diploid human lymphocytes.

Autoradiography, as an experimental technique, is hampered by two problems. The first problem is concerned with background grain development and the second with radiation scatter. If the proper precautions, controls and considerations are made, both of these problems can usually be surmounted and meaningful results can be obtained.

The background grain estimation for the present study, which was derived from the unlabelled ascites

cells, was found to be so small (0.43% of the light microscope level results and 0.45% of the electron microscope level results) and evenly distributed that it was considered to be negligible. Mitochondrial grains were also considered negligible in view of the fact that few (approximately 24 grains) were observed in the electron microscope autoradiographs.

Due to beta particle scatter within the distance between the source and the emulsion, it is possible to have silver grains develop at a distance from any labelled source. Therefore in the present study, the possibility arose of having cell surface-associated silver grains which could have resulted from labelled DNA within the nucleus. This possibility was considered when the grain measurements were made. No cell surface-associated grain was counted unless it was greater than a certain distance (1 μm light microscope level; 5,200 Å electron microscope level) away from the nuclear membrane.

Light and electron microscope level results concerning the presence of surface-associated DNA could not be taken directly from the histograms. Since each band of the histogram represented a portion of cellular area, and since these areas were not equal, corrections were necessary. The ratio of the band areas was determined and corrections were made for both the light and electron microscope results. The corrected light microscope level results do not show a distinct peak at the surface

membrane. These numbers are consistent with a large amount of beta particle scatter from the nucleus into the plasma membrane region since they proceed from a high point inside the plasma membrane to a low point outside the membrane. Due to the thickness of the sections and emulsion used to obtain the light microscope level results, it is very likely that the nuclear buffer region was not large enough to be effective against beta particle scatter from the nucleus into the plasma membrane region. If the buffer region were increased in an attempt to make it more effective, however, the region would nearly encompass the entire cell. Therefore, light microscope autoradiography is not a good method to use to study the presence of cell surface DNA. The light microscope level results obtained from this study are therefore inconclusive; that is, they do not prove or disprove the presence of surface membrane-associated DNA.

The silver grain numbers, corrected for band area differences, obtained from the electron microscope level results show a membrane-associated peak. This silver grain peak demonstrates the presence of cell surface membrane-associated DNA. The more gradual drop-off on the cytoplasmic side of the plasma membrane could be attributed to mitochondrial silver grains. Since the section and emulsion are not nearly so thick in electron microscope autoradiography as in light microscope autoradiography, beta particle scatter does not present as

serious a problem. Thus, the presence of any surface DNA would not be masked by beta particle scatter from the nucleus.

Although the present study demonstrates the presence of membrane-associated DNA, the source remains unknown. Many possibilities can be considered. One source could have involved lysed cells. Labelled DNA from the nucleus of lysed cells could have attached to the cell surface membrane of nearby cells and caused silver grain development at that position. This possibility does not seem likely, however, since an observation of the ascites cells revealed an absence of lysed cells. Furthermore, Anker et al. (3), in their work with human lymphocytes, found that the spontaneous release of DNA from these cells was not due to dead or dying cells since the cell death rate had no effect on the amount of released DNA.

Another source of the membrane-associated DNA could have been virus particles, in view of the fact that many virus particles have been associated with various kinds of tumorigenic cells (13). Some RNA viruses have been shown to contain reverse transcriptase (4,27,29). Kakefuda et al. (14) have infected 3T3/BALB and chicken embryo cells with either murine sarcoma or Rous sarcoma virus particles and have demonstrated by electron microscope autoradiography the reverse transcription of the viral genome at the cell surface membrane.

Although Sodhi and Aggarwal (26) have found virus particles associated with the solid sarcoma-180 tumor, no virus particles were observed during the present study, and no proof of a viral origin is available for the ascites sarcoma-180 tumor (24). Therefore, although it would seem that the cell surface DNA was not associated with virus particles, the possibility that these cells carry a latent viral genome cannot be completely discounted on the basis of the present study.

The release of nuclear DNA seems to be a somewhat general phenomenon occurring in eucaryotic cells since many eucaryotes have been shown to transport their nuclear DNA into the cytoplasm (5,15,16,17), to the plasma membrane (12,18), and into the surrounding medium (3,7,11, 20,21,22). The third possible source of the surface membrane-associated DNA found in this study may have been the release of DNA from the nucleus.

Koch and Pfeil (17) have found a unique class of cytoplasmic DNA in many of the animal cells which they have studied. They have found that both the complexity and labelling kinetics of this cytoplasmic DNA suggest a nuclear origin. Bell (5) has also found cytoplasmic DNA in eucaryotic cells and has suggested that this DNA may be an intermediate in the transfer of information from the nucleus into the cytoplasm.

Lerner et al. (18) and Hall et al. (12), using cell fractionation and membrane isolation techniques, have

demonstrated surface membrane-associated DNA in human lymphocytes. Hall et al. (12) have used 5-bromodeoxyuridine to investigate both the time and place of the synthesis of the membrane-associated DNA and have found that this DNA is synthesized in the nucleus during the S growth phase and then is transported to the plasma membrane.

Eng and Morgan (11) have demonstrated the release of nucleic acids and proteins from both Ehrlich and 6C3HED ascites tumor cells incubated with isotonic solutions of either glycerol, dimethylsulfoxide, or inositol. This release occurred without significant cell disruption, and DNA was believed to comprise approximately 20% of the released nucleic acids. It was suggested that the released nucleic acids may have originated from the nucleus or mitochondria. When Anker et al. (3) incubated human blood lymphocytes *in vitro*, they discovered DNA in the medium. Although they are unsure of the exact origin of this released DNA, they believe it may represent extra copies of part of the genome which, once used for transcription or regulation, may move to the membrane and then be excreted as a cell to cell transfer of genetic information. A similar phenomenon may be involved in the ascites sarcoma-180 cells, but such an occurrence still needs to be proven.

The function of this DNA is also open to speculation since the results of the present study do not offer any

detailed information regarding function. It is possible that the surface-associated DNA acts in the transfer of genetic information between cells. This informational DNA could then spread to other cells through direct cell-cell contact, or it could be released into the medium and picked up by other cells. Once this DNA has spread to a new cell, it could transform it and thus perpetuate the tumor growth (6). This would only be possible if the DNA retains its biological activity during transport. Bendich et al. (6) have shown that tumorigenic DNA does indeed retain its biological activity after being circulated in the blood stream. Therefore, it seems possible that DNA, in a biologically active form, can be transferred to other cells.

Yet another possible function of the surface-associated DNA found in connection with tumorigenic cells has been proposed by Rosenberg (23). Antigens generated at the cell surface of tumor cells after transformation can be detected by the host immune system, and the cells are then presumably destroyed. The tumor cells, however, survive, and Rosenberg has suggested that the surface-associated DNA is important to their survival since DNA is a weak antigen and can mask the stronger antigens produced by the tumor cell. The role of the surface-associated DNA may be different in the case of lymphocytes (18).

Platinum cell surface binding was also used in the present study and was intended to be complimentary to the autoradiography technique since Aggarwal et al. (2) have indicated the possibility that the platinum compound binds to cell surface-associated DNA. The numerous cell surface platinum granules observed in this study are very much different from the heavy dense patches which have been observed earlier on the cell surface membrane of ascites sarcoma-180 cells (2). The reason for this change is not immediately clear since the tissue handling procedures were identical. A possible explanation may be linked to the preparation of this chemically ill-defined platinum compound. The compound used for the present study was prepared commercially, whereas previously the compound was prepared in the laboratory of Dr. Barnett Rosenberg, Department of Biophysics, Michigan State University.

There was not a strong correlation between the platinum surface granules and the surface-associated silver grains. This lack of a strong correlation could be consistent with an infrequent DNA replication period since the opportunity for ^{3}H-thymidine incorporation would be small. It is also possible that the platinum compound does not bind to the surface-associated DNA but is bound to the surface due to some other factor.

Rosenberg (23), however, believes that the platinum-thymine compound may indeed bind to surface DNA. In doing so, he believes, it interferes with the ability of the DNA

to mask the tumor cell antigens, thereby enabling the immune system to destroy the tumor cells.

In connection with the platinum-thymine compound, it is interesting to note that the ascites cells showed surface binding whereas the intestine cells did not. This finding is consistent with indications that platinum-thymine is specific for tumor cells (2).

In conclusion, this study has demonstrated the presence of cell surface-associated DNA in ascites sarcoma-180 cells and brings up questions which cannot be answered from the results of the present study. It would be of interest to determine the quantity, source, and function of this DNA. Answers to these questions may be significant in providing information regarding the nature of tumor systems. Such information is necessary to develop methods for combatting tumor development.

SUMMARY

The presence of nucleic acids on the surface membrane of tumorigenic cells has been indicated using platinum-pyrimidine complexes as electron dense stains. The complexes stain nucleic acid-containing structures such as chromatin, ribosomes and the nucleolus. In tumorigenic cells platinum complexes have been shown to bind in the form of electron dense patches at the cell surface. Pretreatment with DNase removes these surface patches and thus indicates the DNA nature of these patches.

In an attempt to show that the platinum-pyrimidine complexes do indeed bind to DNA at the cell surface membrane of tumorigenic cells, the present study made use of light and electron microscope autoradiographic methods. Ascites sarcoma-180 cells, labelled by intraperitoneal injection of ^{3}H-thymidine into Swiss Webster mice (≃ 0.8 μCi/gm mouse body weight), were removed from the mice, fixed, embedded, and sectioned. Sections were coated with NTB 2 (light microscope autoradiography) or Ilford L4 (electron microscope autoradiography) emulsion and were developed in Microdol X after an exposure period of 3 weeks (light microscope autoradiograms) or 5 weeks (electron microscope autoradiograms). Histograms (silver

grain number versus silver grain location) were made from the data obtained from the autoradiographs.

The results of this study indicate the presence of cell surface-associated DNA. Since the number of silver grains does not closely match the amount of platinum binding, it would seem that either the surface DNA has an infrequent replication time or the platinum compound (platinum-thymine blue) is bound to the cell surface membrane through some factor other than DNA. The results of this study do not allow one to form conclusions on the source or function of the surface-associated DNA; nevertheless, speculations can be made on the basis of work found in the literature. Possible sources include virus particles or the cell nucleus since it has been shown that some cells release nuclear DNA. Possible functions include cell to cell transfer of genetic information or masking of strong antigens present on the cell surface thus enabling tumorigenic cells to escape destruction by the host immune system.

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