FINE STRUCTURAL ANALYSIS OF SARCOMA-180 BEFORE AND AFTER cis-DICHLORODIAMMINEPLATINUM(II) IN SWISS WHITE MICE, IN VIVO AND IN VITRO STUDIES

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
AJIT SODHI
1973

This is to certify that the

thesis entitled

FINE STRUCTURAL ANALYSIS OF SARCOMA-180 BEFORE AND AFTER cis-DICHLORODIAMMINE PLATINUM (II) IN SWISS WHITE MICE, IN VIVO AND IN VITRO STUDIES.

presented by

AJIT SODHI

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Zoology

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Date June 1, 1973

O-7639

ABSTRACT

FINE STRUCTURAL ANALYSIS OF SARCOMA-180 BEFORE AND AFTER cis-DICHLORODIAMMINEPLATINUM(II) IN SWISS WHITE MICE, IN VIVO AND IN VITRO STUDIES

By

Ajit Sodhi

Fine structural studies of Sarcoma-180 show four distinct cell types within the cortical, intermediate and the central regions of the tumor. Long spindle-shaped cells are most common in the cortical region, showing bloated rough endoplasmic reticulum cisternae, clusters of ribosomes and free fibrils concentrated under the plasma membrane. The mononucleate, principal tumor cells in the cortical and the intermediate regions are the dark and the light cells. Dark cells are the smaller of the two and show intense cytoplasmic basophilia in the form of free ribosomes. The light cells in the cortical region show a dense nucleus, well developed rough ER and dense mitochondria, while in the intermediate region the nucleus is usually pale and the mitochondria are less developed with ill defined cristae.

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Wandering leukocytes have multilobed highly electron dense nucleus and a large number of lysosomal bodies, mostly concentrated in the central region. Embedded within the cytoplasm of the leukocytes can be found certain 225-250Å electron dense particles. Cytochemical studies demonstrate the presence of ribonucleoproteins in addition to glycogen within these particles.

Two types of virus particles have been found associated with the cytoplasmic membranes and the nucleus of the various tumor cells. The nucleus associated particles measure about 425Å in diameter while the cytoplasmic membrane associated particles are about 900Å in diameter.

A single intraperitoneal injection of cis-dichlorodiammineplatinum(II) (cis-Pt-II) in a dose of 8mg/kg of the body weight causes complete regression of the Sarcoma-180 solid tumor in Swiss white mice. Fine structural studies after cis-Pt-II treatment show certain morphological changes indicative of the possible mechanism involved in the tumor regression. The mitotic activity of tumor cells is inhibited soon after platinum injection. The appearance of plasma cells, lymphocytes and macrophages within the regressing tumor mass after a 4-6 day period of platinum treatment indicates a possible enhancement of the cellular immune system against S-180 cells and increase in the pyroninphilic cells in the spleens of mice with regressing tumor also suggests the above hypothesis of the

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enhancement of the host immune system. Serum analysis on gel-electrophoresis show a marked enhancement of the bands in α_1 and α_2 regions of globulins.

About 4 days after platinum treatment giant cells with well developed Golgi apparatus and rough endoplasmic reticulum (RER) can be observed within the mass of the tumor. All of them have a single nucleus, but a few electron micrographs may show 3-4 nuclei. Such giant cells, as well as the regular tumor cells after platinum treatment, produce large numbers of lipid globules within the cytoplasm. These lipid globules grow by fusion of smaller globules and ultimately fills the cell pushing the nucleus to one side.

Treatment of Sarcoma-180 ascites cells <u>in vitro</u> with the platinum compound (2μg/ml) also stops the mitotic activity of the cells. The apparent cytototoxic effects can be easily observed after 15 minutes of such treatment, i.e., the formation of a perinuclear band made up of microfilaments 50-60Å thick, originating from Golgi area. Treatment of one hour causes the cytoplasmic organelles to start moving closer to the nucleus, thus is very pronounced after 2 hours when the mitochondria also show a very irregular appearance. The cells recover their usual morphology if allowed to grow on normal medium after a platinum shock of about 2 hours.

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Thus it appears that the cis-Pt-II in therapeutic dose is not cytotoxic enough to kill the S-180 cells in vitro, but it stops the mitotic activity both in vitro and in vivo. Subsequently the host immune system is enhanced and made effective against S-180 cells before the mitotic activity comes back.

FINE STRUCTURAL ANALYSIS OF SARCOMA-180 BEFORE AND AFTER *cis*-DICHLORODIAMMINEPLATINUM(II) IN SWISS WHITE MICE, *IN VIVO* AND *IN VITRO* STUDIES

By

Ajit Sodhi

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Zoology

1973

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ACKNOWLEDGMENTS

I would like to express my thanks to Dr. S. K. Aggarwal for his academic advice as a major professor and providing me with the laboratory facilities to pursue my graduate work.

Thanks are also extended to Dr. J. R. Shaver, Dr. B. Rosenberg, and Dr. W. Tai, for their assistance as committee members.

I sincerely appreciate the love, affection and encouragement given to me by Carol, Ginny, Paul and Vicki during the last months of the thesis preparation.

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LIST OF ABBREVIATIONS

Alb	Albumin
C	collagen
Ch	chromosomes
cis-Pt II	cis-dichlorodiammineplatinum(II)
C V	cytoplasmic vesicle
d	electron dense tumor cell
e·r	endoplasmic reticulum
F	fibroblast
G	Golgi
Gl	glycogen particles
L	lipid globules
Leu	leukocyte
Ly	lysosome
Lym	lymphocyte
M	macrophage
m	mitochondria
mb	midbody
mf	microfilaments
mt	microtubules
n	nucleus

o·n	outer nuclear membrane
P	plasma membrane
PC	plasma cell
RER	rough endoplasmic reticulum
Sa	tumor cell

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INTRODUCTION

Certain platinum compounds completely but reversibly inhibit cell division in Gram-negative rods, but does not inhibit the growth of the bacteria resulting in long filamentous growth (Rosenberg, VanCamp, and Krigas, 1965; Rosenberg, Renshaw, VanCamp, Hartwick, and Drobnik, 1967; and Rosenberg, VanCamp, Grimlley, and Thomson, 1967). Renshaw and Thomson (1967) showed by using ^{191}Pt. that the platinum metal is associated with metabolic intermediates, nucleic acids, and cytoplasmic proteins in the filamentous cells, whereas in inhibited cells, the platinum is combined only with the cytoplasmic proteins. A striking reduction in the rate of development of Sarcoma-180 and leukemia L1210 in mice is effected by a number of platinum compounds, the most active one thus far being cis-dichlorodiammineplatinum(II) (cis-$[Pt(NH_3)_2Cl_2]$) (Rosenberg, VanCamp, Trosko, and Mansour, 1969). More recently Rosenberg and VanCamp (1970) reported that both cis-dichlorodiammineplatinum(II) and cis-Pt(IV) diammine-tetrachloride when injected into mice with large solid Sarcoma-180 tumors can cause successful complete

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regression of these large tumors in 73% to 100% of the animals, with no apparent irreversible damage, in a number of dose schedules. After a period of 4 months, the cured animals did not show any signs of a resurgence of the tumor. Further studies indicated that the high success rate is due to giving the platinum injection based upon individual mouse weight, rather than average value from the cage weight. This was necessary because of the very steep slope in the dose response curve. From their studies Rosenberg and VanCamp (1970) have shown that the most effective dose of cis-dichlorodiammineplatinum(II) is a single i.p injection of 8mg/kg of the body weight of mouse given on 8th day of the tumor implant.

Richard, Sleight and Rosenberg (1970) have shown the inhibition of Dunning ascites leukemia and Walker 256 carcino-sarcoma with cis-dichlorodiammineplatinum(II). A single i.p injection of 2 to 4mg/kg of body weight of cis-dichlorodiammineplatinum(II) on day 1 inhibits the development of Dunning leukemia. A single treatment delayed until day 4 or 7 was sufficient to arrest tumor development and thus resulting into extended survival time. Paracentesis of the peritoneal cavity failed to demonstrate any ascitic fluid or Dunning leukemia cells in any of the day 30 survivors. A challenge dose of Dunning leukemia cells killed all the control rats, whereas it failed to develop in the platinum-treated cured rats.

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Day 3 treatment of intramuscular Walker 256 tumor with a single injection of either 4mg/kg or 2mg/kg of cis-dichlorodiammineplatinum(II) caused a significant regression of tumor development by day 7. Delayed treatment on day 7 also caused a significant regression of tumor development and extended the survival time. Welsch (1971) showed inhibition of growth of rat mammary carcinoma with cis-dichlorodiammineplatinum(II). His investigations showed the above platinum compound to be effective in promoting regression of DMBA-induced mammary tumors in female Sprague-Dawley rats. The dose schedule of cis-dichlorodiammineplatinum(II) most significant in inhibiting mammary tumor growth was 6mg/kg of body weight administered once or 4mg/kg of body weight administered thrice, every 12 days. The former dose was more efficacious than the latter. Pretreatment of DMBA-induced mammary tumor bearing rats with Cytoxan (50mg/kg body weight) markedly enhanced the effectiveness of the platinum compound. Cis-dichlorodiammineplatinum(II) inhibits preferentially and persistently DNA synthesis in Ehrlich ascites tumor cells in vivo (Howle and Gale, 1970), whereas inhibition of RNA and protein synthesis is reversible and less effective. The inhibitory effects of some of the platinum compounds on DNA, RNA, and protein synthesis have been shown by Harder and Rosenberg (1970) in the case of mammalian cells

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in vitro, while the effect of *cis*-dichlorodiammine-platinum(II) on bone marrow cells has been reported by Zak, Drobnik, and Rezny (1972).

According to Harder and Rosenberg (1970) inhibition of DNA synthesis by *cis*-dichlorodiammineplatinum(II) is dose dependent. This inhibition occurs at a concentration of 25μM, which is approximately equivalent to the best therapeutic dose (8mg/kg) for Sarcoma-180 treatment in mice. Although RNA and protein synthesis are also inhibited at 25μM with *cis*-dichlorodiammineplatinum(II), DNA synthesis is selectively inhibited at concentrations of less than 5μM, and is preferentially inhibited at 25μM. These results suggest that DNA synthesis is the primary target of the effective platinum compounds in human AV_3 cells and that the inhibition of RNA and protein synthesis is a secondary effect at higher concentrations. The inhibition of DNA synthesis in XC rat sarcoma cells have also been shown to be irreversible for at least 3 days after the treatment with platinum (Kara, Svoboda, and Drobnik, 1971).

In contrast to hydroxyurea, which is also a tumor inhibitor and induces filamentous growth in *E.coli*, platinum compounds inhibit DNA synthesis at a very slow rate in the therapeutic dose range. For example, at 1mM concentration of hydroxyurea, DNA synthesis in HeLa S-3 cells is reduced to about 10% of the control in 2 hours

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(Kim et al., 1967), whereas in Harder and Rosenberg (1970) studies DNA synthesis is still about 82% of the control in human amnion AV_3 cells treated 2 hours with 25μM of cis-dichlorodiammineplatinum(II). Furthermore, hydroxyurea does not inhibit RNA or protein synthesis in vitro at 1mM (Pfeiffer and Tolmach, 1967; Yarbro, 1968; Young et al., 1967) as do the platinum compounds. One possible explanation of the slow rate of inhibition by platinum compounds is that the applied platinum compound is itself inactive and must be converted intracellularly to an active form, by means of one or many step reaction. Possibly a better explanation of the sequential inhibition of DNA, RNA, and protein synthesis is that the active platinum species binds directly to DNA so that at a low concentration, only DNA synthesis is affected. Then at high concentrations, a higher frequency of platinum-induced lesions might lead to a measurable inhibition of messenger RNA production which in turn would eventually result in a reduction of protein synthesis (Harder and Rosenberg, 1970).

The above-mentioned possibility of interaction of platinum compounds with DNA is also supported by the studies of Horacek and Drobnik (1971). They have shown the interaction of platinum compounds with DNA by using the calf thymus DNA and studying its absorption UV spectrum with and without cis-dichlorodiammineplatinum(II).

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The absorption maximum shifts from 259nm to 264nm. Roberts and Pascoe (1972) have also provided evidence for the cross-linking of the complementary strands of DNA by the platinum compounds in cultured mammalian cells.

A possible action of the cis-dichlorodiammine-platinum(II) in regression of Sarcoma-180 in mice, mediated via immunologic means has been proposed by Conran and Rosenberg (1971). They studied the effect of Zymosan + cis-dichlorodiammineplatinum(II) and Hydrocortisone + cis-dichlorodiammineplatinum(II) on the acceptance of the Sarcoma-180 transplants by white swiss mice and by BALB/c mice, a histocompatible system. Hydrocortisone, an immunosuppressive drug, when used in combination with cis-dichlorodiammineplatinum(II) results in only 40% regressions of tumors, whereas 90% of tumors regressed in animals treated with only platinum compound. Combined Zymosan and platinum therapy on Sarcoma-180 maintained in BALB/c mice, produced tumor regression anywhere from 50%-100%, whereas the treatment with just Zymosan showed much lower percentages. No regressions were observed with cis-dichlorodiammineplatinum(II) treatment in tumor-bearing BALB/c mice. Thus it was apparent that host defenses play an important role in effecting the ultimate regression of Sarcoma-180 in mice treated with cis-dichlorodiammineplatinum(II).

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The involvement of the host immune response in the antitumor activity of cis-dichlorodiammineplatinum(II) is further supported by the fact that swiss white mice cured of advanced Sarcoma-180 tumors with a therapeutic dose of platinum compound completely rejects all further transplants of the tumor up to 11 months later (Rosenberg et al., personal communication).

On the contrary, Khan and Hill (1971, 1971) and Khan, Albayrak and Hill (1972) have shown cis-dichlorodiammineplatinum(II) to be a immunosuppressive drug. They have shown the inhibition of phytohemagglutinin (PHA) induced blastogenesis in human lymphocytes and also the suppression of antibody plaque-forming spleen cells in the mice sensitized to sheep erythrocytes. This property was further investigated and was found that cis-dichlorodiammineplatinum(II) prolonged the life of skin grafts in mice against H_2 histocompatibility barrier.

Kociba and Sleight (1971) studied the toxicologic and pathologic effects of cis-dichlorodiammineplatinum(II) in the male rats. According to them the LD 50 of the antitumor compound cis-dichlorodiammineplatinum(II) was 12.0 mg/kg in the male rat. Histologic, hematologic, and serum chemistry alterations were studied after the intraperitoneal injection of 12.2 mg/kg of cis-dichlorodiammineplatinum(II). Histologic alterations were most pronounced in those tissues having cellular constituents with rapid

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turnover times. Generalized lymphoid depletion, intestinal epithelial injury, and bone marrow depression were most severe 2-4 days after injection. Sloughing of the renal tubular epithelium also occurs at the same time, possibly in conjunction with the urinary excretion of the metabolites of the drug. Rats surviving the intoxication had regeneration of the cellular constituents of affected tissues. Panleukocytopenia, reticulocytopenia and platelet depression were most severe 3 days after injection, and were followed by regenerative increases.

Recently Vonka, Kutinova, Drobnik and Brauerova (1972) treated EB3 cells with the cis-dichlorodiammine-platinum(II) at the 1-10μm concentration and showed an increase in cell reactivity in the indirect immuno-fluorescence test with human sera possessing Epstein-Barr virus (EBV) antibody. The increase was usually twofold to threefold and was observed after 3-7 days. Addition of cytosine arabinoside, at the concentration of 10μg/ml, prevented the enhancement; this indicated that DNA synthesis was necessary for the effect.

The present investigation was undertaken to study the effect of cis-dichlorodiammineplatinum(II) on the fine structure of Sarcoma-180 in Swiss white mice and to establish the possible role of cis-dichlorodiammineplatinum(II) in causing the regression of Sarcoma-180 tumors in mice.

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Before the mechanism of action of cis-dichlorodiammine-platinum(II) can be understood at the cellular level it becomes important to know the fine structure of the Sarcoma-180 tumor. The only histological descriptions of Sarcoma-180 known are by Worley and Spater (1952) and Stewart, Snell, Dunhan and ScWyer (1959), while a brief account of fine structural studies was reported by Aggarwal, Sodhi, and VanCamp (1971). Also, Merekalova (1970) has reported the multiplication of the Friend leucosis virus in the cells of Sarcoma-180 and Lee, Krsmanovic and Brawerman (1971) have published few electron micrographs of Sarcoma-180 cells showing the presence of leukosis viral particles.

Shohat and Joshua (1969) studied and provided evidences for the role of immunologic mechanism in the spontaneous regression of Sarcoma-180 which may run from 5-20%. They showed that surviving mice after a spontaneous regression become completely resistant to subsequent implantation of Sarcoma-180 and partially resistant to challenge with Ehrlich's ascites carcinoma tumors. Whole body X-radiation of mice prior to tumor inoculation reduced the incidence of regression of Sarcoma-180 10-30%. Serological studies using the passive cutaneous anaphylaxis technique, complement fixation and tanned haemagglutination techniques demonstrated the presence of antibody against Sarcoma-180 in the serum of mice with

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spontaneously regressing tumors. Passive transfer of immunity to normal mice could not be achieved by intraperitoneal injection of immune serum. However, Sarcoma-180 growth in normal mice was completely inhibited by intraperitoneal injection of spleen cells from immune mice or by parabiosis with immune mice. The above observation as well as the presence of mononuclear pyrinophilic cells in the regressing tumors point to the possibility of cell-bound antibodies as responsible for the spontaneous rejection of Sarcoma-180. However, no precipitation lines were observed in agar gel diffusion between Sarcoma-180 saline extracts and the sera of Sarcoma-180 resistant mice (Shohat and Joshua, 1969).

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MATERIALS AND METHODS

Sarcoma-180, originally obtained from Mr. S. Polly of NIH was implanted into random bred specific pathogen free Swiss white mice according to CCNSC protocols. This original tumor line passed through 18 transplantations in ICR mice through six further transplantations in random bred, specific pathogen free, Swiss white mice before being used for these studies.

The cis-dichlorodiammineplatinum(II) used in these tests were synthesized and purified in Dr. Rosenberg's laboratory (Dept. of Biophysics, Michigan State University).

The day of tumor implant was taken as day 0 and on day 8 after implant, animals bearing healthy tumors were given a single intraperitoneal injection of cis-dichlorodiammineplatinum(II) in physiological saline in a dose of 8mg/kg of the body weight. Controls were given the single injection of physiological saline. Animals from both control and treated batches were sacrificed every second day till the tumors completely regressed in the treated animals or till the death of controls.

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For electron microscopy, tumor tissue from three animals in each batch was fixed in 3% cacodylate buffered glutaraldehyde (pH-7.3) at 4°C for 60 minutes. After washing in 6% sucrose solution the tissue was post fixed in 1% buffered osmium tetroxide (pH-7.3) for one hour. After dehydration in an acetone series, embedding was completed in epon-araldite mixture (Mollenhauer, 1964). For light microscopic studies, 1μ thick sections were cut using an LKB ultratome III and were stained with 1% methylene blue in 1% borax solution. Electron micrographs were prepared of thin sections (about 500 Å thick) sectioned with ultratome III and collected on 100 mesh copper grids and stained with uranyl acetate and lead hydroxide or lead citrate. Observations were made with a Hitachi-HUllE electron microscope operated at 75 kv.

Spleen.--The spleens were taken out from the above sacrificed mice (controls and treated) and parts of the spleen were fixed, embedded and sectioned for electron microscopical studies. 8μ thick sections of freshly taken cut spleen were cut on a cryostat and stained with methyl green/pyronin-Y stain (Pearse, 1968) to observe the pyroninphilic cells.

Lipids.--1μ thick plastic sections were stained for lipids, using alcoholic Sudan Black, Nile Blue and Sudan III and IV (Pearse, 1968).

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Acid-phosphatase.--For localization of acid-phosphatase the Naphthol AS phosphate-hexazonium pararosaniline (HPR) method was used (Barka and Anderson, 1962). The working medium was made up as follows:

Compound A	Compound B
1.0 ml pararosanilin solution. (1g pararosanilin hydrochloride dissolved in 20 ml distilled water and 5 ml concentrated HCl. Warm gently and filter.)	5 ml veronal acetate buffer pH-5.6.
	12 ml distilled H_2O.
	1.0 ml substrate (dissolve 10mg napthol AS-TR phosphate in 1.0 ml N,N-dimethylformamide.)
0.8 ml 4% Sodium nitrate.	
1.6 ml 4% Sodium acetate.	

Each compound was mixed separately and kept at 4%C; B was poured into A just before incubation of the tissue. The pH was adjusted to 5.6 with 1N-NaOH. The final volume was brought to 25ml with distilled water. Pieces of tumor tissue (1mm cubes) were incubated at 4°C for 60 minutes, washed twice with distilled water and processed for electron microscopy as already described.

Carbohydrates.--For the ultrastructural localization of carbohydrates, the modification of the PAS procedure devised by Hanker et al. (1964) was used. Ultra thin sections were picked up on 100 mesh gold grids (previously coated with formvar film). The sections were

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exposed to 1% aqueous periodic acid for 8 minutes at room temperature. The sections were next rinsed in distilled water. The diglycols of glycogen and similar polysaccharides converted to aldehydes by periodic acid were next reacted with thiosemicarbazide. The sections were left in a saturated aqueous solution of thiosemicarbazide (pH 3) for 30 minutes. After rinsing the grids again, they were exposed to OsO_4 vapor for 45 minutes. Subsequently the grids were rinsed, dried and observed. Controls were run where sections were exposed to thiosemicarbazide and subsequently to osmium vapor, but no pretreatment with periodic acid.

Extraction of RNA.--Extraction of RNA was done with 1% RNase (Sigma) solution in water at pH-6.5 by immersing golden grids with thin sections directly in the nuclease solution (Anderson, W. A. and Andre, T., 1968).

Tissue culture studies.--Sarcoma-180 ascites cells were cultured in Eagle's basal medium (BME) with Earle's salts (GIBCO, Grand Island, N.Y.) containing 2x amino acids with glutamine and 10% fetal calf serum in disposable Falcon plastic tissue culture flasks and tubes at 37°C. Cis-dichlorodiammineplatinum(II) in physiological saline was added to the culture medium in concentrations of 5 parts per million (5ppm) or 2µg/ml of the culture medium. Inoculation of the medium with

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platinum was done through a 0.22μ millipore filter. Cells were treated with the platinum compound for varying time intervals of 15 minutes, 30 minutes, 1 hour and 2 hours. Samples from treated and nontreated control cell cultures were fixed for electron microscopic studies. Platinum-treated cells and nontreated cells from each culture were also washed three times with BSS and maintained in normal culture medium with 2x amino acids for 24-36 hours before being fixed for EM studies.

Isolation of C-type virus particles.--Cell-free extracts were prepared and concentrated for virus particles according to Moloney (1960). The parts of pellets of such extracts were fixed and sectioned for electron microscopic studies to determine the presence and concentration of viral particles. A 10% suspension of tumor tissue in 0.153 M potassium citrate, containing 1.5mg of hyaluronidase (Sigma) per 100 ml, was allowed to digest for 1 hour at room temperature, with occasional mixing. After digestion, the tissue suspension was homogenized in a Waring blender or in a Potter and Elvehjem type homogenizer. The homogenate was then cleared of nuclei and cell fragments by centrifugation at 2,300 x g for 20 minutes at 4°C in a Sorvall refrigerated centrifuge. The supernatant (S_1) was pipetted off, care being taken not to disturb the sediment. Fraction S_1 was recentrifuged at 2,300 x g for 20 minutes

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to insure a more complete separation of the cell fragments. Supernatant (S_2) was pipetted off and centrifuged at 10,000 x g for 1-2 minutes to remove mitochondrial fragments. To sediment the small particulate fraction from the recovered supernatant (S_3), was centrifuged at 30,000 x g for 1 hour in a Spinco ultracentrifuge with rotor #40. The pelletized fraction (P_1) containing active virus was then resuspended in 0.05 M, pH-6.8 sodium-citrate buffer.

Suspension of the pellet was facilitated by use of Potter and Elvehjem homogenizer. The suspended material was recentrifuged at a force of 5,000 x g for 10 minutes. The middle third of supernatant (S_4) was recovered by means of a capillary-tipped pipette and set aside in an ice bath. The pellet (P_2) was washed in the remaining supernatant fluid and resuspended. An additional volume of buffer was added and recentrifuged at 5,000 x g for 10 minutes and the supernatant (S_5) pooled with fraction (S_4). The final product, composed of the pooled suspensions (S_4+S_5), is a concentrated particulate fraction containing the biologically active virus.

For serological and immunological studies with gel-electrophoresis and gel-double diffusion method, the mice were implanted with Sarcoma-180 on day zero and treated with platinum on day 8 of implant. Three mice from treated and control batches were sacrificed every 2nd day as in the early experiments. Animals were

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anesthetized very lightly with ether and their necks were cut with a sharp scissor and blood collected in sterile teflon-coated test tubes. These tubes with blood were kept at 4°C in the refrigerator for 24 hours and serum was collected from the top of the blood clot and stored at -20°C in small sterile vials. The serum collected was used for sereological and immunological studies.

Gel-electrophoresis.--Serum proteins were analyzed according to Clarke (1964), with a Hoefer EF 301 gel electrophoresis unit, using a Hoefer PS 101 power supply unit at 4m Amp. current/tube.

Preparation of Gel:

(a) 2 vol. of the following solution:
Acrylamide monomer - 30.0g.
N,N'-methylene bisacrylamide - 1.0g.
Distilled water - 123ml.

(b) 1 vol. of 0.28% vol/vol of Temed (n,n,n', n'-tetramethylethylenediamine solution.)

(c) 4 vol. of 0.14% wt/vol. of Ammonium persulfate.

(d) 1 vol. glycine-tris mixture:
Glycine - 29g.
Tris - 6.00g.
Water - 980ml.

Electrolyte was prepared in 1:10 ratio by diluting the stock prepared from Glycine=29g, Tris=6:0g, N-HCl=5.0ml. and 975 ml. of water. Final pH-8.1. 0.5% Bromo Phenol Blue in 1% acetic acid was used as tracking-dye.

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To the empty wells at the top of the vertically supported gel tubes was added up to 0.2 ml. of 2-5% Urea or Sucrose solution containing 200-400 micrograms of serum proteins (3 to 5 microliter of serum). After the run proteins were stained and fixed by immersing them for about 30 minutes in 0.1% Naphthol Blue Black (Sigma) in 7% Acetic acid. Stained gels were cleared of residual dye by destaining in 3% acetic acid and stored in 1% formalin.

Preparation of Agar Plates.--1% solution of agar in saline to which Merthiolate had been added was made by melting agar in autoclave. About 25 ml. of agar solution was poured in 90 mm x 15 mm glass petri dishes. Before pouring the agar, 1/4" wide strips of filter paper were folded in such a way that the strips touched the bottom of the male half of the petri dish and the other end hangs over the side. About 6 of such strips were arranged around each male half dish. When the melted agar had cooled, the filter paper strips were kept wet with distilled water. The wells were made in the solidified agar by using commercially available templates. For cutting the wells, a length of brass or copper tubing 1/2" in diameter was inserted through the agar to the bottom of dish. The cut-out pieces removed, and two drops of melted agar were placed in the bottom of each well to seal the bottom of the hole. The wells were filled with appropriate antigens and serum.

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Antigen Solution.--Homogenized extract of Sarcoma-180 in physiological saline.

After the wells were filled with serum and antigen solution, the plates were kept at room temperature from 8 to 24 hours and then stored in the refrigerator at 4°C.

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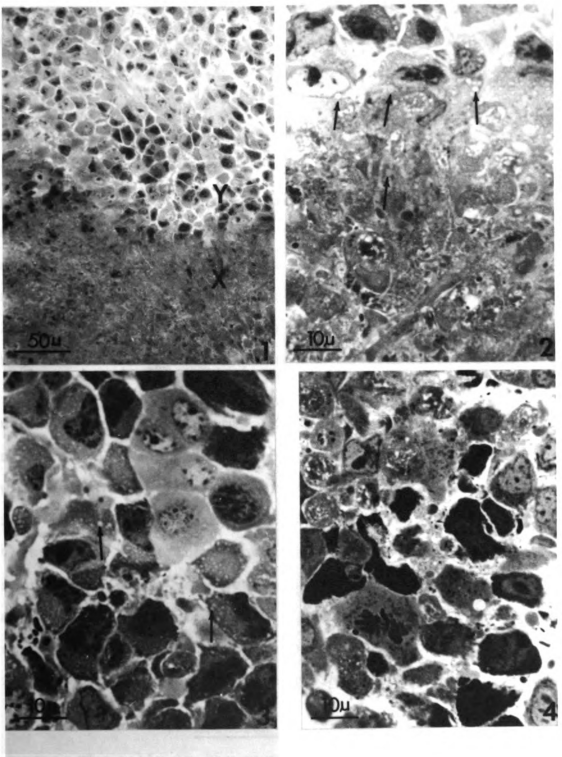
RESULTS

Light Microscopy

At the light microscopic level the tumor seems to lack a definite capsule and presents an alveolar pattern. In section, one can easily distinguish three regions: (1) central or necrotic region (Figs. 1, 2) that form about two-thirds of the tumor; (2) the cortical region consisting of thin strands of connective tissue stroma surrounding the relatively larger mass of tumor cells; (3) the intermediate layer, cells of which can be easily distinguished by the presence of large amounts of lipids in their cytoplasm (Fig. 2).

The viable tumor cells both in the cortical region and the intermediate region are about the same size, averaging about 15μ in maximum diameter but varying greatly in shape. They may be rounded, polygonal or sometimes elongated (Figs. 3, 4). The elongated cells are more common in the cortical region. Most nuclei are rounded, ovoid or bean-shaped. Mitotic figures can be observed both in the cortical as well as in the

Figs. 1-4. Light micrographs showing the gross morphology of Sarcoma-180 tumor at different magnifications. Fig. 1. Interface between the intermediate region and the necrotic region. The areas marked X and Y are shown enlarged in figures 2 and 3 respectively. Fig. 2. Showing different morphology of the cells in the intermediate and the necrotic region. Note the relative abundance of lipids (arrows). Fig. 3. Showing two types of tumor cells (light and dark) in the intermediate region varying greatly in their shape. Fig. 4. Same as figure 3 and a mitotic figure.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intermediate region (Fig. 4). Based on the staining intensities after methylene blue, two types of cells can be easily distinguished: (1) the light cells and (2) the dark cells, staining darker possibly due to the large amounts of cytoplasmic basophilia (Fig. 4). The nucleus in the dark cells appears larger in size and normally contains a single large nucleolus. The ratio of dark basophilic cells to light cells may vary from section to section but it appears that the number of dark cells increases as one goes from the cortical region toward the necrotic region. In addition, one can always find a few wandering leukocytes that are much more common in the necrotic region than in the cortical or the intermediate regions.

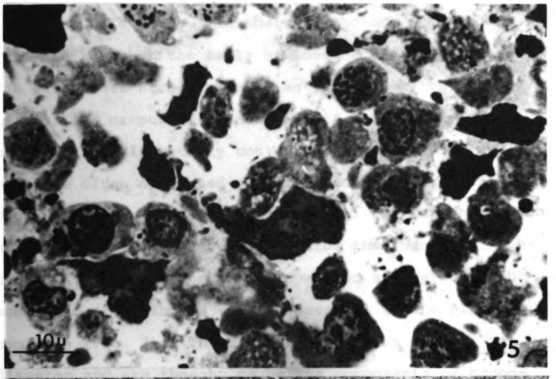
In the intermediate region cells close to the necrotic area show accumulations of lipids, tend to become globular and smaller in size and often lose contact with each other. The nuclear chromatin shows condensation into intensely staining spheres (Fig. 5). Soon after, these cells enter into a necrotic process that must be short-lived since very few intermediatory stages can be noticed. In late necrosis the chromatin material does not stain and the nuclear and plasma membranes break down, forming a pool of cytoplasm containing cellular organelles.

Electron Microscopy

The cells in the cortical region are more or less elongated (Fig. 6), and are often drawn out at both ends

Fig. 5. Light micrograph of a section through the medullary part of the necrotic region. Note the rounded isolated cells and the condensed chromatin in the nuclei.

Fig. 6. An electron micrograph of a spindle type cell from the cortical region of the tumor. Note the irregular elliptical nucleus (n) and the bloated cisternae of the endoplasmic reticulum. C, collagen.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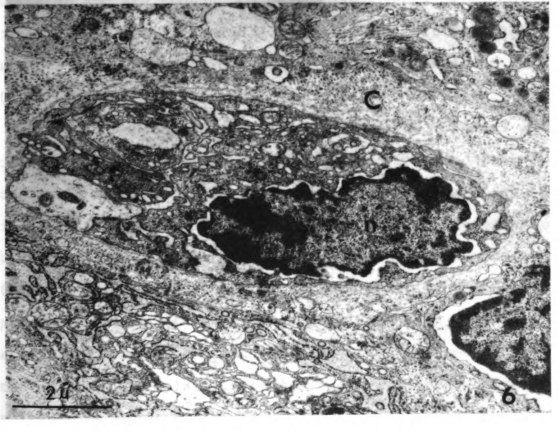
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into a spindle shape. In their morphology, these cells resemble somewhat the cells of loose connective tissue. The elliptical nucleus measuring 3μ x 7μ may be central in position or pushed to one side of the cell. The nuclear outline is usually contoured with the outer nuclear membrane often separated from the inner nuclear membrane. One can observe a well developed Golgi complex and a much more extensive endoplasmic reticulum than usual. In the cortical cytoplasm one can always find clusters of ribosomes and free fibrils ruhning parallel to the plasma membrane (Fig. 7). The fibrils range from 50Å-80Å in diameter and are thought to be the components of tropocollagen. Often the spindle cells in the cortical region seem to be embedded in bundles of collagen fibers (Figs. 6, 7).

The mononucleate, tumor cells in the cortical and the intermediate regions range from electron transparent to highly electron dense cells (Fig. 8). The majority of these cells are held together by the loose connective tissue but often cells may develop desmosomal connections (Figs. 9, 10). To develop an intercellular junction the plasma membranes of two cells approximate each other within a distance of 200Å. The intercellular space and the adjacent cytoplasm increase in density. The cells multiply by mitotic division (Figs. 11, 12). After division the daughter cells may not separate but may

Fig. 7. Part of a spindle cell in a longitudinal section from the cortical region of the tumor. Note the microfilaments (mf) in the cytoplasm running parallel to the plasma membrane (p). C, collagen.

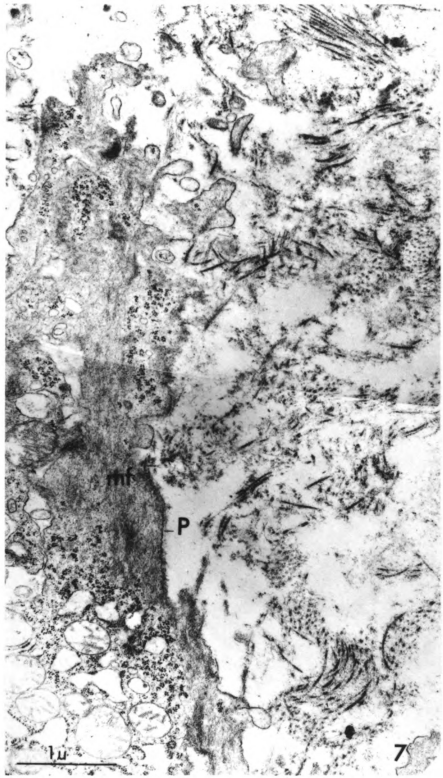
mf
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Fig. 8. A composite electron micrograph showing variations in the electron density of the various principal tumor cells. Note the elongated pseudopod-like processes from various cells extending considerable distance (arrows).

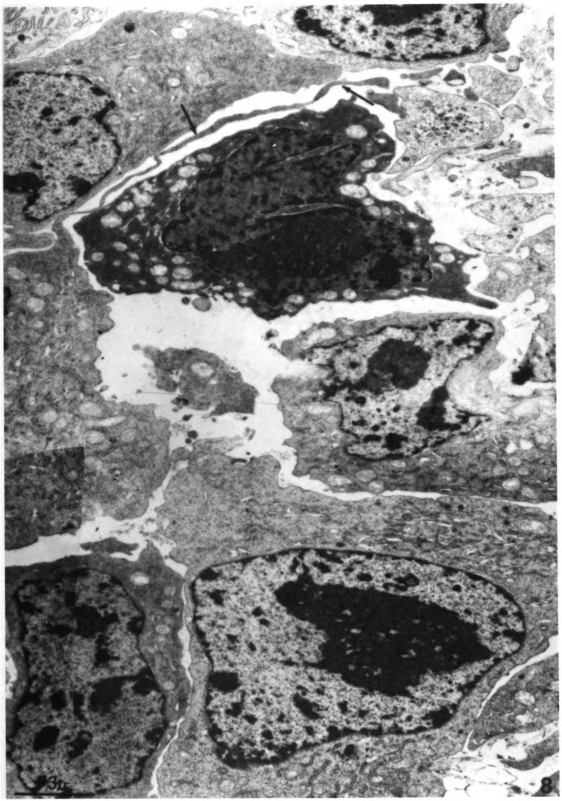
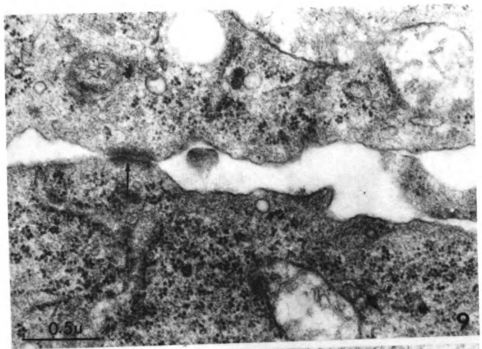


Fig. 9. An intercellular junction between two tumor cells (arrow).

Fig. 10. An electron micrograph showing the light and dark cells of Sarcoma-180. Note the presence of intercellular junction (arrow). The ribosomes are abundant and freely dispersed in the dark cell but are clustered in the light cell. Arrow-heads indicate a projection from the outer nuclear membrane into the cytoplasm, G, Golgi complex; m, mitochondrion; n, nucleus.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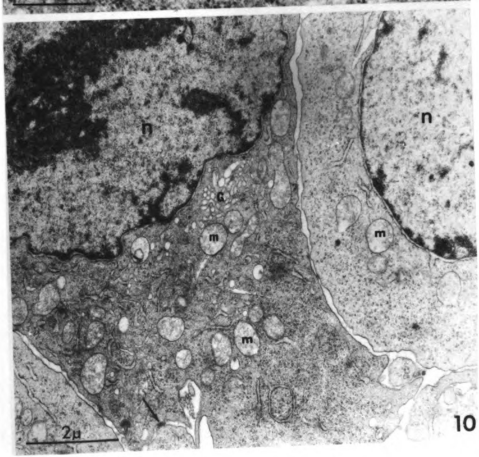
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m
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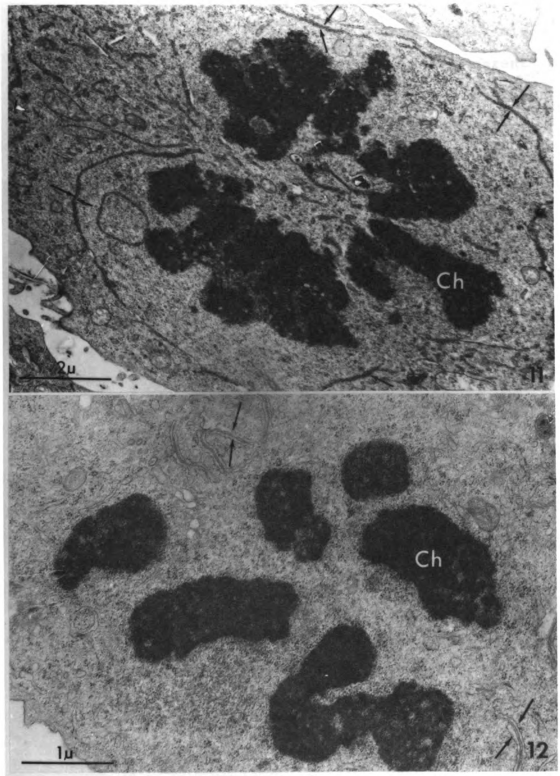
2μ
10

Fig. 11. A cell in an advanced prophase stage showing the rupture of the nuclear membrane, portions of which appear as "paired cisternae" with ribosomes attached on the outer surface (arrows). Ch, chromosomes.

Fig. 12. Late prophase showing discrete chromosomes (Ch) and the "paired cisternae" without ribosomes (arrows).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remain held together by the midbody connection (Figs. 13, 14). In some cases one of the cells may be highly electron dense while the other may be electron transparent (Fig. 13) suggesting a possible change in their functional morphology. The tumor cells assume bizarre shapes, often sending out long pseudo-pod-like projections which extend considerable distances in between the other cells (Fig. 8). Often membrane-bound microvilli project from the cortical cytoplasm all over the cell surface, being more apt to occur at the tips of the cytoplasmic processes. The microvilli are highly variable in size and are usually without any cytoplasmic organelles other than few microfilaments.

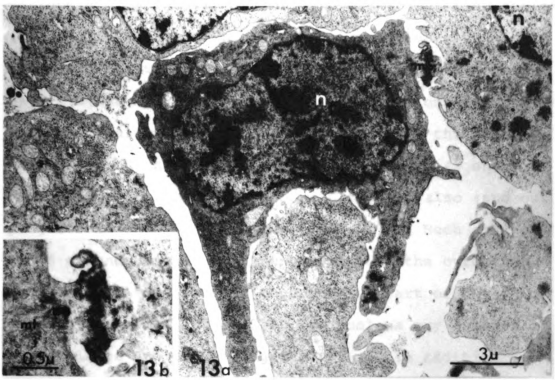
The dark cells are usually smaller in size as compared to the light cells, since there are lesser amounts of cytoplasm in the dark cells. The nuclear cytoplasmic ratio varies from 1.24 in the dark cells to 0.83 in the light cells. Due to the very bizarre shapes these cells assume, it is very difficult to make a volumetric estimation. However, area measurements of cross-sectioned cells in a random section show dark cells to be $85\mu^2$ and light cells to be $130\mu^2$.

Nucleus

The nucleus is highly variable in size and shape. It may be highly irregular, oval or rounded. The nuclear membrane may sometimes show deep cytoplasmic

Fig. 13. (a) Late telophase showing two daughter cells each with completely reformed nucleus held together by midbody (mb) connection. Note one of the daughter cells is dark while the other is lighter in its electron density. Midbody connection is shown further enlarged in Figure 3b. mt, microtubules, n, nucleus.

Fig. 14. Higher magnification of a midbody between other cells showing numerous microtubules (arrowheads) and lysosome-like bodies (arrows).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invaginations (Figs. 8, 15, 16, 17) which when sectioned obliquely give the appearance of nuclear inclusion bodies (Fig. 17). However, actual inclusion bodies always have a nuclear limiting membrane. The inner nuclear membrane may invaginate to form fınger-like projections or "intranuclear canalicule" that may extend 3μ into the nucleus (Fig. 18). Such projections may sometimes also show branching. The outer nuclear membrane may also send out projections into the cytoplasm (Fig. 19). Such projections extend considerable distances into the cytoplasm and may acquire ribosomes thus forming part of the endoplasmic reticulum (Fig. 19). The nucleus may contain a single large nucleolus (Figs. 8, 20) or in addition may have a number of small nucleoli (Figs. 8, 15). The nucleolus is usually centrally placed but may sometimes be attached to the inner nuclear membrane (Fig. 19). The highly electron dense nucleolus may have light fibrillar areas (Figs. 8, 15, 17) or the nucleolar material may form a network spreading all through the nucleoplasm (Fig. 16). Chromatin material is usually associated with the marginal zones of the nucleus, surrounding the nucleolus (Fig. 15) and is often present as small clumps all through the nucleus (Figs. 8, 15, 18, 19). In rare cases the chromatin may be associated with the nucleolar material forming a rope-like structure twisted on itself in the nucleus (Fig. 16).

Fig. 15. A dark cell with two nucleoli (nu) with condensed chromatin associated around them. Arrows point toward light fibrillar areas within the nucleolus. The nuclear membrane is irregular often invaginating into the nucleus.

Fig. 16. A light cell showing the nucleolus and the chromatin together forming a rope-like structure twisted all through the nucleus.

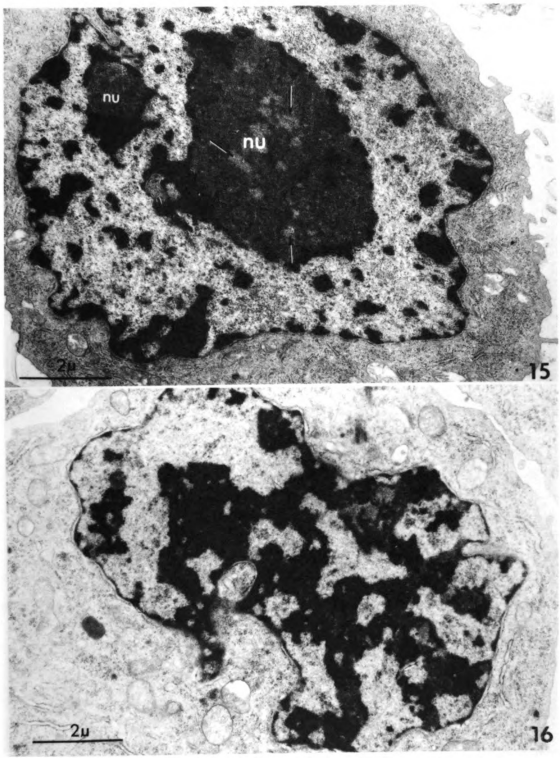
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Fig. 17. Nuclear membrane invaginations cut in cross sections giving the appearance of nuclear inclusions (arrows). Note the 4 daughter centrioles. nu, nucleolus.

Fig. 18. Inner nuclear membrane extending into the nucleus to form "intra-nuclear canalicule" (arrowheads). Arrows point to the nuclear pores in surface view. m, mitochondria.

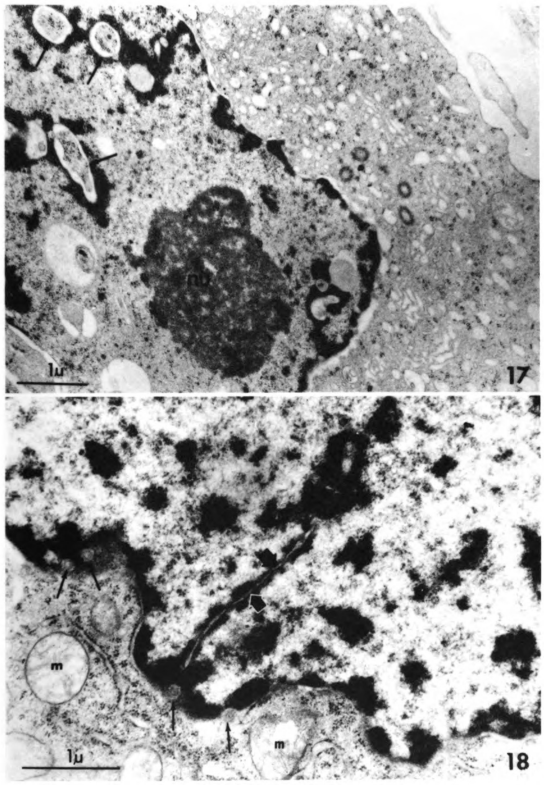
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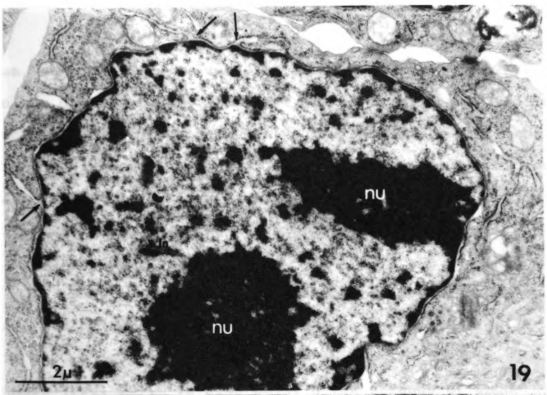
Fig. 19. Showing a continuity between the outer nuclear membrane and the cisternae of the endoplasmic reticulum (arrows). In, intra-nuclear canalicule; Nu, Nucleolus.

Fig. 20. Compact nucleolus (nu) in a dark tumor cell. Nucleolus associated chromatin (arrow), a pair of centrioles (c) and the mitochondria (m) concentrated on one side of the nucleus. Insert shows a cross section through the centriole consisting of 9 bundles of 3 tubules each.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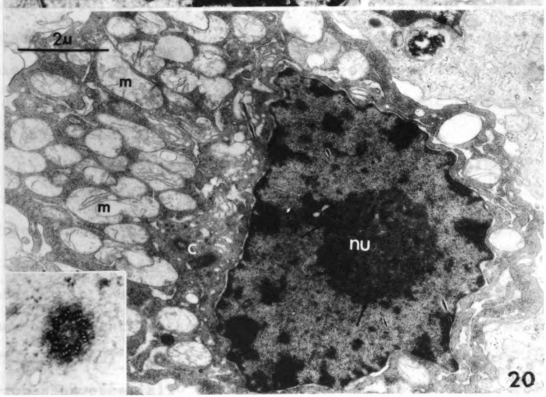
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Cytoplasmic Membrane Systems

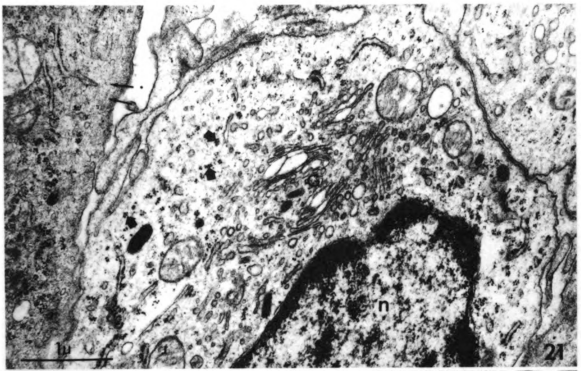
The Golgi complex consists of 4-5 stacked cisternae, surrounded by a number of small vesicles (Fig. 21). In the dark cells the cisternae may be considerably swollen to form groups of large vesicles that are confined to the juxta nuclear position (Figs. 20, 22). The Golgi vesicles may also extend far beyond into the cortical cytoplasm. Within the Golgi complex may also be found membrane-limited dense bodies (Fig. 21).

The rough-surfaced endoplasmic reticulum seems to have its origin from the outer nuclear envelope (Figs. 10, 19) and is much more developed in some cells than in the others (Fig. 10). In some cells a few strands of endoplasmic reticulum may be markedly distended. Such dilated cisternae usually contain flocculent electron dense material (Fig. 23). The smooth-surfaced endoplasmic reticulum is undeveloped and scanty.

The ribosomes may be attached to the endoplasmic reticulum or may be present free in the cytoplasm. The free-floating ribosomes far exceed the membrane-bound ribosomes. Within the cytoplasm the ribosomes may be present in the form of clusters (Figs. 10, 18, 19, 23) or may be present in the monosomal form (Figs. 20, 22, 24, 25). The monosomal form is most abundant in the cytoplasmic processes especially those from the dark cells (Figs. 22, 24, 25). Except for large quantities of ribosomes such

Fig. 21. Well developed Golgi system (G) situated on one side of the nucleus (n). Numerous microfilaments (arrows) are present under the plasma membrane of another cell. d, dense body; polysomes (arrowhead).

Fig. 22. Light and dark cells. Processes (P) from the dark cells are devoid of cytoplasmic organelles except for ribosomal particles. Golgi complex is mostly juxtanuclear in distribution.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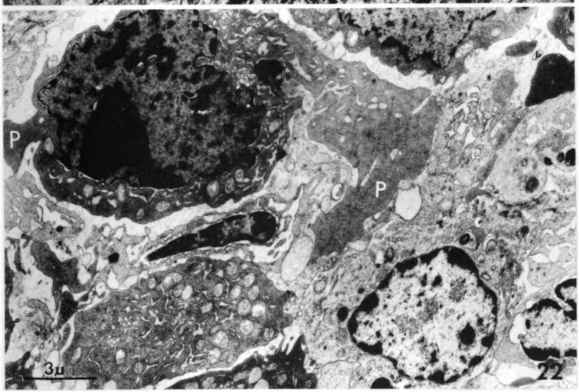
P
P
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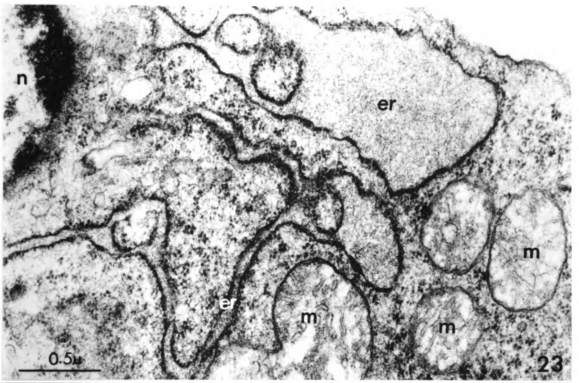
Fig. 23. Bloated cisternae of the endoplasmic reticulum (e.r.) full of flocculent material. m, mitochondria; n, nucleus.

Fig. 24. Cytoplasmic extension of a dark cell full of ribosomes.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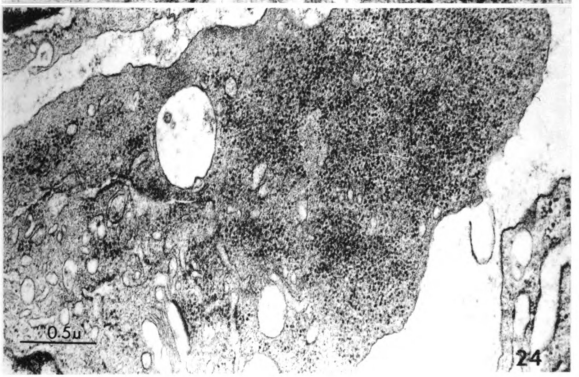
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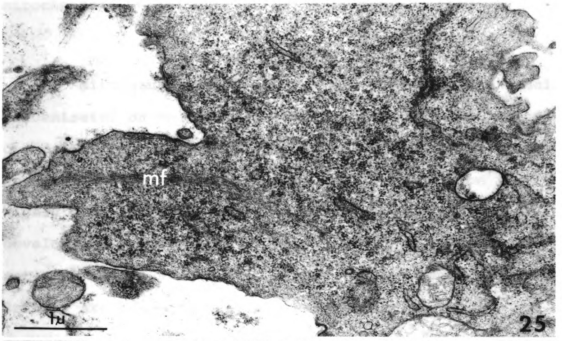
Fig. 25. Cytoplasmic process from a dark cell with ribosomes and bundle of microfilaments (mf).

Fig. 26. Microfilaments (arrows) present loose in the cytoplasm often crisscrossing each other. l. lipid globules; m, mitochondria; n, nucleus.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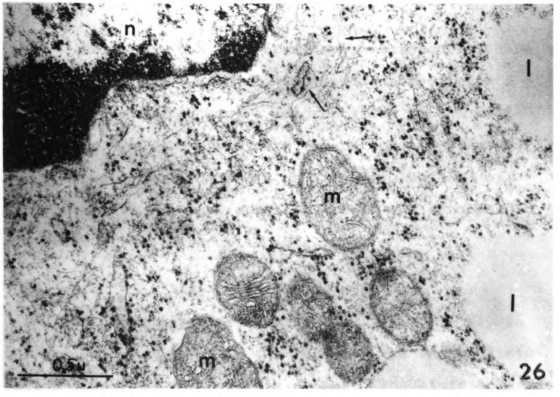
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cytoplasmic processes are devoid of other organelles like mitochondria, Golgi complex or endoplasmic reticulum.

Mitochondria

Mitochondria vary in size and shape and are usually concentrated on one side of the nucleus (Fig. 20). Mitochondria with elongated to spherical profiles are most abundant. Usually the matrix of the mitochondrion is highly electronlucent and the cristae are not well developed. Often a section through a mitochondrion may not have any cristae (Figs. 10, 20, 25). Whenever the cristae are present, they are very irregular.

Microfilaments

Except at the time of mitosis microtubules have not been observed. However, nonbanded microfilaments of about 60Å diameter occupy a major portion of the cell cytoplasm. The microfilaments may be loosely arranged often crossing each other (Figs. 7, 21). Bundles of microfilaments are more abundant within the cytoplasmic processes of the dark cells (Fig. 25).

Lipid Inclusions

Lipid spheres of variable size occur mostly in the necrotic region or in the cells of the intermediate region closer to the necrotic region. The lipid spheres are not membrane-bound and are not very electron dense

thus probably indicating their saturated nature. As a cell enters the necrotic region, the size and number of lipid spheres increases.

Ultrastructure of Cells During Mitosis

The process of mitosis has been studied in wide variety of tumors cells grown in tissue culture (Buck, 1961; Yasuzumi, 1959; Selby, 1953). Information on the mitotic activity of solid tumor cells in their natural environment has been scanty. During the course of the present studies we have observed anywhere from 2 ro 5 centrioles (Figs. 17, 20). Each centriole consists of 9 radiating groups of tubules arranged to form a cylinder (Fig. 20 insert). Each radiating group of tubules is in turn formed of 3 tubules. Migration of a set of centrioles sets the stage for the process of mitosis. The chromatinic mass condenses into discrete chromosomes and the nuclear membrane breaks into pieces. Such pieces distend but still maintain the shape of the nucleus (Fig. 11). The broken nuclear membranes may contact each other to form paired cisternae. Such paired cisternae may also be seen in between the chromosomal mass (Fig. 11). The paired cisternae consist of 4-unit membranes. The inner two membranes are smooth surfaced and may be closely applied to each other or sometimes be separated by a zone of 100Å (Fig. 12). The outer two membranes are studded with

ribosomes. No pores can be observed in the paired membranous stacks. The paired cisternae range from 0.5 to 6 in length. During metaphase the paired cisternae lose their ribosomes from the outer membranes and tend to become swollen thus mingling more easily with the Golgi membrane system (Fig. 12). Subsequently, in late metaphase such paired cisternae are no longer discernible. Nucleolar masses persist in close association with the chromosomes in the early stages of prophase but gradually disappear during metaphase. The Golgi components are well defined and persist all through mitosis. The mitotic spindle is formed by microtubules of 200Å in diameter.

During late telophase the daughter nuclei are formed by the formation of new membranes appearing around the chromosomes. Cytokinesis takes place by an invagination of the plasma membrane stopping short at the spindle. Numerous spindle tubules in the intercellular bridge region accumulate dense granules on their surface thus forming a typical midbody (Fig. 13a, b). Within the region of the midbody and radiating out on both sides can be observed numerous membrane enclosed elongated electron dense bodies (Fig. 14). The nature and function of these structures is not clear.

Necrotic Cells

In the necrotic region can be observed different stages of necrosis--from isolated rounded up cells to an

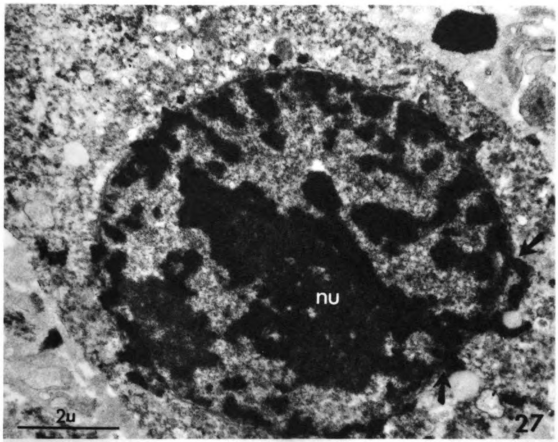
amorphous mass of disintegrated cells. Usually at the onset of necrosis the cells tend to become circular and the cellular organelles become more or less smudgy. The ribosomes are no longer discernible. The nuclear chromatin starts oozing out into the cytoplasm (Figs. 27, 28) and gets distributed evenly into the cytoplasm. The lipid globules either get dissolved or get converted into myelin-type figures. The nucleolus that is quite electron dense in the beginning becomes more fibrillar and rapidly loses its electron density (Figs. 27, 28) and the cell cytoplasm usually becomes filled with small vesicles (Fig. 28). The nuclear and the plasma membranes may break down and release all the contents into the mass of the tumor. The necrotic cell may also become surrounded by one or more phagocytic cells (Figs. 29, 30) and ultimately be engulfed. A phagocytic cell may have as many as 5 necrotic cells in its phagocytic vacuoles (Fig. 31).

Leukocytes

Some white blood cells could be observed in the main body of the tumor tissue mostly being centered in the necrotic region. These leukocytes have a characteristic morphology with a multilobulated nucleus condensed granular chromatin, and scarce small mitochondria embedded in a coarse-grained cytoplasmic matrix with few endoplasmic cisternae. In addition, there are certain β-glycogen type particles (225Å - 250Å in diameter) assembled into

Fig. 27. Cell in the initial stages of necrosis. The condensed chromatin oozing out (arrows) into the cytoplasm and the nucleolus beginning to loose its electron density (clearer in figure 28).

Fig. 28. Necrotic cell showing cytoplasm full of vesicles. Nucleolus (nu) is less electron dense and more fibrillar; Ch, chromatin; l, lipid globules.


nu
2u
27

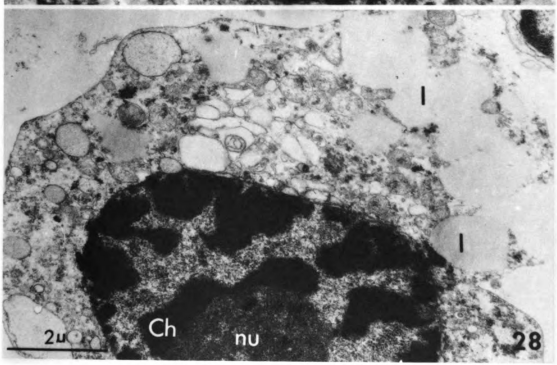
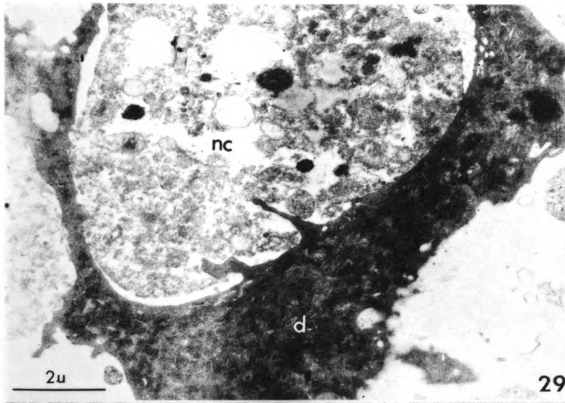

l
l
Ch
nu
2u
28

Fig. 29. A necrotic cell (nc) being phagocytized by another highly electron dense tumor cell (d). Note no cellular organelles can be made out in the necrotic cell.

Fig. 30. A necrotic cell (nc) surrounded by three electron dense cells (d) probably in the process of being engulfed. The nucleus of the necrotic cell has lost its electron density and portions of its nuclear membrane.



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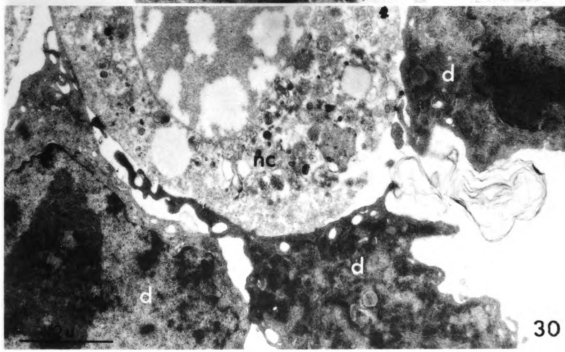

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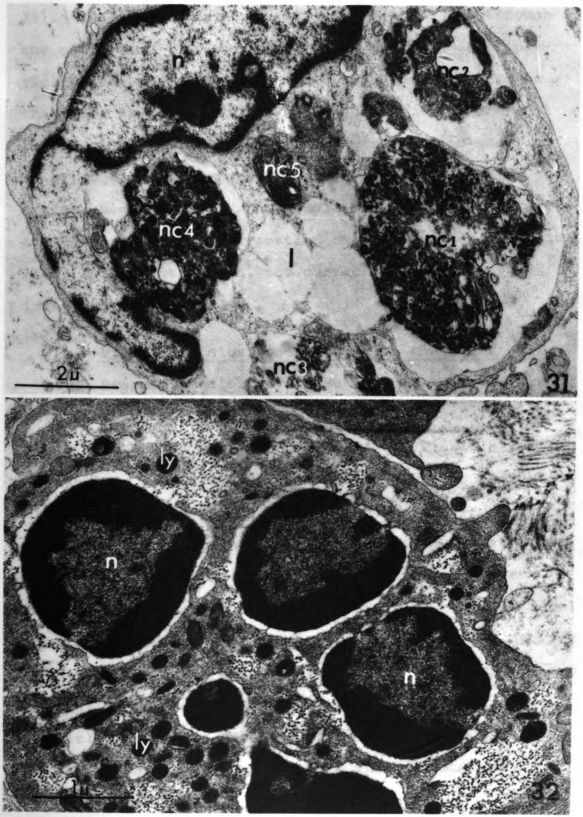
Fig. 31. Phagocytic cell from necrotic region showing 4-5 phagocytic vacuoles containing necrotic cells (nc_1-nc_5). l, lipid globule; n, nucleus.

Fig. 32. Leukocyte with its multilobulated nucleus cut to give an appearance of 4 small nuclei (n). The glycogen particles are present in agglomerations in the perinuclear area and are shown at a higher magnification in figure 35. ly, lysosomes.


n
nc2
nc5
nc4
nc1
nc3
2µ
31
ly
n
n
ly
2µ
32

irregularly shaped islets within the cytoplasm (Figs. 32, 35). These particles may also be dispersed all through the cytoplasm. However, when these electron-dense particles are present in agglomerations, the cytoplasm in that area is always electron transparent. Cytochemical studies have indicated that these particles in addition to glycogen also contain ribonucleoproteins. Salivary amylase digestion does not completely extract these particles nor does RNase digestion obliterate them from view. After either extraction, the particles present a very irregular outline.

Virus Particles Associated With the Tumor Cells

Present studies have revealed two types of virus particles (Figs. 33-34, 36-38) associated with the cytoplasmic membranes and the nucleus of the viable Sarcoma-180 cells. Particles found within the nucleus measure about 425Å (Figs. 33, 36) and have been observed in very few cells. These particles lack a limiting membrane and tend to form clusters in the peripheral zone of the nucleus. Each particle shows a dense core of about 300Å with a lighter zone surrounding it. Although the nuclear viral particles may form clusters, they never achieve any crystalline or semicrystalline appearance. Since these particles have been observed in very few cells it is inferred that their formation and the life must be very

Fig. 33. Chromatin-associated 425Å virus particles present mostly in clusters. Area enclosed by the rectangle is shown at a higher magnification in figure 36.

Fig. 34. 200-300Å nuclear associated particles probably stages in the formation of mature 425Å nuclear virus particles described in figure 33. Ch, chromatin; nu, nucleolus.

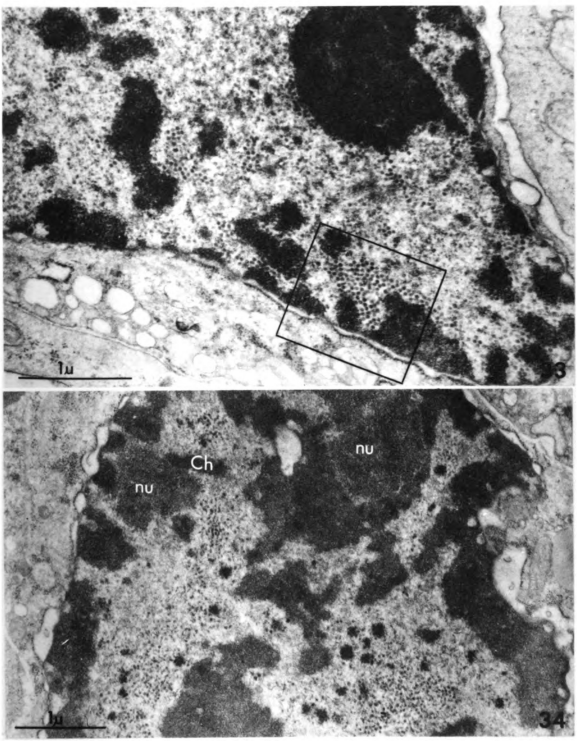
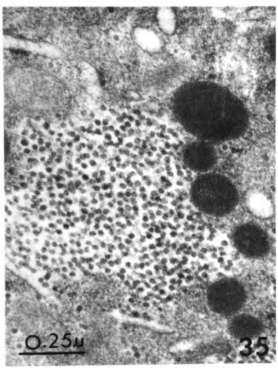

1μ
3
Ch
nu
nu
1μ
34

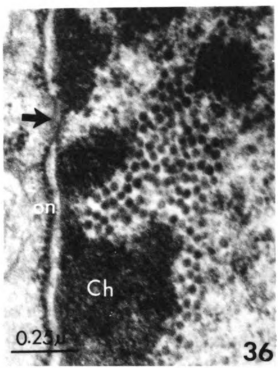
Fig. 35. β-glycogen type particles at higher magnification showing individuality of the particles.

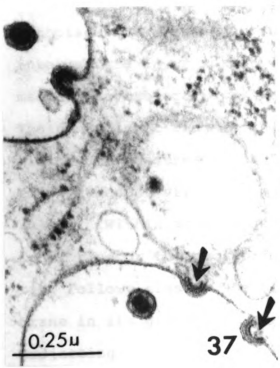
Fig. 36. Enlarged view of the nuclear virus particles. Arrow indicates a nuclear pore. o.n., outer nuclear membrane; ch, chromatin.

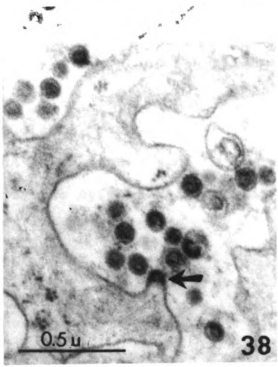
Fig. 37. Mature virus (900Å) particles within the cytoplasmic vacuoles. Various stages in the formation of these particles can also be noticed at the vacuolar membranes (arrows).

Fig. 38. Formation of 900Å virus particles at the plasma membrane (arrow). Note number of mature virus particles outside the cell.


0.25μ
35


en
Ch
0.25μ
36


0.25μ
37


0.5μ
38

short. Although, no developmental stages in the formation of these particles have been observed, but have observed particles ranging from 200-300Å in diameter (Fig. 34) in some cells. Such particles may be scattered in the nucleoplasm or may form clumps. The size of the particles in clumps is larger than that of those scattered in the nucleoplasm. These particles might represent the developmental stages in the formation of 425Å particles.

The membrane associated particles are about 900Å in diameter and are often seen budding and maturing at the surface of the plasma membrane and within the bloated cisternae of the endoplasmic reticulum (Figs. 37, 38). Each mature virus particle consists of an outer coat of cytoplasmic membrane surrounding a dense ring with an intermediate layer in between. The inner and the intermediate layers are separated by a light-zone of about 60Å. The dense inner ring is the nucleoid portion of the virion and measures about 500Å in diameter. In the initial stages of virion formation the cytoplasmic membrane bulges. Within this bulge the formation of the dense inner ring or the nucleoid part takes place. The inner ring follows closely the bulge in the cytoplasmic membrane in its growth and one never finds the inner ring projecting into the cytoplasm beyond the edge of the bulge (Fig. 37). The number of these virus particles increases with the age of the tumor and one can find far

more particles after 20 days of tumor implant than after 10 days.

Injection of cell free extracts of Sarcoma-180 (0.05ml/mouse) rich in membrane-bound virus particles into the newly born mice induces leukemia in 40% of the animals and the animals die within 3 weeks of such injections.

Light and Electronmicroscopy of Regressing S-180 Tumor After cis-dichlorodiammineplatinum(II) Treatment

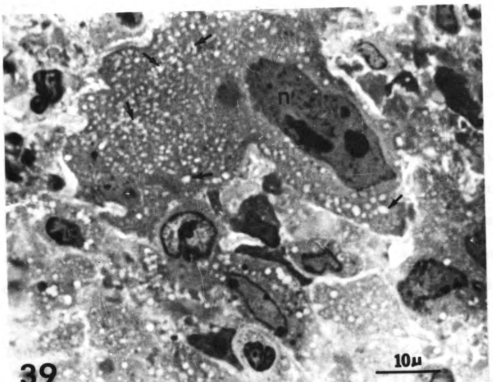
Tumor in Cis-Pt-II treated mice completely disappears by 12 days of the treatment, whereas the control animals are dead by that time, i.e., about 20 days after the tumor transplant. The platinum-treated animals develop an immunity to all the future transplants.

Mitotic Activity.--Within two days of the treatment with platinum compound, no mitotic figures are observed in the tumor mass. Otherwise, cells do not show any morphological changes from cells from nontreated tumors. However, mitotic activity starts reappearing within the regressing tumor mass by day 10 of the treatment.

Giant Cells.--By the 4th day of Cis-Pt-II treatment, quite a few very large cells can be observed within the mass of the tumor. 1μ thick sections under light microscope shows a single large nucleus (Figs. 39, 40), whereas electron micrographs may show a single very

Fig. 39. Light micrograph of a 4-day platinum-treated tumor showing giant cell with single nucleus (n) and cytoplasm filled with small lipid globules (arrows).

Fig. 40. Light micrograph showing a giant cell from the tumor mass treated with platinum for 4 days, with irregular nucleus (n) enclosing a cytoplasmic vesicle (cv). Lipids--arrows.



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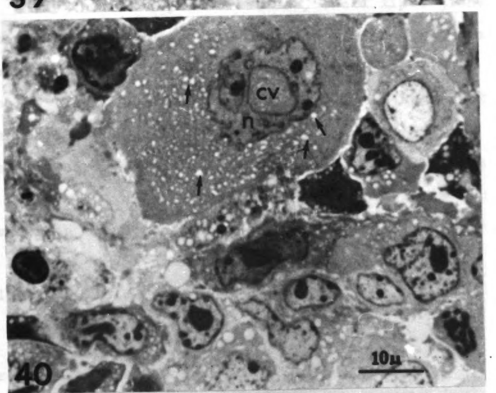

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irregular, undulating nucleus with enclosed cytoplasmic vesicles (Fig. 41) or may show 2-3 nuclei. Such cells have very well developed Golgi membranes spread throughout the cytoplasm. Rough endoplasmic reticulum (RER) is also well developed and 50-60Å thick microfilaments can be observed in the cytoplasm. In addition, the cytoplasm is filled with small droplets of lipids without any limiting membranes (Fig. 41). The amount of such lipid globules increases gradually in both the giant cells and regular tumor cells after Cis-Pt-II treatment. Most of the giant cells show 3-6 centrioles present on one side of the nucleus (Fig. 41).

By the 6th day of treatment, small lipid globules in both giant and regular tumor cells are observed coming together, fusing and forming larger and larger lipid globules (Fig. 42). During this period, the number of observable giant cells decreases and by day 8 of the treatment hardly any such large cells are observed. But one can observe a large number of very large lipid droplets within the cytoplasm pushing the nucleus to one side and leaving only a small cytoplasmic band around the nucleus and lipid droplet (Fig. 43).

Cells of Reticuloendothelial System.--During the regression of the tumor after Cis-Pt-II treatment, certain cells of the reticuloendothelial system (lymphocytes, plasma cells and macrophages) make their appearance in the

Fig. 41. Electron micrograph of a giant cell from treated tumor for 4 days, showing the irregular undulating nucleus with pockets of cytoplasmic vesicles (cv), well developed Golgi (G), lots of lipid droplets (arrows) and 5 centrioles near the lower end of the nucleus (arrow-heads).

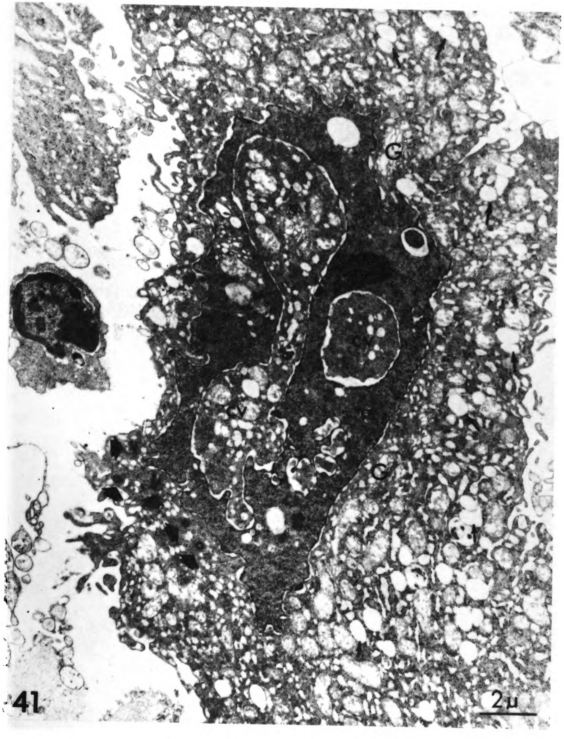
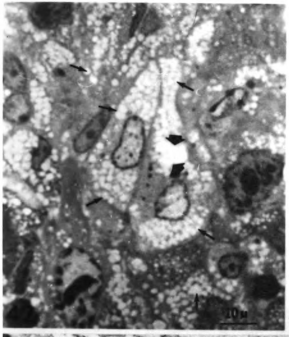
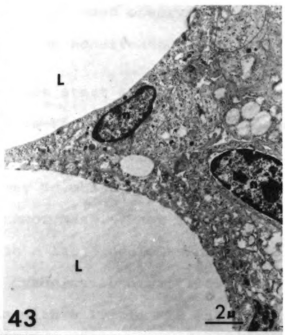

G
41
2μ

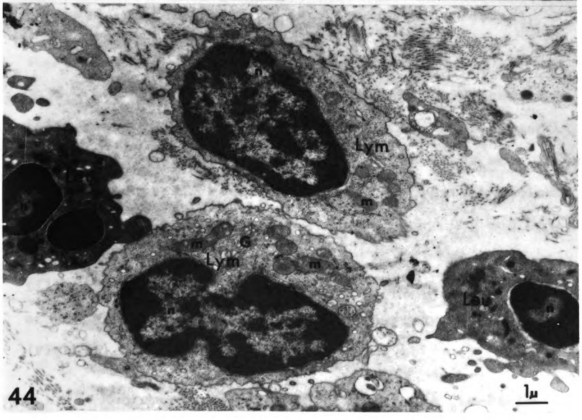
Fig. 42. Light micrograph showing many regular tumor cells after 6 days of platinum treatment. Note the cytoplasm filled with lipids (arrows) and the fusion of smaller lipid globules into larger bodies (arrowheads).

Fig. 43. Electron micrograph of S-180 tumor treated for 6 days, showing large lipid droplets (L) pushing the nucleus to one side of the cell. n, nucleus.

Fig. 44. Electron micrograph of part of tumor treated for 6 days, showing lymphocytes (Lym) and parts of leukocytes (Leu). n, nucleus; m, mitochondria; G, Golgi.


10μ


L
L
43
2μ


n
Lym
m
Lym
m
Lym
44
1μ

tumor mass in a sequential manner. Such cells of the reticuloendothelial system have not been observed at any time in the mass of the tumor from non-treated animals.

Lymphocytes.--Lymphocytes start appearing within the tumor mass by 6th day of Cis-Pt-II treatment and their number increases progressively until the tumor is almost completely regressed. They may be observed in groups or dispersed singly within the tumor mass. They show the characteristic morphology with a large nucleus surrounded by a thin rim of cytoplasm. Cytoplasmic organelles are few and only 2-3 mitochondria with a little RER may be observed in a thin section of a lymphocyte (Figs. 44, 48, 50).

Plasma Cells.--Plasma cells with their characteristic morphology appear by the 8th day of the treatment. Their number also increases gradually but stays much lower than lymphocytes. Plasma cells show a spherical nucleus, surrounded by a large volume of cytoplasm. RER and Golgi membranes are very well developed. RER almost completely fills the cytoplasm in the form of concentric cisternae around the nucleus filled with a flocuated material (Fig. 45).

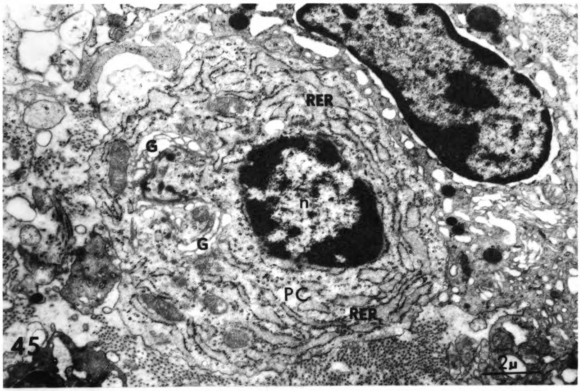
Macrophages.--By the 6th day of the Cis-Pt-II treatment, the tumor starts showing a definite visible regression and at the same time a few macrophages start

appearing in the tumor mass (Figs. 46, 47, 48). Macrophages have well developed RER, golgi and show many lysosomal bodies spread within their cytoplasm. By day 8 of treatment quite a few of these macrophages are observed closely associated with the tumor cells which show degenerative morphology, cytoplasmic organelles including ribosomes are dispersed, mitochondria become spherical and filled with granular material, chromatin loses its electron density and fills the nucleus with evenly dispersed fibrillar material. The plasma membrane degenerates, particularly where it comes in close contact with the macrophage surrounding it (Fig. 46). During this period, macrophages may also be observed releasing lysosome like bodies (rich in Acid-phosphatase) in the extracellular spaces or into the loose matrix produced by the debris of degenerating or dead tumor cells (Fig. 47).

White Blood Cells.--The number of leukocytes increases tremendously from 6th day of the treatment to the 12th day when the tumor has completely regressed and leaves an empty scar. Leukocytes can be observed wandering among the tumor cells and by 10th day of treatment converge into the areas of regressing tumor filled with loose semisolid debris of the cells and show active phagocytic activity (Figs. 44, 48, 49, 50).

Fig. 45. Electron micrograph of a plasma cell (PC) in regressing tumor mass after platinum treatment. Note the spherical nucleus (n), well developed rough endoplasmic reticulum (RER) and Golgi (G).

Fig. 46. Electron micrograph showing two processes of macrophage (M) closely encircling a degenerating tumor cell (Sa) in the tumor mass after platinum treatment. Arrows point to the degenerating plasma membrane where the surface of macrophage comes in contact with the tumor cell. Ly, lysosome; n, nucleus.


RER
G
n
G
PC
RER
45
2μ

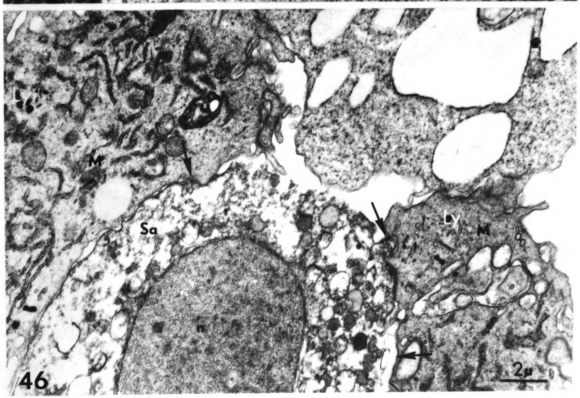
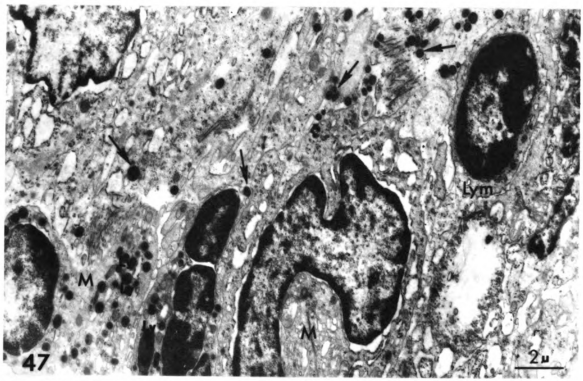
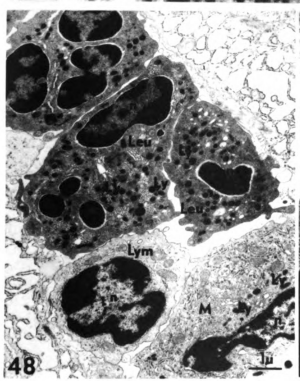

M
Sa
Ly
M
46
2μ

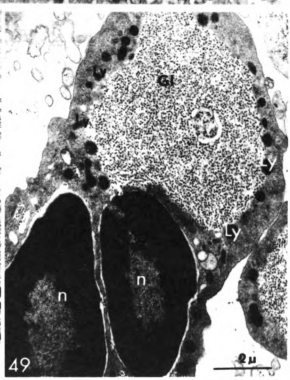
Fig. 47. Electron micrograph of tumor mass after platinum treatment, showing a lymphocyte (Lym) and macrophages (M) with lysosome-like bodies (Ly). Arrows point to the large number of such bodies released into the matrix.

Fig. 48. Electron micrograph from treated tumor for 10 days with platinum, showing leukocytes (leu), lymphocyte (Lym) and a portion of macrophage (M). n, nucleus; Ly, lysosome.

Fig. 49. Electron micrograph of a leukocyte from regressing tumor after 10 days of platinum treatment, with its cytoplasm completely filled with glycogen particles (Gl). Ly, lysosome; n, nucleus.


Lym
M
M
47
2μ


Lym
M
48
1μ


Gr
Ly
Ly
n
n
49
2μ

Glycogen Particles.--The amount of 225 Å - 250 Å glycogen particles within the cytoplasm of leukocytes increases progressively from day 6 of the treatment to day 10 and by day 10 of treatment glycogen particles may completely fill the cytoplasm (Fig. 49). The number of such particles in the leukocytes from non-treated tumor stays constant.

By day 8 of the treatment another interesting phenomenon is observed among the tumor cells. The plasma membranes of adjoining cells dissolve or come together, join and form small vesicles, thus leading to the formation of a syncitial mass (Fig. 51).

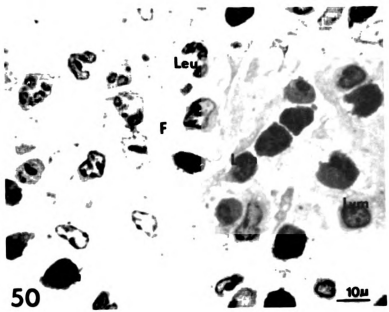
Light and Electronmicroscopy of Spleen from Platinum Treated and Non-Treated Mice

Within two days of platinum treatment the spleen shows a shrinkage in its mass, which is followed by a depression in the pyroninophilic cells up to 6 days. After that the spleen starts to enlarge again and shows an increase in the pyroninphilic cells up to 10-11 days of the treatment (when compared with the controls). Fine structural studies also show an increase in the number of plasma cells in the spleen of the treated animals about 8-10 days after treatment (Fig. 52).

Fig. 50. Light micrograph showing many leukocytes (Leu) and lymphocytes (Lym) wandering in the regressing tumor mass after 12 days of platinum treatment. Note the elongated fibroblast (F) coming into the area.

Fig. 51. Insert--Light micrograph of synsitial mass formed in the tumor after 8 days of platinum treatment. L, lipids; n, nucleus.

Fig. 51. Electron micrograph of a similar synistial mass showing the complete dessolution of plasma membrane of one cell and partial dessolution of plasma membrane of the other (arrows), and the formation of vesicles due to the joining of adjoining plasma membranes (arrowheads). n, nucleus; m, mitochondria.



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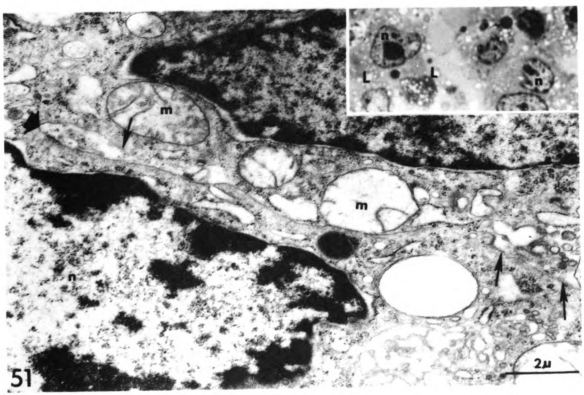

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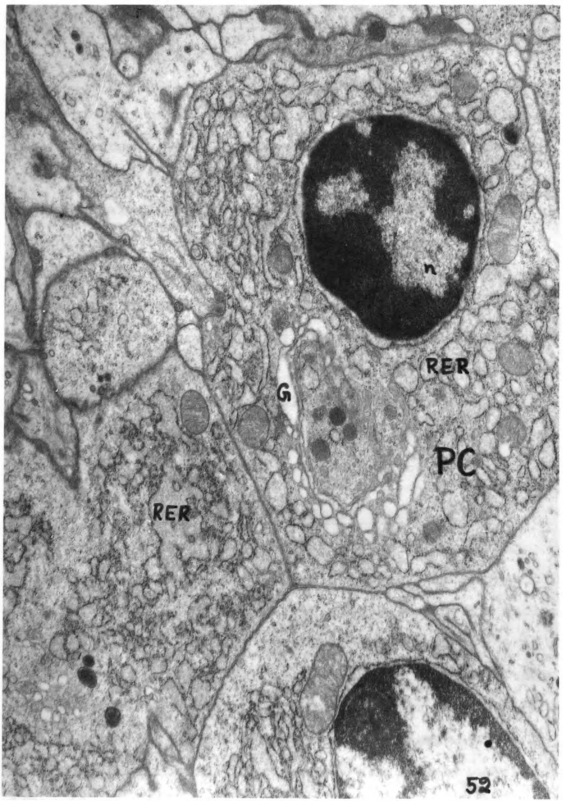
Fig. 52. Electron micrograph of spleen from 10 days platinum-treated mouse with regressing tumor. Note the plasma cell (PC) with well developed rough endoplasmic reticulum (RER). n, nucleus; G, Golgi.


n
RER
G
PC
RER
52

Effects of Cis-dichlorodiammineplatinum(II) on Sarcoma-180 Ascites Cells in Vitro; Light and Electron Microscopical Studies

The majority of S-180 ascites tumor cell population consists of lightly staining cells with a few dark cells, macrophages and polymorphic leukocytes. One can always find a spectrum of cells ranging between the lightly stained and dark cells. The nuclei in the majority of the cells are rounded to oval with one to two nucleoli. In a random section one can always find one or two mitotic figures (Fig. 53). At the electron microscope level the morphology of the Sarcoma-180 ascites cells is very similar to the solid Sarcoma-180 cells. The cellular organelles are loosely arranged all through the cell cytoplasm (Fig. 54). The cells usually show microvilli with filamentous cores on the surface.

Effect of cis-Pt-II in Vitro

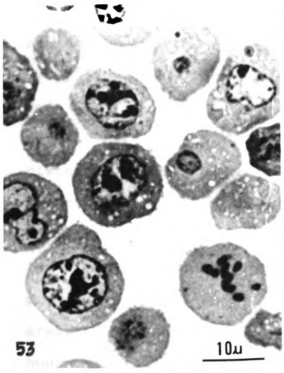
Fifteen to thirty minutes of cis-Pt-II (5PPM) treatment induces the concentration of all the microfilaments (60-70Å) into a discrete band around the nucleus (Figs. 55, 56). The origin of this band seems to be at the region of Golgi apparatus. The Golgi cisternae that are normally concentrated in a juxtanuclear position tend to separate out and along with the Golgi vesicles get uniformly distributed in and around the filamentous band. After 60 minutes of treatment the filamentous band around

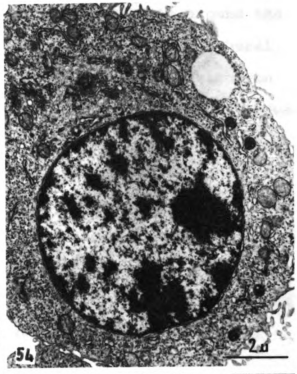
Fig. 53. Light micrograph of a random area from a 1μ thick section of a random block of Sarcoma-180 Ascites tumor cells. Note the normal morphology of the cells. Arrow points towards a cell in mitosis.

Fig. 54. Electron micrograph of a representative Sarcoma-180 ascites cell grown on normal medium.

Fig. 55. Light micrograph of Sarcoma-180 ascites cell after 15 minutes of cis-Pt (II) $\overline{(NH_3)_2}Cl_2$ treatment. Note the perinuclear band of (arrows) microfilaments.

Fig. 56. Electron micrograph of a Sarcoma-180 ascites cell showing the perinuclear band of microfilaments after 15 minutes of cis-Pt (II) $(NH_3)_2Cl_2$ treatment. Note the inward movement of the cellular organelles.


53
10μ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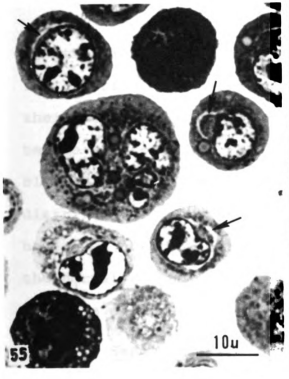
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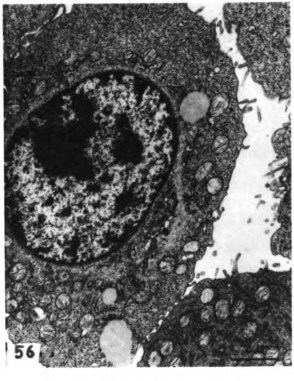

56

the nucleus disappears and the mitochondria that still appear normal in their morphology concentrate around the nucleus in the form of a distinct band. The peripheral cytoplasm shows a considerable decrease in its electron density. The chromatin material within the nucleus shows a granular and more uniformly dispersed appearance. The cisternae of the RER tend to become distended. This effect is very pronounced after 2 hours of platinum treatment (Fig. 57). The mitochondria shrink, become irregular in outline and are very electron lucent (Fig. 58). The progressive decrease in electron density in the peripheral areas of the cytoplasm is also very pronounced after 2 hours of platinum treatment.

The cells after varying intervals of such platinum treatment, if allowed to grow back on normal medium for a period of 24-36 hours, show a complete recovery of their normal morphology (Fig. 59). The mitochondria become spherical and the RER cisternae no longer appear bloated. The cellular organelles in general become evenly distributed throughout the cytoplasm. No mitotic figures have been observed after the recovery of the cells in the normal medium, but the replication of the centriole is quite common (Fig. 60).

Serum Analysis with Gel-electrophoresis

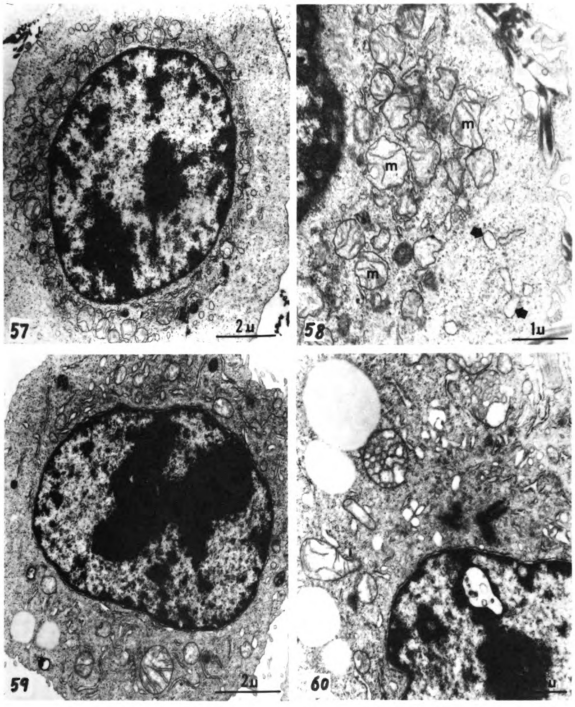
Serum protein analysis did not show any appearance of new bands or disappearance of old bands after cis-Pt-II

Fig. 57. Sarcoma-180 ascites cell after 2 hours of cis-Pt (II) $\overline{(NH_3)_2Cl_2}$ treatment. Note the perinuclear band of cellular organelles and the clear cortical cytoplasm.

Fig. 58. Part of the Sarcoma-180 ascites cell shown in Fig. 5, at a higher magnification. Note the shrunken irregular mitochondria (m) and the bloated RER cisternae (arrowheads).

Fig. 59. Sarcoma-180 ascites cell treated with cis-Pt (II) $\overline{(NH_3)_2Cl_2}$ for 2 hours and allowed to grow on normal medium for 36 hours. The cellular organelles recover their normal morphology and distribution.

Fig. 60. Part of a cis-Pt (II) $(NH_3)\overline{2Cl_2}$ treated cell allowed to grow on normal medium for 36 hours showing the replicated centriole (C). Note some of the mitochondria have irregular cristae.


57
2u
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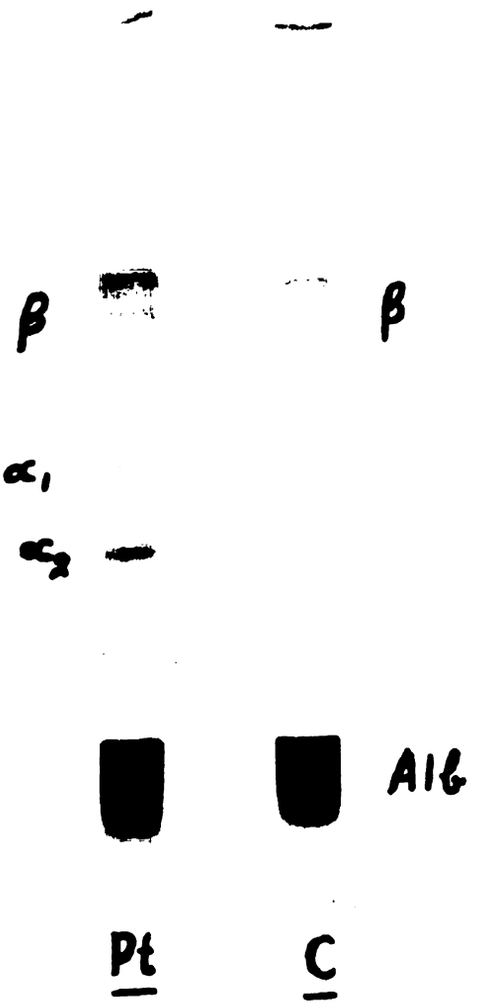
treatment. But from day 4 of platinum treatment to 10th day there was a marked enhancement of the α_1 and α_2 bands (Fig. 61). After 10 days of treatment the concentration of α_1 and α_2 bands starts dropping and comes back near to the non-treated mice serum by the 16th day of treatment.

No precipitation lines were observed in the agar gel diffusion between the S-180 saline extracts and the serum from platinum treated mice during and after regression of solid S-180.

Fig. 61. Serum analysis of control and 10 days platinum-treated mice with disc electrophoresis. Note the enhancement of protein bands in α region. Alb = albumin, Pt = cis-Pt (II) $(NH_3)_2Cl_2$ treated, C = control.



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DISCUSSION

Light microscopic studies by Worley and Spater (1952) on Sarcoma-180 have indicated three distince regions varying with the amount of lipids and the comparative development of the Golgi apparatus. Present studies with the electron microscope indicate the tumor to be alveolar in appearance. Each alveolus does show three distinct regions, again showing an increase in the lipid contents of the cells as one goes from the cortical region toward the central part. It seems true that the increase in the lipid contents of the tumor cell somehow relates to the process of necrosis. The maximum number of lipid bodies can be observed in the cells adjacent to the necrotic region. The cells in the necrotic region are usually rounded up with their lipids either extruded or possible converted into myelin-type figures. Similar myelin figure formations have been described by Weinberger and Banfield (1965) in the reticulum cell sarcoma. Weinberger and Banfield (1965) classified the process of necrosis into necrobiosis and coagulative necrosis. Coagulative necrosis has been further shown to contain

three distinct cell types designated A, B and C. However, no correlation has been made between these various cell types. In Sarcoma-180 the process of necrosis is very similar to coagulative necrosis but certain intermediary stages in the process are not suggestive of different cell types.

In addition to the spindle-shaped fibroblasts in the cortical region the tumor tissue has two distinct cell types. The larger of the two is less electron dense while the smaller is highly electron dense. Both cell types contain fibrils. The smaller, darker cells usually have the fibrils in the form of bundles while the larger and less electron dense cells have a more even distribution of fibrils. The two distinct cell types possible are different stages in the life cycle of the same cell or may represent one cell type which originates the other type. Indeed, present observations have shown a dark and a light cell still held together by the midbody connection indicating that two cell types are actually derived from a single parent cell by mitosis, or, it may indicate a fusion of two cells.

Only one type of desmosomal connection between the various cell types of Sarcoma-180 was identified. These desmosomal connections probably support the structural integrity of the tissue. Clarke (1970) has described two types of desmosomal connections in the

3-methylcholanthrene-induced mouse sarcomas. It is possible that the second type of intercellular junction may have escaped attention. Tight junctions have not been observed. Lack of tight junctions or specialized attachments are usually taken into indicate a lesser degree of cell-to-cell exchange. However, contact-mediated communication between cells cannot be ruled out (Clarke, 1970). According to Martinez-Palomo et al. (1969), lack of tight junctions may represent a morphological basis for the modification of intercellular communication and exchange of growth regulating substances.

Ultrastructural studies have revealed the presence of flattened canaliculi within the nuclei of the Sarcoma-180 cells. The canaliculi are attached to the inner nuclear envelope at one end and the other end is usually embedded into the mass of the nucleolus. The space of the canalicular system shows continuity with the perinuclear cisternae of the nuclear envelope, which in turn show continuity with the cisternae of the endoplasmic reticulum. Such a canalicular system has been described for numbers of rapidly growing and malignant tumors: the transplantable Novikoff hepatoma cells (Karasaki, 1970), Ehrlich ascites tumor (Yasuzumi and Sugihara, 1965), Yoshida ascites hepatoma (Locker et al., 1968), Rous sarcoma (Bucciarelli, 1966) and 6C3HED ascites lymphoma (Levine et al., 1968). A possible role in the transport

of nucleolar products via the canalicular system has been suggested by a number of authors (Babai et al., 1969); Terzakis, 1965; Clyman, 1963). Terzakis (1965), in the case of the human endometrium, suggested that the channels probably represent a passage for the ribosomal subunits from the nucleolus into the cytoplasm. Transport of RNA precursors from the perinuclear space into the nucleolus has also been suggested (Karasaki, 1970). The present studies have shown some flocculent material within the canalicular system and the role of this system in the nucleus remains to be worked out.

Present studies have revealed the existence of "paired cisternae" of the endoplasmic reticulum and the stacked remnants of the nuclear membranes during the mitotic cycle only. Paired cisternae have been described in some cell types during interphase (Kumegawa et al., 1968; Flickinger, 1969), in others during the mitotic cycle (Buck, 1961; Kelley, 1971) both in normal cell types (Flickinger, 1969; Murray et al., 1965) and tumor cells (Chang and Gibley, 1968; Leak et al., 1967). According to Hanaoka and Friedman (1970) paired cisternae are a common feature of rapidly proliferating cells. Different interpretations have been presented as to the origin of these paired cisternae. According to Chang and Gibley (1968) fragments of nuclear envelope contact each other to become stacked. According to Barer et al. (1961), the stacking of segments is achieved through

periodic flaking off of certain parts of the nuclear envelope and reformation at the place of flaking. The flaked off portion is stacked on the previously shed portion. Yet according to Kelley (1971) the nuclear envelope is doubled prior to disruption during metaphase. Present studies tend to support the latter interpretation since just after the breakage of the nuclear envelope at prophase it is almost always doubled. Again, paired cisternae can be made out in between the chromosomes. If the nuclear membrane portions double by folding over to form the paired cisternae then the area enclosed by such cisternae will be much less than is actually indicated in the electron micrographs. The paired cisternae disappear completely after metaphase and are never observed during the interphase stages. The new nuclear membranes after cell division probably arise from the vesicular system.

The arrangement of ribosomes in different cell types in different regions of Sarcoma-180 definitely suggests a difference in their metabolic activity. The cells in the cortical region have well developed cisternae of the endoplasmic reticulum with ribosomes forming polysomes. These cells are probably active in the synthesis of collagen.

The dark cells that are actively engaged in the phagocytosis of the necrotic cells always show ribosomes

evenly spread in the cytoplasm, whereas similar dark cells present in the intermediate region do show polysomes in their cytoplasm. Similarly, the light cells that have a well developed rough surfaced endoplasmic reticulum in the intermediate region show poorly developed endoplasmic reticulum with sparse to no ribosomes attached to its membranes. It seems obvious from the distribution of these cells and from the arrangement of the ribosomes that the viable cells in the intermediate region are probably involved in protein synthesis, while the cells in the necrotic region are destined to die and are probably being starved out by the surrounding cells. Lee, Krsmanovic and Brawerman (1971) have shown in their studies of Sarcoma-180 in tissue culture that the addition of nutrients to the starved cells leads to a rapid conversion of ribosomal monosomes into polysomes. However, these authors made no effort to differentiate the various cell types and it is very difficult to correlate this work with theirs. The very fact that there are two distinct cell types present in the tumor indicates a difference in their functional morphology. Yet, the exact role of each cell type in the intermediate region is not clear. In the necrotic region, however, the dark cells are mostly involved in the process of phagocytosis.

Two types of virus particles have been demonstrated in association with the Sarcoma-180 cells. The

cytoplasmic membrane associated 900Å viral particles are very similar to Leukemia type C virus in their morphology. Association of leukogenic virus particles with Sarcoma-180 has been shown earlier by Merekalova (1970). Lee, Krsmanovic and Brawerman (1971) have also published electron micrographs showing leukemia type viruses associate with Sarcoma-180 cell membranes. It is very hard to establish if these particles are the causal agent for the tumor. According to Merekalova (1970) the leukosis virus is just latent in the tumor cells and the transplantation of cells that produces the tumor may or may not produce leukosis. Present observations tend to support the above observations since injections of cell free extracts from Sarcoma-180 into newly born mice do produce leukemia within 10 days without the formation of tumor. The mice die within 3 weeks of such injections, so no observations could be made on the development of the tumor. It is possible that these particles may be capable of tumor production but the animals die of leukemia long before the tumor can be induced. However, injections of cell free extracts into adult mice did not produce either leukemia or the tumor even after 3 months showing immunity toward such injections. The actual formation of C type virus can be observed at the cytoplasmic membranes and has been described earlier. It is believed that the inner electron dense ring measuring 500Å with electron lucent center is

actually the viral RNA which is tightly twisted into a coil as has been proposed by Kakefuda and Bader (1969). This coiled RNA in turn is further coiled into the hollow sphere against the inner surface of the intermediate membrane. The hollow sphere after reaching a maximum size is pinched off as a mature virion.

The nucleus associated particles measure about 425Å in diameter and resemble very much the polyoma virus in their morphology. Although large numbers of serial sections under the electron microscope have been screened but such particles as yet have been observed in very few cells. It is possible that these particles exist all the time without being morphologically identifiable. It has already been pointed out (Dourmashkin, 1962) that only small numbers of cells presumably infected by polyoma virus produce viruses at any one time. The difficulties in studying the sequence of development of these virus particles through their life cycle using the electron microscope have been pointed out. The presence of these particles in small numbers of cells has made it difficult to isolate these particles. It is hard to say if this virus is the causal agent for Sarcoma-180. Polyoma type-D particles have been noted in the cytoplasm of the infected cells by several workers (Banfield et al., 1959; Bernhard et al., 1959). However, present studies failed to show any particles resembling polyoma viruses in the cytoplasm.

The virus particles when present outside the cell are usually enclosed by a common cytoplasmic membrane after the lysis of the cell. The mechanism of infection with these viruses is not yet understood.

Thus far two types of viruses--RNA virus particles and presumably DNA nuclear-associated particles have been observed within the same tumor cell type. On the basis of these studies it is hard to decide if the tumor is due to either of these two viruses or whether one or the other is acting as a helper virus.

In addition to the two types of virus particles in the viable tumor cells, the polymorphonuclear leukocytes wandering in the tumor mass show 225Å particles within the granular matrix of the cytoplasm. Wanson and Tielemans (1971) have not only demonstrated but have actually isolated similar particles that are 35-45 mμ in diameter from the ascitic polymorphic leukocytes of rabbit. Wanson and Tielemans (1971) have shown these particles to be glycogen in nature. Present studies indicate such glycogen particles in the leukocytes to be partially digested by RNase or salivary amylase. After such digestion, although the size of each particle stays the same, one can notice the etching effect all over the structural unit. Accumulations of such particles are common in front of the nuclear pores on the cytoplasmic side but these particles have not been observed within the nucleus.

Present studies have shown that cis-Pt-II has multiple effects on the cells of the solid Sarcoma-180 in mice with a single i.p. injection. The sequential changes observed with the electron microscope on the morphology of S-180 cells during regression such as inhibition of mitotic activity, formation of giant cells, accumulation of large amounts of lipids, appearance of cells of reticuloendothelial system, dissolution of plasma membranes, resulting in the formation of syncitial mass and the appearance of large numbers of active leukocytes, collectively seems to be the cause of regression of the S-180 tumor after cis-Pt-II on the morphological details of tumor cells including the inhibition of mitotic activity seems to be of secondary importance. The primary effect is the possible enhancement of the host immune system against S-180 cells as indicated by the appearance of lymphocytes, plasma cells and macrophages in the regressing tumor mass after platinum treatment. Of course, the inhibition of mitotic activity is important to check the growth of the tumor initially, until the host immune system is made effective. And by the time mitotic activity comes back, the immune system is quite active and tumor is not able to grow back. The studies of Conran and Rosenberg (1971) with cis-Pt-II + zymosan and cis-Pt-II + hydrocortisone on the acceptance and rejection of S-180 transplants by Swiss white mice and BALB/c mice also support

the hypothesis of the enhancement of the host immune system after platinum treatment. This hypothesis is also further strengthened by the fact that mice treated and cured for S-180 tumors and Dunning ascites leukemia do not take up any further transplantations (Rosenberg et al.; personal communication, Richard, Sleight and Rosenberg, 1970).

On the contrary, Khan and Hill (1971; 1971) and Khan, Albayrak and Hill (1972) have shown cis-Pt-II to be a immunosuppressive drug. The inhibition of phytohemagglutinin (PHA) induced blastogenesis in human lymphocytes, the suppression of antibody plaque forming spleen cells in the mice sensitized to sheep erythrocytes and the prolongation of the life of skin graft in the mice against H_2 histocompatible barrier after cis-Pt-II treatment probably is not due to the immunosuppressive action of platinum compound as suggested by the above authors. All the above studies of Khan and Hill (1971; 1971) were short term, where they only saw the effect of cis-Pt-II on inhibition of blastogenesis, of lymphocytes and antibody plaque forming spleen cells up to 3-6 days after platinum treatment. Similarly studies of Khan, Albayrak and Hill (1972) on the survival of skin grafts against H_2 histocompatibility barrier have shown that the drug is most effective during the initial phases of graft rejection and maximum prolongation was seen when the drug was given at the time of transplantation. A delay in the administration of

platinum compound led to abolition of the effect. The drug was also effective when given prior to transplantation. Thus it seems the platinum compound is really not a immunosuppressive drug, but being a strong antimitogenic compound it would also inhibit the blastogenesis of lymphocytes induced by PHA and cellular elements required for the graft rejection, thus leading to the prolongation of the life of the graft.

Present investigations; studies of Roberts and Pascoe (1972); Gale, Howle and Walker (1971); Harder and Rosenberg (1971) and the toxicologic investigations of Kociba and Sleight (1971) have shown that cis-Pt-II is a strong mitotic inhibitor, particularly of the cells of rapidly proliferating tissues, but this inhibition is reversible in a few days. Thymus and spleen shrinks during the first 4-6 days of platinum treatment in tumor-bearing mice. But after that, there is a regeneration and the spleen and thymus enlarges by day 12 of treatment. The light microscopical studies do show a depression in the population of pyroninphilic cells in the spleens of platinum-treated animals, but their number gradually increases from 6th day of the treatment. Kociba and Sleight Sleight's (1971) studies also clearly show an initial drop in the population of lymphocytes and neutrophils in male rats treated with different doses of cis-Pt-II for

first three days, but show a gradual increase above the normal population from 5-20 days of treatment.

Macrophages appearing in the mass of the tumor after cis-Pt-II treatment, might be are acting as immune macrophages, directly destroying the target (tumor) cells by phagocytizing them or by releasing certain cytotoxins over the surface of the tumor cells by releasing lysosome like bodies. Similar types of interactions of immune macrophages with target cells have also been studied by Chambers and Weiser (1972) in C57BL/6 mice that had recently rejected a primary Sarcoma I ascites tumor and were given a second i.p. dose of Sarcoma I cells.

The appearance of a large number of leukocytes in the mass of regressing tumor becomes necessary for the removal of the cellular debris. The increase in the number of glycogen particles within their cytoplasm probably is due to their increased metabolic activity.

Appearance of giant cells after platinum treatment is clearly due to the inhibition of mitotic activity but not of growth, i.e., protein synthesis, which is further supported by the presence of number of centrioles in a single cell. Light microscopical studies show beyond doubt that these giant cells have a single nucleus. The 3-5 nuclei seen in giant cells in the electron microscope is probably due to the thin sectioning of single very irregular nucleus at a number of places.

The continuous accumulation of lipids in both the giant and regular tumor cells after platinum treatment may stop the metabolic activities of the cells, thus resulting in their disintegration. This probably is the reason that giant cells disappear completely within 3-4 days of their appearance. At this point it is interesting to note the similarity between the process of necrosis in nontreated tumor, which is preceded by increase in lipids.

Fine structural studies of the tumor cells in vitro after Cis-Pt-II treatment show a change in their morphology, such as the appearance of perinuclear band of microfilaments and concentration of cellular organelles around the nucleus, etc. The present in vitro studies also show that the platinum compound when used in concentrations equivalent to the therapeutic dosage in the adult (8.0mg/kg or 5PPM) for shorter periods of up to 2 hours is not toxic enough to kill the Sarcoma-180 cells. Cells treated with platinum compound for up to 2 hours when allowed to grow on normal medium, recover their normal morphological details, although no mitotic activity is observed even after 36 hours. However, the replication of centrioles is observed. It is therefore, concluded that the platinum compound when used at a therapeutic dose level inhibits mitotic activity but is not toxic enough

to kill the Sarcoma-180 cells *in vitro* when treated for short periods.

It has been shown in *in vivo* studies by Hoeschele and VanCamp (1971) that a large percentage of the injected platinum compound is excreted within 2 hours of the initial injection and that there is no selective uptake of the drug in Sarcoma-180 tumor tissue. This means that the concentration of the platinum compound *in vivo* is extremely low when compared to the concentration used *in vitro* studies, thus clearly showing that the regression of the tumor cannot be caused by the direct cytotoxicity of the *cis*-Pt-II, but must be mediated via some other means. The present investigations also support the hypothesis that the platinum compound somehow reacts and exposes the surface antigens or changes the antigensity of the tumor cells resulting in an increased cellular immune response by the host, as also has been proposed by Rosenberg (1971).

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