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Bovine Ocular Squamous Cell Carcinoma : In Vitro Investigations of a Viral Etiology

Mary A. Anson

BOVINE OCULAR SQUAMOUS CELL CARCINOMA:

IN VITRO INVESTIGATIONS OF A VIRAL ETIOLOGY

BY

MARY A. ANSON

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IN VITRO INVESTIGATIONS OF A VIRAL ETIOLOGY

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable as meeting the thesis requirement for this degree, but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

_____ Thesis Advisor / Date

_____ Head, Microbiology Department Date

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INTRODUCTION

Bovine ocular squamous cell carcinoma, commonly known as cancer eye, is a common and economically important problem to the U.S. cattle industry. This neoplasm is particularly significant in aged breeding stock. The tumor usually originates from the squamous epithelial cells of the corneal-scleral junction and the nicitating membrane of the eye. Cancer eye lesions progress from small pin point plaques to large tumors involving the eye and surrounding tissue. This disease causes partial or total loss of vision in the affected eye and the cancer may metastasize. Economic loss results from a shortened productive life span of the animal plus condemnation or reduction in salvage value of the carcass at slaughter. The U.S. cattle industry annually loses an estimated $20 million due to bovine ocular squamous cell carcinoma (27).

During a two-year survey of bovine neoplasms in Denver abbatoirs, USDA Meat Inspection veterinarians reported 62% were squamous cell carcinomas, 4% were early squamous cell carcinomas, 5% were premalignant epidermal papillomas, and 10% were premalignant epidermal plaques (38). The lesions usually progress sequentially from epidermal plaques to papillomas to carcinomas with reported malignancy rates of 91% (41).

The exact etiology of the disease is unknown. High nutritional levels, prolonged exposure to ultraviolet irradiation or sunlight, genetic susceptibility, age, and lack of pigment in the eye and peri-orbital skin are implicated in the incidence of cancer eye (4).

Initiation of the disease may involve the interaction of a virus with the above factors. Investigations seeking potentially oncogenic viruses possibly associated with cancer eye have been limited.

The objectives of this research were to: investigate the properties of tumor cell cultures derived from bovine ocular squamous cell carcinoma and its precursor lesions, determine if viral agents are present in or can be induced in tumor cell cultures, and attempt to demonstrate viral antigens in the tumor cell cultures.

LITERATURE REVIEW

Cancer of the eye (ocular squamous cell carcinoma) was recognized as a bovine disease in the latter part of the nineteenth century (31). The neoplasm has been reported in several breeds, including Ayrshire, Brahman, Brown Brahman, Brown Swiss, Charolais, Durham, Guernsey, Hollandensa, Holstein, Javanese Mongolian, Jersey, Normandy, and Shorthorn, but the Hereford breed has been implicated in most reports (3,14,37,39). The disease is not geographically limited and occurs in cattle in Africa, Asia, Australia, Europe, Great Britain, North and South America, and New Zealand (3). Age susceptibility patterns of cancer eye are similar to those of other cancers. The disease seldom occurs in young cattle under four years of age, but peaks in incidence in animals aged seven to nine years (14). During a 20 year nutrition experiment involving 435 range beef cows, Anderson et al. found a definite association between high nutritional levels and an increased rate of cancer eye in cattle six to nine years of age (5). Irritation of the eyes by dust, sand, chemical, or insects have been proposed as factors contributing to the disease (4,44).

Studies of genetic traits associated with susceptibility tó ocular squamous cell cell carcinoma have concerned the degree of pigmentation of the corneoscleral membrance and the eyelid. Anderson concluded that the genetic effect on susceptibility is mediated primarily through pigment (3,4). Cancer eye lesions may occur in any animal lacking pigment in the nicitating membrane, corneoscleral junction or eyelid.

Sunlight or more specifically the ultraviolet (UV) component of

sunlight also pays a role in the development of ocular squamous cell carcinoma. Anderson and Skinner have reported that the frequency of cancer eye is proportional to annual hours of sunshine and increases with higher altitude or lower latitude (2). Studies have shown that a carcinogenic response in mice is not elicited by the whole spectrum of sunlight but is limited to wavelengths of 2800 to 3200 angstroms (15). UV irradiation of cell monolayers has been demonstrated to enhance susceptibility to viral transformation (32).

Herpesviruses have been reported to transform cells in vitro (29,43). Malignant transformation of hamster embryo cells 96 days after inoculation with infectious bovine rhinotracheitis (IBR) virus at an input multiplicity of less than one was noted by Michalski and Hsiung (35). It has been shown that UV irradiation of oncogenic viruses does not decrease the oncogenic potential of these viruses as rapidly as it decreases the cytopathic effect (23,24). Transformation of hamster embryo fibroblast cells after infection by UV irradiated herpes simplex virus type 1 (HSV-1) or UV irradiated herpes simplex virus type 2 (HSV-2) was demonstrated by Duff and Doller in 1973 (22). UV inactivated herpesviruses have been shown to transform some mammalian cells (13,30,43). A search of the literature revealed no reports on transformation of bovine cell monolayers by herpes viruses.

A human disorder, xeroderma pigmentosum, resulting in ocular squamous cell carcinoma is caused by an enzymatic defect in excision and repair synthesis of DNA damaged by UV light. In 1972, Cleaver

et al. demonstrated no difference in UV sensitivity or in the level of DNA repair synthesis between cultured conjunctival cells from Hereford and Angus cows (18). Therefore, cancer eye in Herefords is apparently unrelated to xeroderma pigmentosum and is not attributable to a defect in DNA repair.

In 1956, Russell et al. described, from clinical and histopathological examinations of 830 abnormal eyes, the pathological anatomy of bovine ocular squamous cell carcinoma and its benign precursor lesions (44). A definite sequence in lesion appearance was noted and three phases were described: (1) plaques are opaque areas of hyperplastic epithelium, (2) papillomas represent a connective tissue core with multiple frond-like projections, (3) invasive carcinomas are large, protruding, polypoid masses. Ocular tumors originate most frequently at the corneal-scleral junction or limbus of the eyeball. Although the lesions usually occur sequentially, it has been observed that they may occur spontaneously or may undergo disappearance, reappearance and a great degree of bilaterality and multiplicity (22). The usual progression of lesions through a plaque-papilloma-carcinoma sequence resembles the pattern of tumor development in the virus induced Shope papilloma of rabbits (47).

Early attempts by Loeb to transplant cancer eye lesions were unsuccessful (31) as were Wynne's transmission experiments with tumor extracts (53). Russell et al. described the occurrence of intranuclear, spherical eosinophilic inclusions in all three types of eye lesions (44). Electron micrographs by Dmochowski et al. in 1958

revealed these "inclusions" to be nucleoli with shape and size abnormalities (21). These researchers experienced difficulties in obtaining primary cell culture growths of cancer eye neoplasms. Sykes et al. in 1961 observed characteristic changes including cytoplasmic vacuolization, cytoplasmic inclusions, and multinucleated cells, in cell cultures derived from bovine ocular squamous cell carcinoma and its precursor lesions (53). No virus isolations were reported. During an etiologic study of infectious bovine keratoconjunctivities (IBKC) in 1964, Sykes et al. reported testing 2 herpes-like viruses isolated from a plaque lesion and from a carcinoma (54). These two isolates along with infectious bovine rhinotracheitis (IBR) virus and a herpesvirus isolated form cattle with IBKC, when inoculated into the conjunctiva of yearling Herefords, subsequently induced symptoms of IBKC. Significantly less severe signs were produced by the carcinoma isolate. No serological evidence was presented to identify the plaque and carcinoma herpes-like viruses as IBR virus. No further work on these isolates has been reported. IBR virus and IBKC virus have been shown to be identical (16). In a limited study of bovine eye tumors from a single abbatoir, Taylor isolated IBR virus from 15 of 32 eye tumors but from none of 20 apparently normal eyes (55). Several researchers have been unsuccessful in attempts to isolate viruses from bovine ocular squamous cell carcinomas and precursor lesions (27,53,57).

After initial infection, herpes viruses may establish long term latent infections in their hosts. Recrudescences follow stress

situations such as exposure to sunlight or wind, fever, or hormone treatment (29).

Herpesviruses, the only DNA viruses which cause malignant tumors in their natural hosts, have been linked to tumors of fowl, frogs, primates, and possibly human cervical cancer (30). From studies of HSV-2 in cervical cancer cell cultures, Aurelian has suggested that some or all of the tumor cells harbor the viral genome in a partially repressed state (9).

Virus expression has been induced in several types of non-virus producing tumor cell cultures. HSV has been released from spinal ganglion grown in organ culture (50) and human cervical carcinoma cells cultivated at a high pH (11). Glucocorticoids have been shown to stimulate murine type-C virus from non-producing BALB/K3T3 mouse cells (59) and to cause Burkitt lymphoma cells to produce Epstein-Barr virus (EBV) (30).

Recent additions to the bovine herpesvirus group include: DN599, isolated by Mohanty et al. (36) in 1971, FTC, isolated by Smith et al. (48) and V11, isolated by Van Der Maaten (56). Potgieter and Mare determined that these three viruses were antigenically identical (40). Neutralization tests revealed no serological relationship to IBR.

Viral antigens have been detected by immunofluorescent techniques in cells transformed by polyoma virus, SV-40, adenovirus, and herpesviruses (26). Evidence of a common antigen between Marek's disease virus, EBV, IBR virus, and bovine ocular squamous cell carcinoma

preparations has been established by Evans et al. (25). Similar studies involving herpesvirus DN599 have not been performed.

MATERIALS AND METHODS

Medium

Immediately after excision, ocular tumors were placed in Hank's balanced salt solution (HBSS) (Grand Island Biologic Co., Grand Island, N.Y.) containing penicillin (1,000 units/ml), streptomycin (1,000 ug/ml), kanamycin (1000, ug/ml), and amphotericin B (50 ug/ml). Initial growth medium was Eagles Minimal Essential Medium (10% MEM) (Grand Island Biologic Co., Grand Island, N.Y.) in Earle's balanced salts supplemented with nonessential amino acids, 10% fetal calf serum, 0.01% pyruvic acid, 0.03% L-glutamine, 0.5% lactalbumin hydrolysate, and containing similar concentrations of the above mentioned antibiotics. After two initial cell passages, similar growth medium containing lower concentrations of penicillin (100 units/ml), streptomycin (100 ug/ml), and kanamycin (100 ug/ml) was used. This growth medium was also employed for culturing bovine fetal spleen (BFS), bovine fetal adrenal (BFA), bovine conjunctival (BCJ), bovine corneal scleral (BCS), rabbit kidney (RK), and rabbit spleen (RS) cells. For maintenance medium (2% MEM), the fetal calf serum concentration was reduced from 10% to 2%. Hepes buffer (5.9%) (ICN Pharmaceuticals, Inc., Life Sciences Group, Cleveland, Ohio) was added to growth medium for the high pH experiment. The pH was raised to 8.4 using 1 N NaOH.

Plaque medium consisted of equal volumes of double strength Eagles's MEM supplemented with 0.01% pyruvic acid and melted 1.4% Ion agar (Colab Laboratory Inc., Science Park, Glenwood, Ill.) containing (40 ug/ml) DEAE dextran. The mixture was equilibrated to 45 C prior to use as an overlay.

Cell Cultures

Normal conjunctival cells (BCJ) for culture were obtained from a one month old Hereford crossbred calf. Normal corneoscleral (BCS) were taken from a slaughtered cow. Tumors were collected from the eyes of cattle slaughtered at abattoirs in Huron and Sioux Falls, S.Dak. Similar tumors were obtained from cattle on a Hereford ranch near Lake Andes, S. Dak.

Cell cultures used in this study were derived from adrenal and spleen cells collected from bovine fetuses (BFA,BFS). Rabbit kidney and spleen (RK,RS) cell cultures were obtained from a young New Zealand White rabbit. Each cell culture was propagated and sub-cultured using methods described by Merchant et al. (34). Stock cultures of each cell type were prepared by suspending trypsinized cells in growth medium containing 7.5% dimethyl sulfoxide (Fisher Chemical Co.) and stored at -70 C.

Explant Cultures (EC)

Gelfoam (Upjohn Co., Kalamazoo, Mich.) gelatin sponge was cut into 1x2x3 mm rectangles and saturated with 10% MEM for 1 hr. Each gelfoam raft was placed in a 60 mm petri dish (Falcon Plastics, Oxnard, Calif.) and a 1-2 mm^3 section of tissue was applied on the raft. Five ml of 10% MEM was added and the cultures were incubated at 37 C under 5% CO_2. Medium was changed every 7-14 days and the decanted medium was stored at -70 C.

Slide Culture Method

Chambered tissue culture slides (Lab-Tek Products Division,

Miles Laboratories, Inc., Naperville, Ill.) were seeded with 1.5-2.0 ml of growth medium containing a sufficient number of cells to form a monolayer in 24 hrs. These cultures were incubated at 37 C as previously described for tissue culture.

Virus Isolation Attempts

A portion of each ocular tumor was ground with fine silica sand and mixed with maintenance medium to make an approximate 10% suspension. The suspension was centrifuged at 3,000 xG for 20 min. at 4 C. The resulting supernatant was filtered through a 0.45 micron membrance filter (Swinny - 25 Syringe Filter, Millipore Corp., Bedford, Mass.) and inoculated onto monolayers of FBS and FBA cells. The cell cultures were incubated as previously described and examined daily for cytopathic effect (CPE). If there was no CPE in 10 days, cultures were frozen, thawed, and an aliquot inoculated onto fresh FBA and FBS cells. After four blind passages without CPE, cultures were considered negative for virus.

RK, RS, BCJ, BCS, and BFS cell monolayers were inoculated with medium removed from tumor cell and organ cultures. Inoculation and incubation of cell cultures were carried out as previously described. Monolayers were observed daily for cellular changes. At 7-14 days the cultures were subjected to three cycles of freezing and thawing and reinoculated onto fresh monolayers. An alternate procedure involved harvesting the culture fluid and reinoculating it onto fresh monolayers.

Staining Procedures

Acridine orange (AO) stain: Chambered slide cultures of tumor

cells or BFS cells and BCS cells previously inoculated with medium removed from tumor cell and explant cultures were fixed in Carnoy's fluid for 20 min. (33). Rehydration was by sequential dips in 80%, 70%, and 50% ethanol and distilled water. The slides were placed in McIlvaine's buffer pH 3.8 for 8 min. and then stained for 5 min. in 0.01% acridine orange solution. The monolayers were rinsed 2-3 minutes in two changes of McIlvaine's buffer, blotted and mounted in fresh buffer (33). Slides were examined immediately with a fluorescence microscope and photographed.

Hematoxylin and eosin stain: Slide cultures were fixed in Carnoy's fluid 20 min and rehydrated by sequential dips in 95%, 80%, and 70% ethanol and distilled water. The slides were then stained with hematoxylin and eosin (7).

Enzyme Digestion Test

Chambered slide cultures of tumor cells or BFS cells and BCS cells previously inoculated with medium removed from tumor cell and explant cultures were used. Cell cultures were flooded with 0.02% pepsin in acetate buffer at pH 2.0 for 1 hr at 37 C. Excess buffer was removed by blotting. Mayor's procedure was followed by applying 0.01% DNase in 0.025 M Veronal buffer containing 0.003 M $MgSO_4$ to the slide cultures for 30 min at 37 C (33). Enzyme digestion was detected by subsequent staining with acridine orange as previously described.

Serum Neutralization (SN) Test

Serum neutralization titers were determined using the microtiter

method described by Black (12).

Indirect Immunofluorescence Test (IIF)

Slide cultures of BCJ cells were infected with herpes virus DN599 and incubated as previously described until cytopathic effect reached 50%. Cells were fixed for 20 min in cold acetone (-20 C). The serum antibody levels against herpes virus DN599 of cows with ocular tumors were determined by twofold dilutions beginning with a 1:2 dilution in phosphate buffered saline pH 7.2 . The infected cell cultures were flooded with each serum dilution and incubated for 2 hrs at 37 C. The procedure described by Assaf et al. was then followed (8).

Slide cultures of tumor cells were tested for the presence of IBR and DN599 antigens using known positive antisera by the method of Assaf et al. (8).

Electron Microscopy (EM)

Ultrathin sections: Cell cultures were scraped from the surface of the culture flasks with a rubber policeman. Cells were fixed in Veronal-acetate buffered 3% glutaraldehyde (Polysciences, Inc., Warrington, Penn.) pH 7.2-7.4 overnight at 4 C (20). After two rinses in Veronal-acetate buffer, specimens were post-fixed in Veronal-acetate buffered 2% osmium tetroxide for 1 hr. The osmium tetroxide was removed by three 5 min. rinses in the buffer. Dehydration included five sequential changes for 5 min. each of 50% acetone, 75% acetone, 100% acetone, 100% acetone, and 100% acetone. Cells were infiltrated with a 1:1 mixture of acetone and plastic (Spurr)

(Polysciences, Inc., Warrington, Penn.) for 1 hr. This was removed and the plastic added for overnight infiltration at room temperature. Cells were embedded in plastic and placed in a 60 C oven until polymerized. Thin sections were cut on a LKB Huxley ultramicrotome (LKB Instruments, Rockville, Md.) and collected on 300 mesh carbon and colloidion-coated grids. The thin sections were stained with aqueous 2% uranyl acetate for 20 min and rinsed in freshly boiled CO_2-free distilled water. The sections were then stained for 30 sec. with lead citrate. Several rinses in CO_2-free H_2O were used to remove excessive lead citrate. The grids were examined in a Hitachi HU-12 electron microscope.

Negative stain: Cells and medium were harvested by a freeze-thaw cycle followed by centrifugation at 140,000 xG for 30 min at -40 C in an International Preparative Ultracentrifuge Model B60 (International Equipment Co., Needham Heights, Mass.). The cell pellet was resuspended in 0.2 ml of sterile water. A 4.0% solution of phosphotungstic acid, pH 6.5, 0.1% bovine serum albumin, water and cell suspension was mixed 1 drop/ 1 drop/ 20 drops/ 2 drops, respectively. The suspension was sprayed with a nebulizer (Pelco Corp., Corp., Austin, Tex.) onto a 300 mesh carbon and colloidion-coated grid. The grid was immediately examined in a Hitachi HU-12 electron microscope.

Ultraviolet Light Irradiation (UV)

UV of slide cultures: Medium was decanted from slide cell culture monolayers. Each slide culture was placed on a platform

shaker with a General Electric germicidal lamp G1578 13 inches above the shaker. Cultures were rotated at 100 RPM at room temperature and irradiated for 20 sec. Maintenance medium was added and cultures were incubated for 5-7 days prior to staining. Culture medium was removed, stored at -70 C and later inoculated onto RK and RS cells. If no cellular changes were noted in 14 days, the cells were scraped and inoculated onto fresh RK or RS monolayers.

Plaque Assay

Cultures of BFS, BCJ or BCS cells were propagated in 60 mm petri dishes. When confluent monolayers were formed, the growth medium was removed and 0.5 ml of tumor cell culture medium was inoculated onto duplicate plates. This medium was adsorbed 1-1/2 hr at 37 C. Excess inoculum was decanted and 5 ml of agar overlay was added to each plate. After the agar was allowed to solidify, plates were inverted and incubated at 37 C in a humidified, 5% CO_2 atmosphere and observed daily for plaque formation. After removal of the agar overlay, equal volumes of 10% crystal violet and phosphate buffered formaldehyde pH 7 were added to fix and stain the cells. The stain was removed after 5 min and the plaques were counted.

Virus Induction Experiments

Glucogenic coricosteroids: Tumor cell cultures were propagated as previously described in 60 mm petri plates.

IdU (5-iodo-2'-deoxyuridine) (Calbiochem, San Diego, Calif.) was dissolved in 0.2 N NaOH at a 40 mg/ml concentration and was then diluted with growth medium to a final concentration of 40 ug/ml.

Dexamethasone (Sigma Chemical Co., St. Louis, Mo.) was initially dissolved in 95% ethanol at a concentration of 10^{-3} M and diluted with growth medium to 10^{-6} M. For virus induction, cell cultures were treated with 40 ug IdU per ml for 24 hr within 1 day after plating. Cell cultures were treated with dexamethasone (10^{-6} M) for 24 hrs at daily intervals for five days after induction with IdU. Each reagent was removed by washing the cell cultures three times with 5 ml of fresh 2% MEM. After dexamethasone treatment, the culture medium was harvested every 24 hr for 5 to 6 days and fresh 2% MEM was added to each culture. Harvested medium was stored at -70 C. Potential virus yield was assayed by the plaque method previously described.

High pH: Trypsinized tumor cells, BCJ and BCS cells were suspended in pH 8.4 growth medium, seeded in 25 cm^2 flasks and incubated at 37 C without CO_2. Following monolayer formation pH 8.4 maintenance medium was added and incubation continued. Cultures were observed daily for cellular changes. Culture medium was harvested weekly, stored at -70 C and fresh maintenance medium was added. When cultures underwent degeneration they were subjected to three cycles of freezing and thawing and the supernatants were stored at -70 C. Supernatants were later inoculated onto RK and RS monolayers and observed daily. If no cellular changes were noted in 14 days, the cells were frozen, thawed, and reinoculated onto fresh RK monolayers.

RESULTS

Virus Isolation

No viral agents were recovered from tissue homogenates of the 31 bovine ocular plaque, papilloma, and carcinoma lesions processed during this study.

Cell Cultures

Cell cultures were initially obtained from 16 of 17 bovine ocular tumors and from normal conjunctival and corneoscleral tissues. The sources and characteristics of these cultures are summarized in Table I. Uniformity in growth and epitheloid morphology were characteristic of normal BCS and BCJ cell cultures (Fig. 1). Evidence of transformation or altered cellular morphology, such as irregular growth, was noted in all tumor cell cultures. Cultures in the first passage exhibited multinucleated giant cell formations, cytoplasmic vacuolization, and feathering of the outer cytoplasmic membrane (Fig. 2). Palisading of cells was consistently observed in cultures 2, 3, 5, and 7 (Fig. 3). Tumor cell cultures at each passage level exhibited the following peculiarities: cytoplasmic vacuolization, cytoplasmic inclusions, ruffled cytoplasmic membrances, and pleomorphic nuclei of variable size with prominent nucleoli (Fig. 4). These characteristics were not observed in normal BCJ and BCS cell cultures at passage levels similar to those of the tumor cell cultures. A well-defined, fibrillar cytoplasm was noted as unique to tumor cell culture #7 (Fig. 5). Syncytial formations were frequently observed in #7 (Fig. 6).

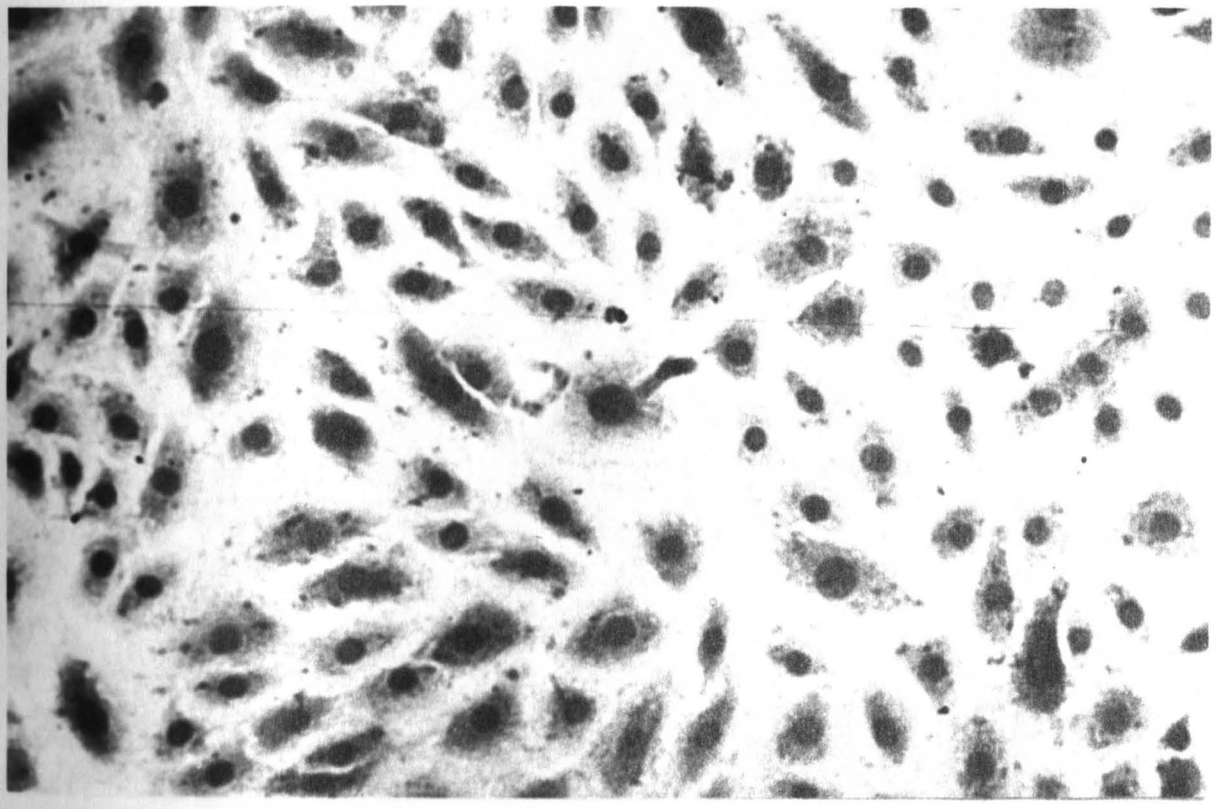
Table I. Cell Culture

ID. #	Current Passage Level	Tumor or Tissue Cultured	Tumor Location	Comments
1A	40	plaque	sclera	viral particles on EM
2	28	papilloma	cornea	palisading cells
3	21	papilloma	corneal-scleral junction	palisading cells
5	25	papilloma	entire eyeball	palisading cells
7	31	early carcinoma	eyeball	palisading cells fibrillar cyto-plasm syncytia
32	36	nicitating membrane	C-S* papilloma	enlarged cells
99	36	nicitating membrane	C-S papilloma	few vacuoles
NBCJ	34	corneal-scleral junction	-----	uniform morpology and growth
NBCS	28	conjunctiva	-----	uniform morpology and growth

C-S*, corneoscleral

Figure 1. Characteristically uniform morphology of normal bovine conjunctival cell monolayer. Hematoxylin and eosin stain. X 215.

Figure 2. First passage bovine ocular tumor cell culture #5,. Feathering of the cytoplasmic membrane (M) and cytoplasmic vacuole were noted in the multinucleated (arrows) giant cell. X 550.



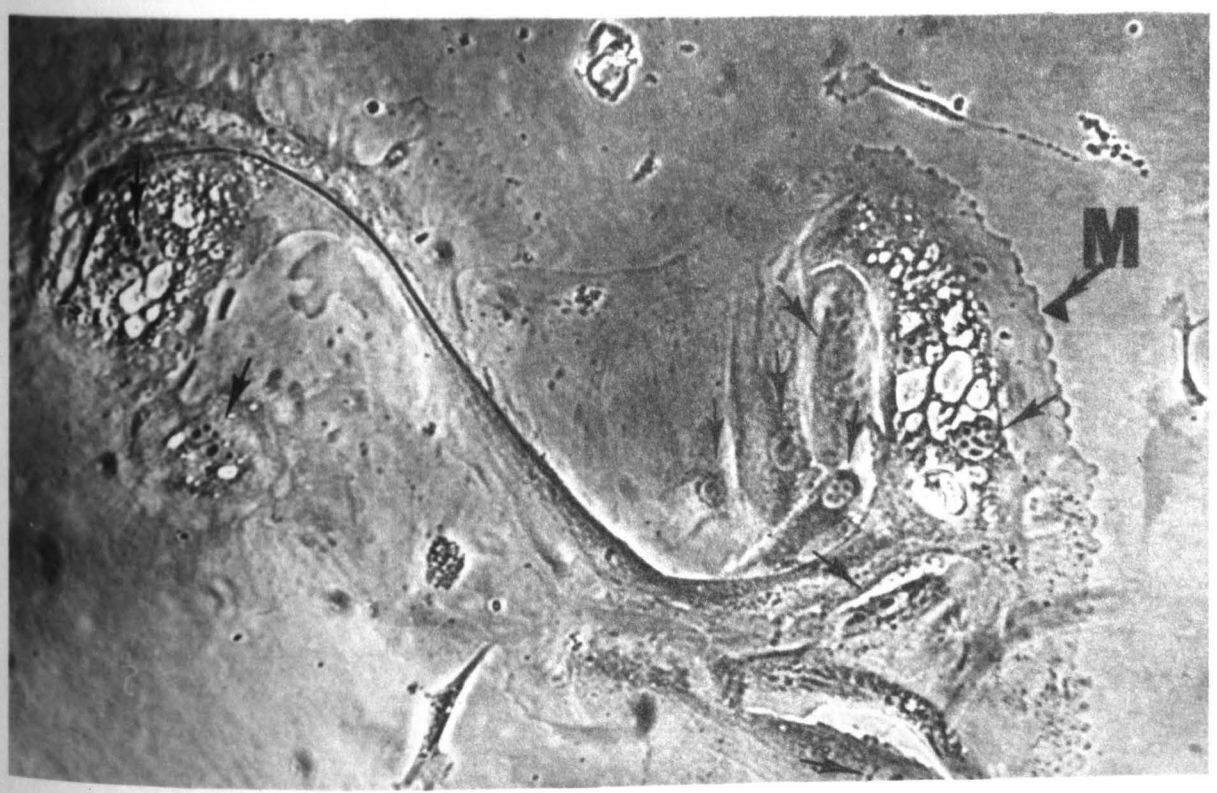

M

Figure 3. Bovine ocular tumor cell monolayer #3 exhibiting palisa
of cells. Hematoxylin and eosin stain. X 215.

Figure 4. Bovine ocular tumor cell culture #1A illustrating cyto-
plasmic vacuolization (cv), cytoplasmic inclusions (i), ruffled
cytoplasmic membranes, and pleomorphic nuclei. Similar cellular
changes were noted in each bovine ocular tumor cell culture.
Hematoxylin and eosin stain. X 530.

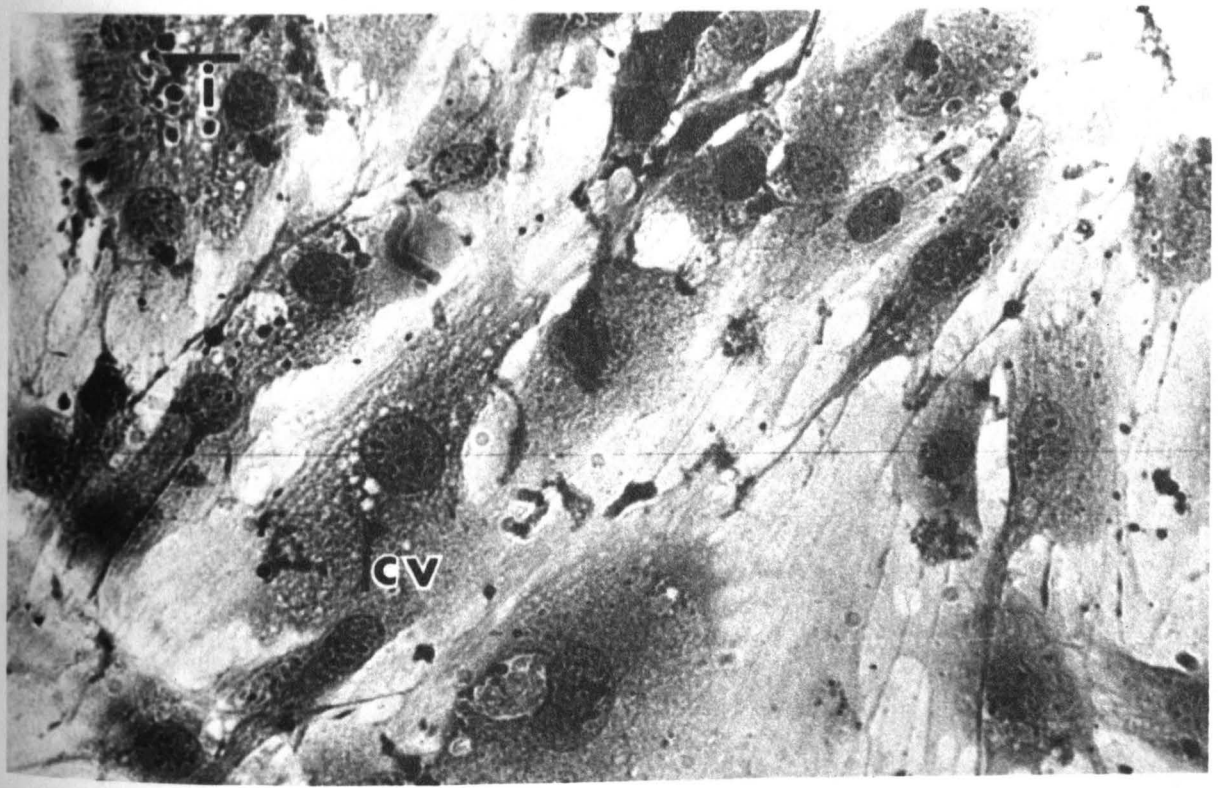
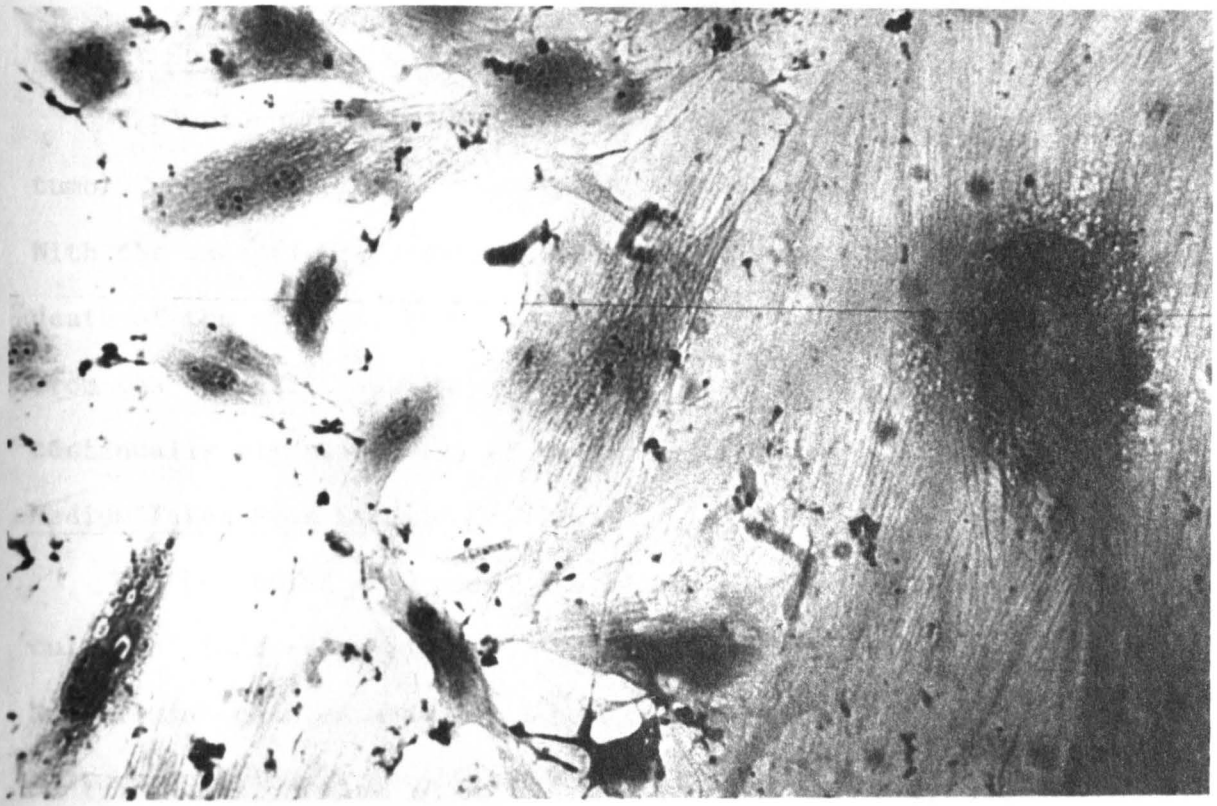
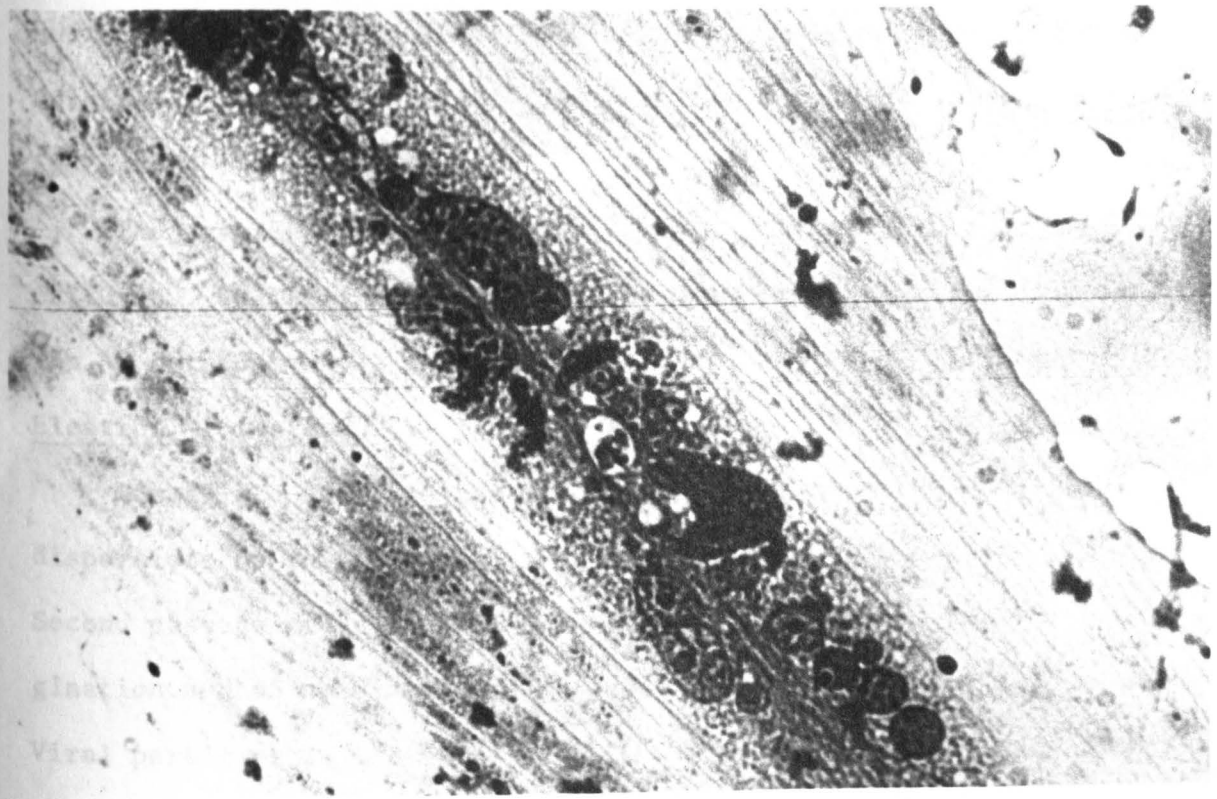

i
CV

Figure 5. Bovine ocular tumor cell culture #7 exhibiting distinct, fibrillar cytoplasm unique to this culture. Hematoxylin and eosin stain. X 215.

Figure 6. A syncytium or multinucleated giant cell frequently observed in bovine ocular tumor cell culture #7. Hematoxylin and eosin stain. X 530.





Explant Cultures

Explant cultures of normal conjunctival tissue and of 12 ocular tumors were maintained. Increases in tissue size were observed. With the exception of tumor culture #32, cell migration and subsequent death of the explants occurred at 50 to 100 days. The tumor explant from cow #32 still remains in culture after 14 months with cells continually migrating from the growing tumor.

Medium Taken From Explant Cultures

The EC medium from all cultures inoculated onto BFS slide cell cultures produced cytoplasmic vacuolization and cytoplasmic inclusions. No cellular changes were noted in control BFS monolayers. BFS slide cultures previously inoculated with this EC medium were stained by AO procedure. Green-staining inclusions indicative of double stranded nucleic acid were revealed. The inclusions were numerous and of variable size (Fig. 7). Similar cytoplasmic inclusions were not noted in control FBS cells (Fig. 8).

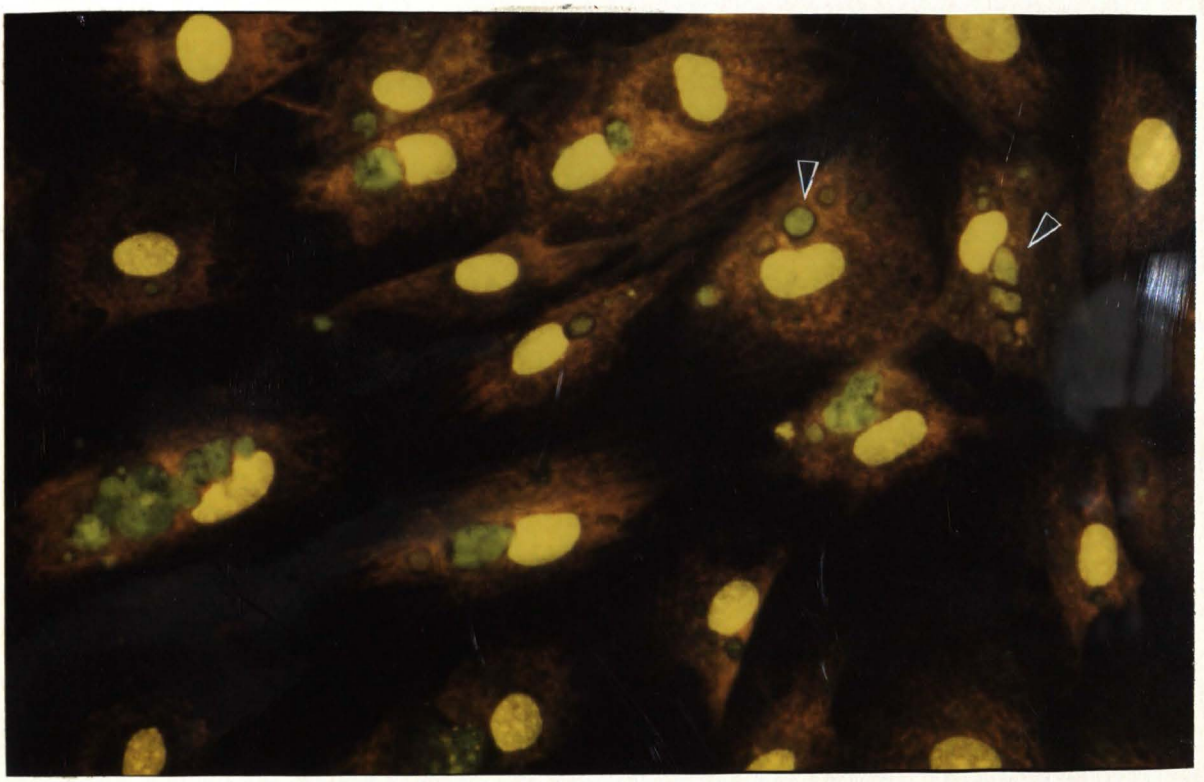
The same EC medium was again inoculated onto BFS slide cell cultures. Cultures were subjected to DNase digestion and stained by the AO method. DNase eliminated the green-staining inclusions (Fig. 9).

Electron Microscopy

Normal BCJ cells in the second passage had nuclei with regular dispersions of chromatin and uniform cytoplasmic organelles (Fig. 10). Second passage cells derived from tumor #1A exhibited chromatin margination and 45 nm diameter microtubules within the nuleus (Fig. 11). Viral particles 24 and 68 nm in size and bacteria 600 nm were observed

Figure 7. Bovine fetal spleen monolayer inoculated with medium from bovine ocular tumor explant culture #106. Green-staining cytoplasmic inclusion bodies (arrow) indicative of double stranded DNA were evident. Acridine orange stain. X 530.

Figure 8. Control bovine fetal spleen monolayer. No inclusions noted. Acridine orange stain. X 800.



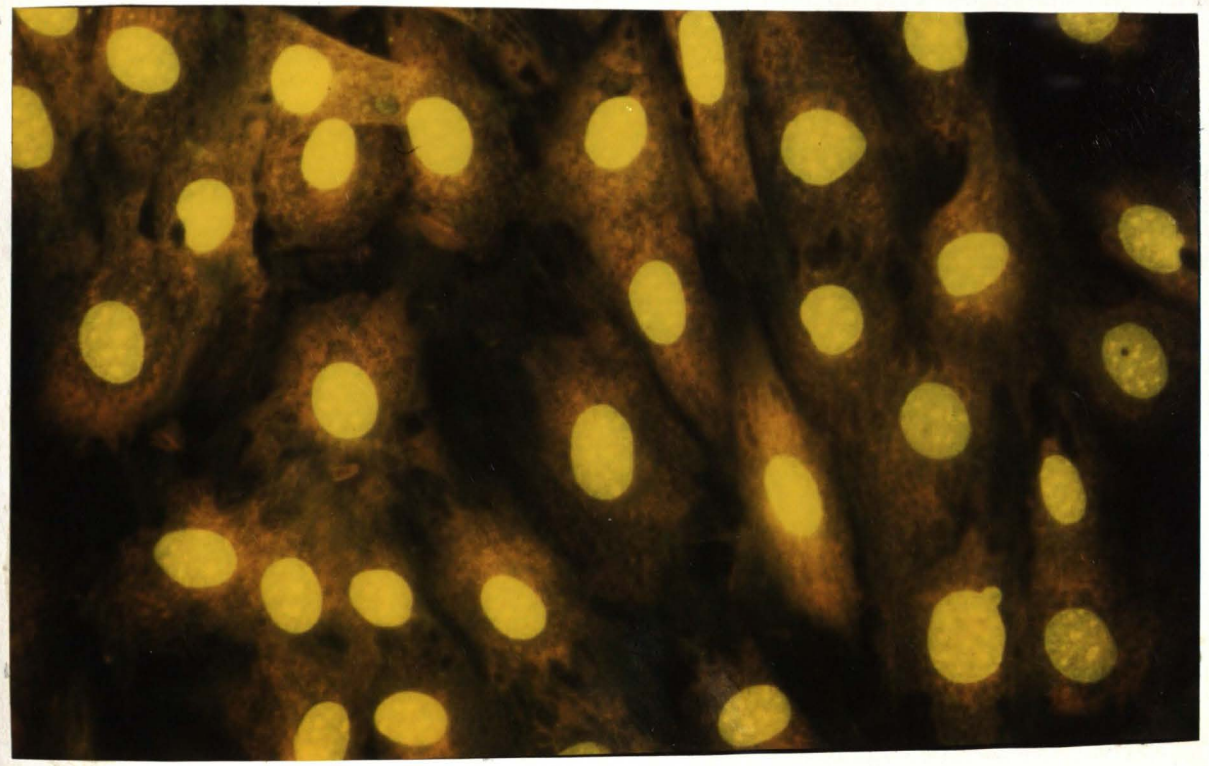


Figure 9. Fetal bovine spleen monolayer inoculated with medium from bovine ocular tumor explant culture #106. DNase treatment eliminated green-staining cytoplasmic inclusions. Acridine orange stain. X 530.

Figure 10. Electron photomicrograph of a normal bovine conjunctival cell monolayer. Uniformity of cytoplasmic organelles and regular dispersions of chromatin in the nucleus (N) were evident. X 27,500.

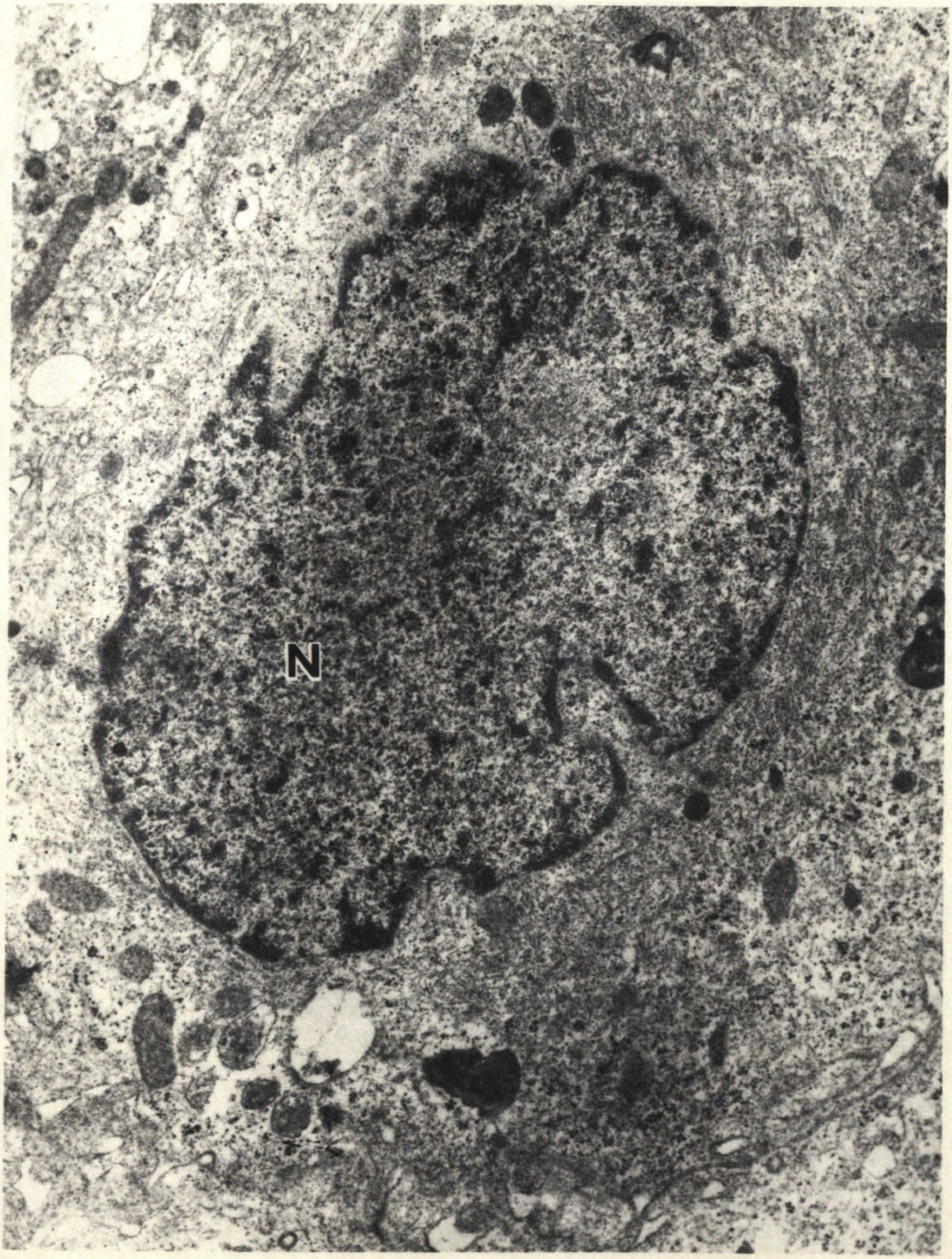
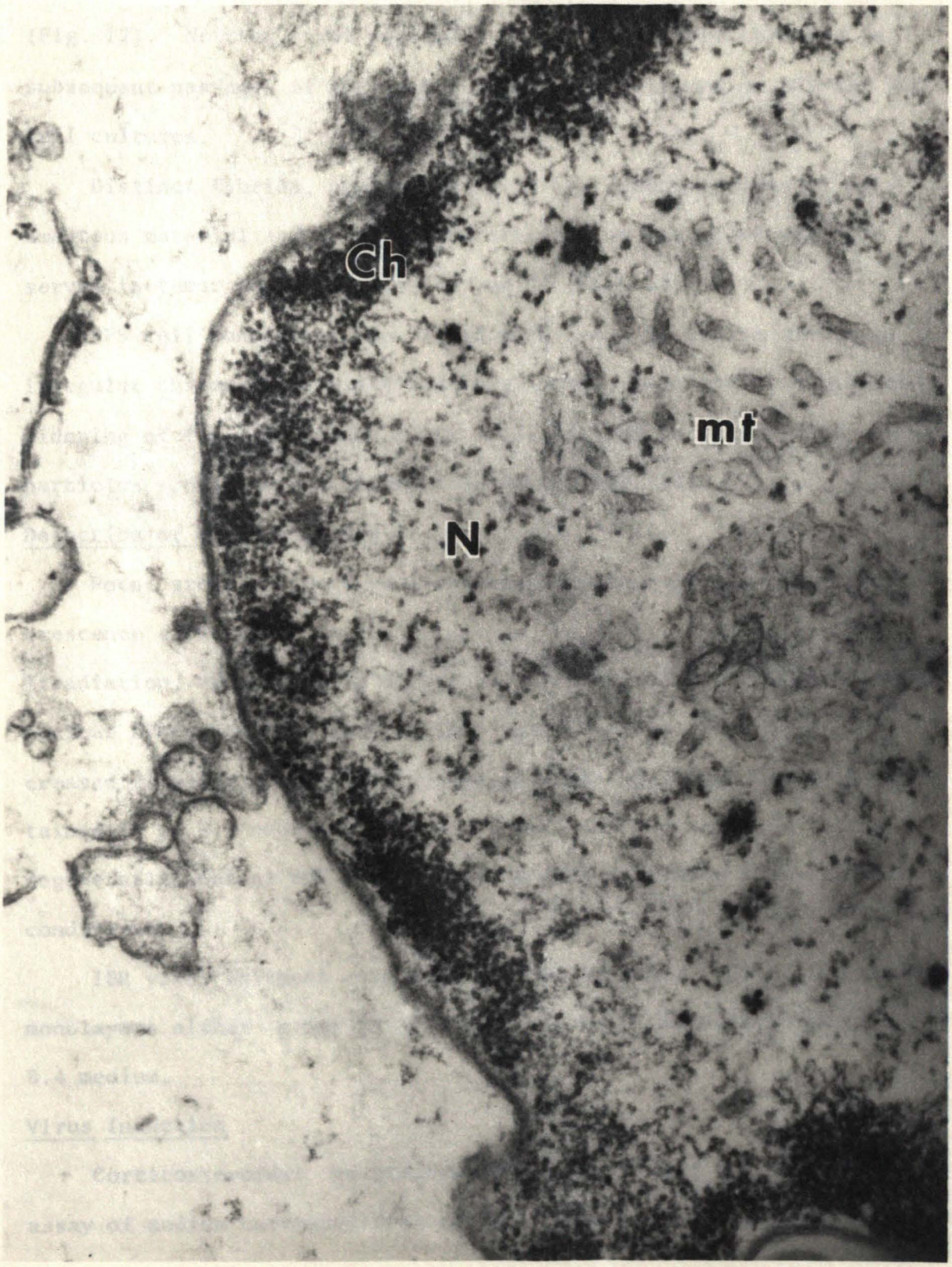
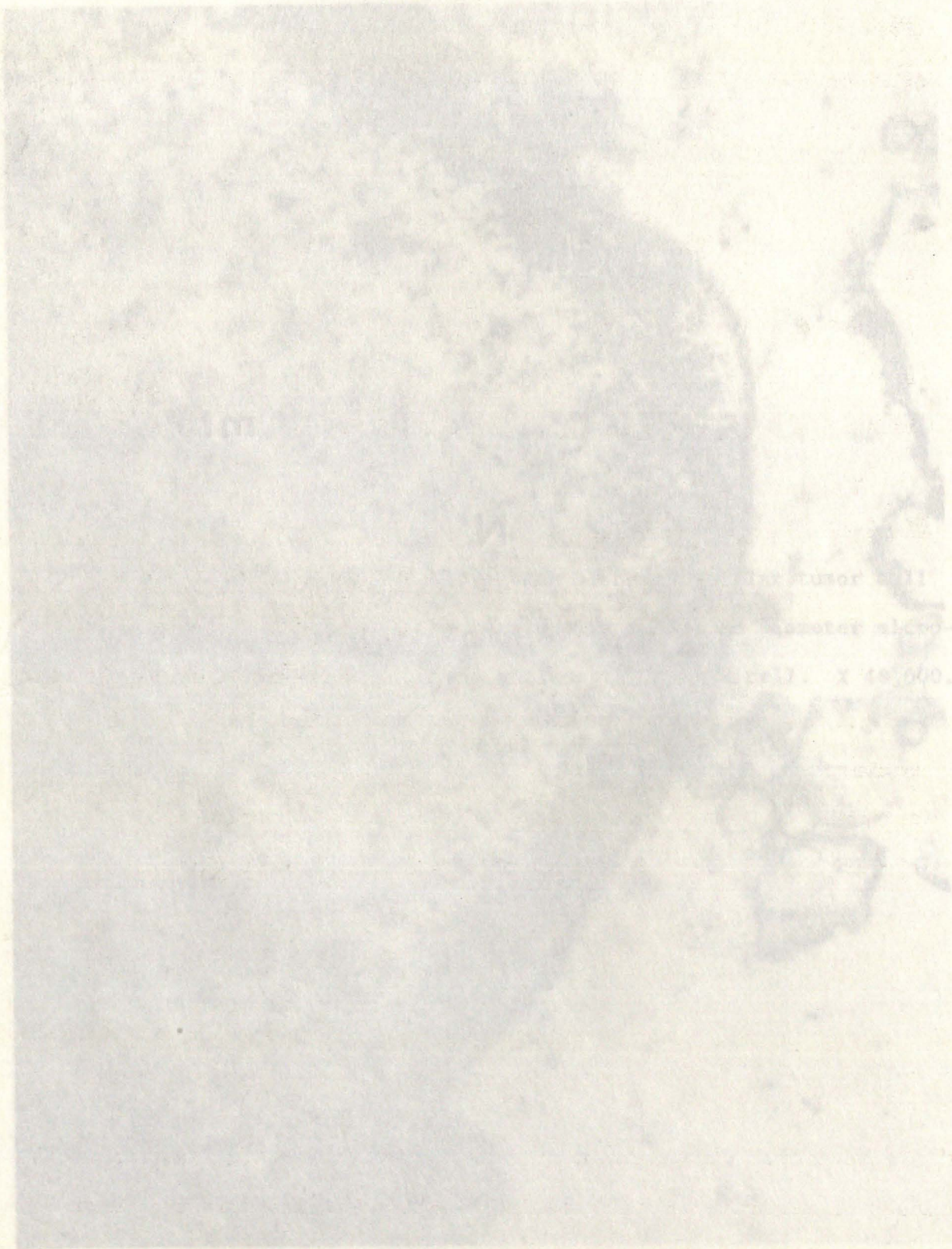

N

Figure 11. Electron photomicrograph of bovine ocular tumor cell culture #1A. Chromatin (Ch) margination and 45 nm diameter microtubules (mt) were noted in the nucleus (N) of the cell. X 48,000.


Ch
mf
N



(Fig. 12). Neither viral particles nor bacteria were observed in subsequent passages of #1A, other tumor cell cultures or BCJ and BCS cell cultures.

Distinct fibrils, approximately 18 nm, concentrations of filamentous material and small unidentified 45 nm particles were observed in tumor cell cultures #1A and #5 (Fig. 13).

BFS cell monolayers inoculated with tumor EC medium displayed irregular chromatin clumping and margination, membrane accumulations, widening of the perinuclear space (Fig. 14) and intracisternal particles approximately 60 nm in diameter (Fig. 15).

Detection of Viral Antigens

Focal areas in tumor cell cultures displayed perinuclear fluorescence extending into the cytoplasm (Fig. 16). Following UV irradiation, approximately 95% of the cells exhibited enhanced perinuclear fluorescence extending into the cytoplasm (Fig. 17). Increased fluorescence was also noted in tumor cell cultures maintained in pH 8.4 medium. No fluorescence was demonstrated in the degenerating normal BCS and BCJ cell monolayers under these stress conditions.

IBR virus anitgens were not detected in tumor, BCS or BCJ cell monolayers either prior to or subsequent to UV irradiation or pH 8.4 medium.

Virus Induction

Corticosteroids: No plaques were demonstrated in the plaque assay of medium harvested from IdU and dexamethasone treated tumor

Figure 12. Electron photomicrograph of bovine ocular tumor cell culture #1A. Viral particles (V) 24 nm and 68 nm in diameter and 600 nm diameter bacteria (B) were evident. X 41,250.

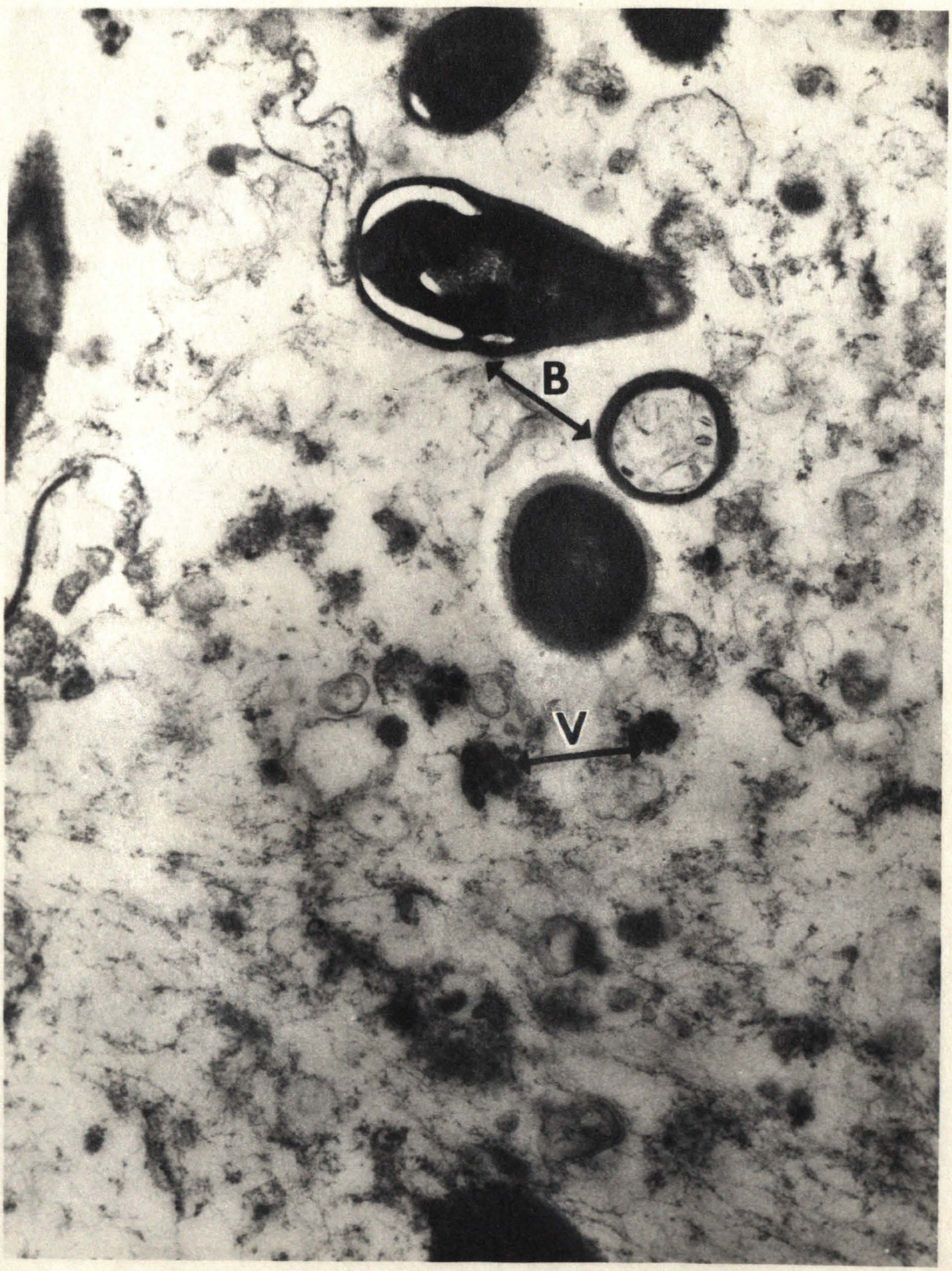

B
V

Figure 13. Electron photomicrograph of bovine ocular tumor cell culture #5,. Evident in the cytoplasm were 18 nm fibrils (f), filamentous concentrations (fm), and unidentified 45 nm diameter particles occurring singly and in aggregates. X 55,000.

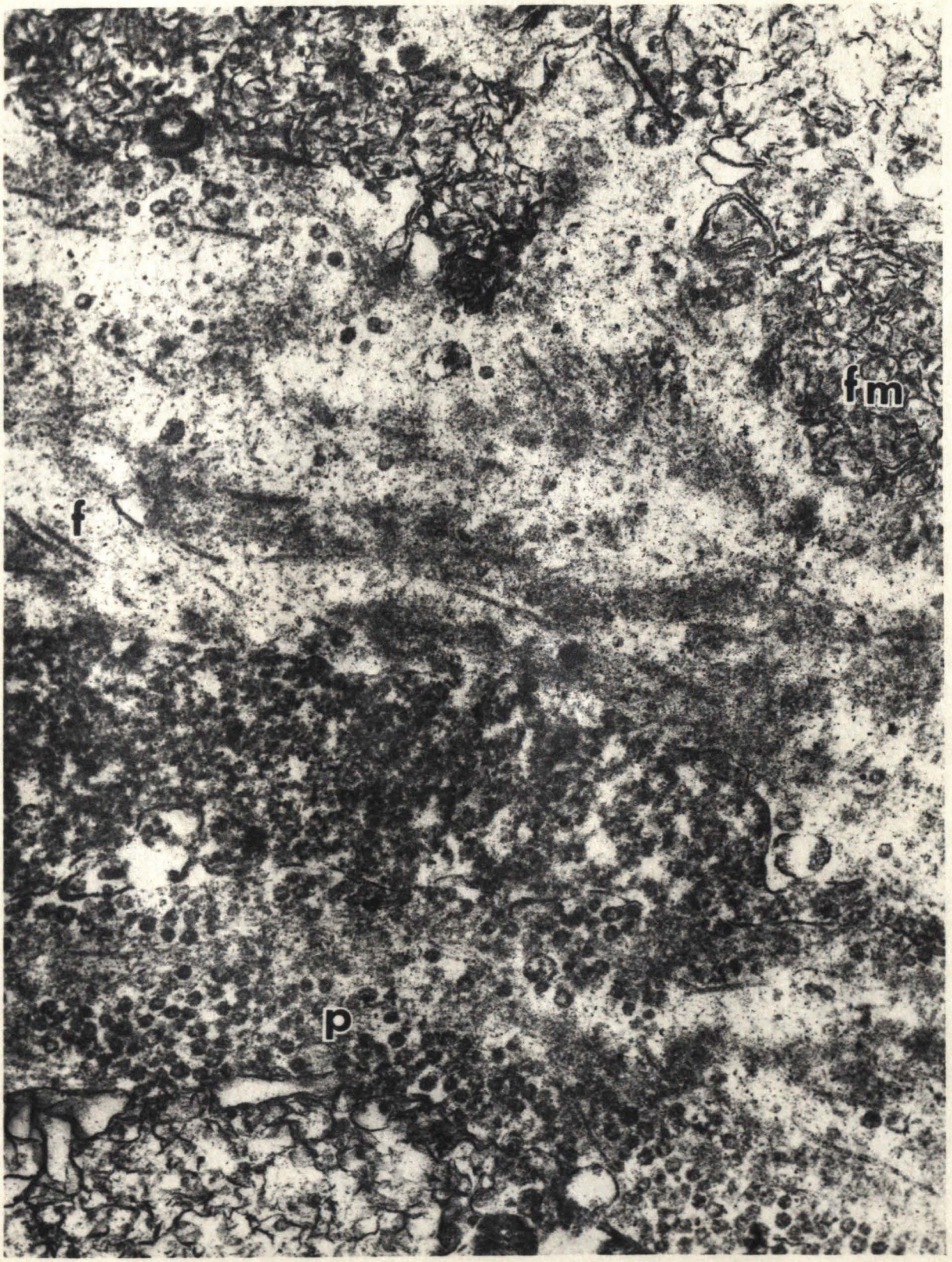

fm
f
p

Figure 14. Electron photomicrograph of bovine fetal spleen monolayer inoculated with medium from bovine ocular tumor explant culture #2. Cytoplasmic membrane accumulations were noted, as were chromatin (Ch) clumping and widening of the perinuclear space (*). X 46,000.

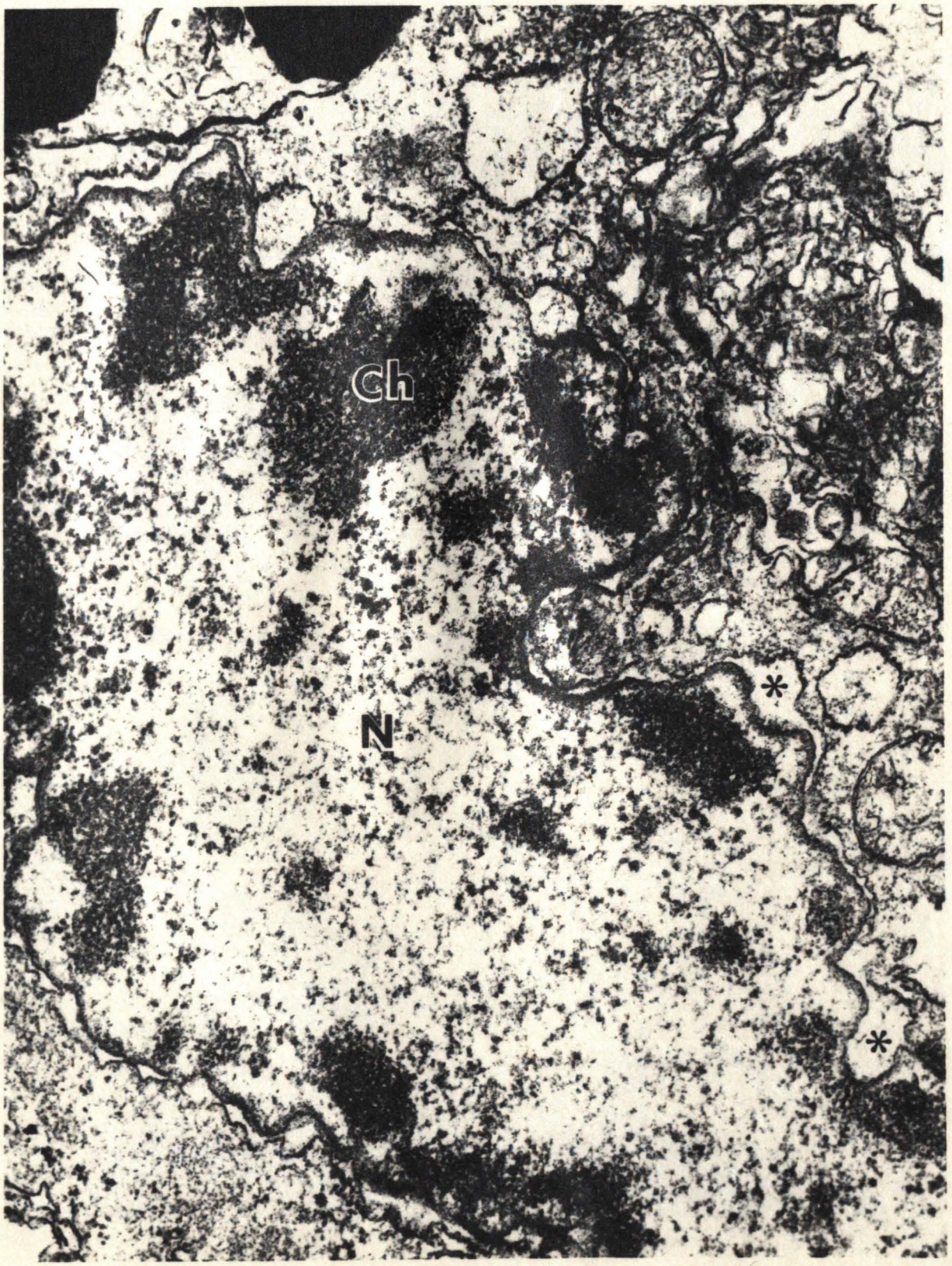
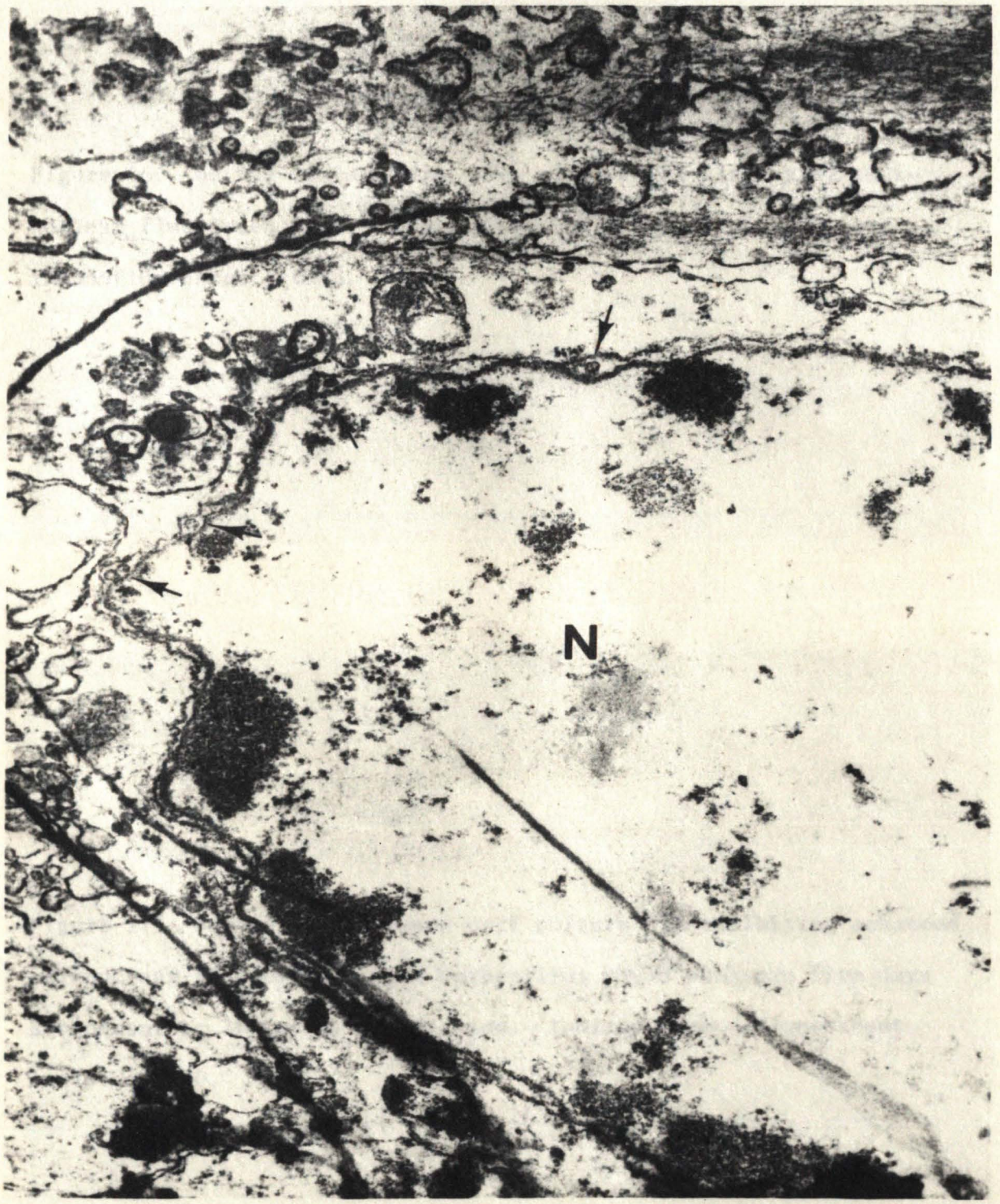

Ch
N
*
*

Figure 15. Electron photomicrograph of bovine fetal spleen monolayers inoculated with medium from bovine ocular tumor explant culture #106. Chromatin clumping was evident in the necleus (N) as were intracisternal particles (arrows) approximately 60 nm in diameter. X 41,250.


N

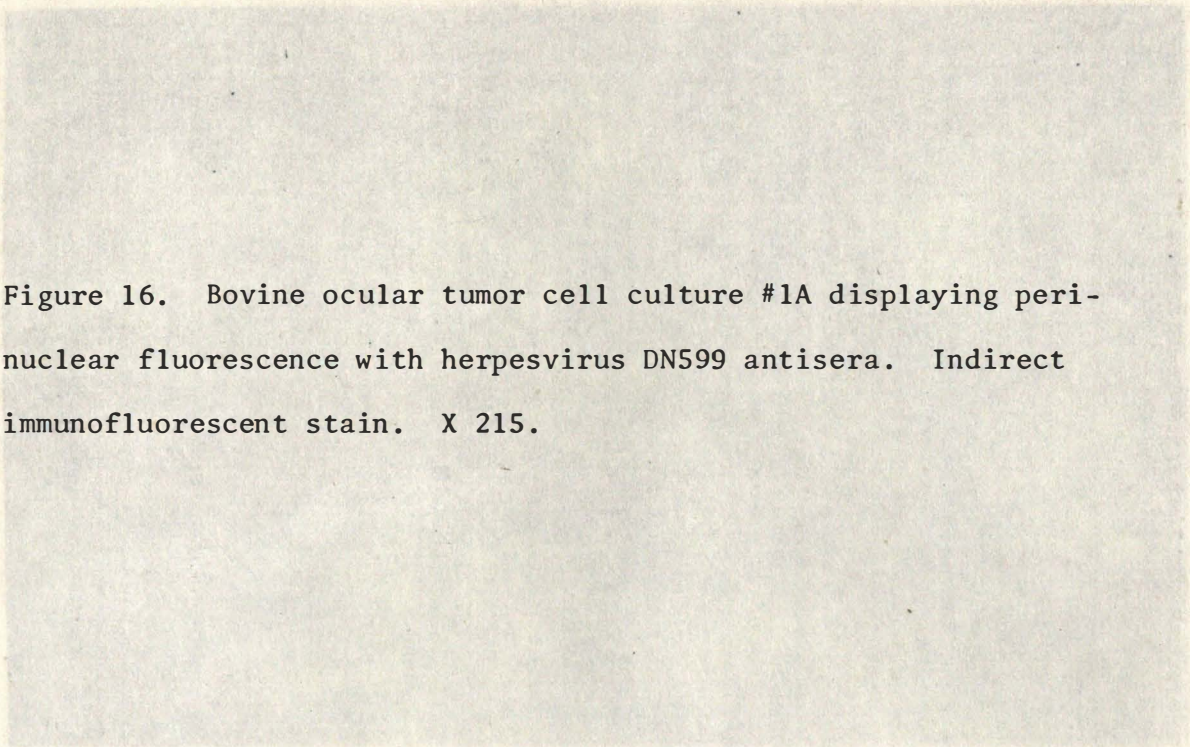


Figure 16. Bovine ocular tumor cell culture #1A displaying perinuclear fluorescence with herpesvirus DN599 antisera. Indirect immunofluorescent stain. X 215.

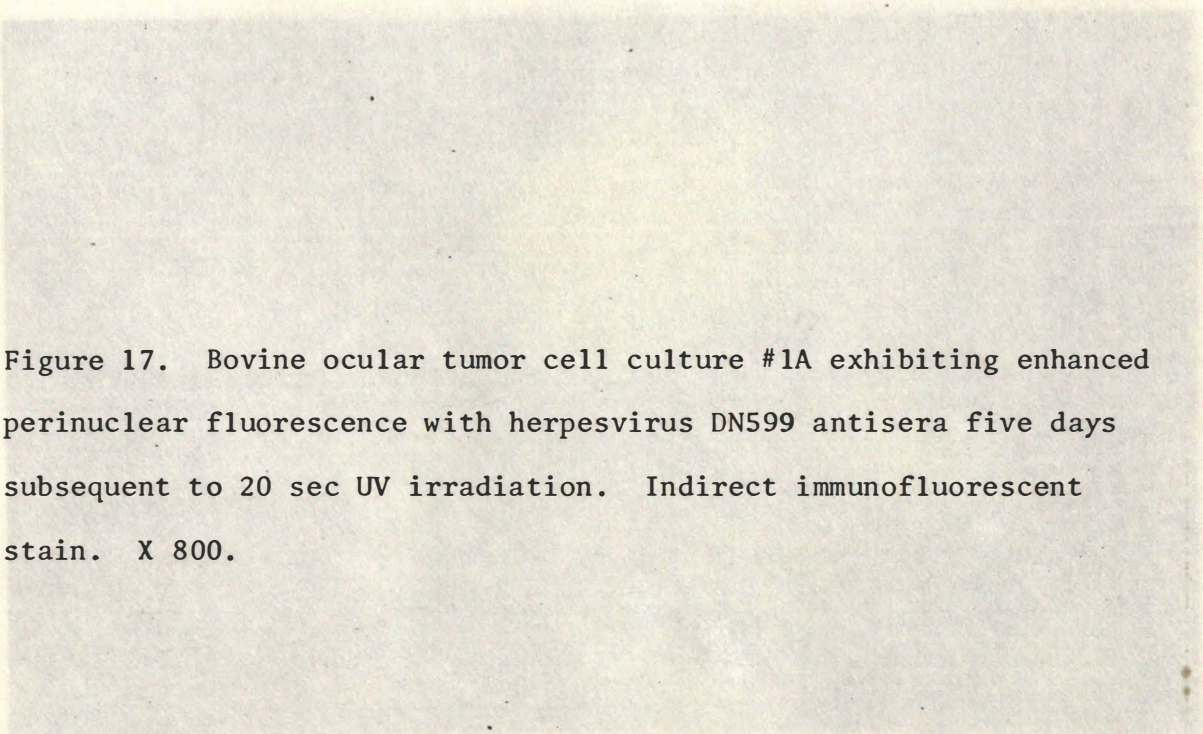
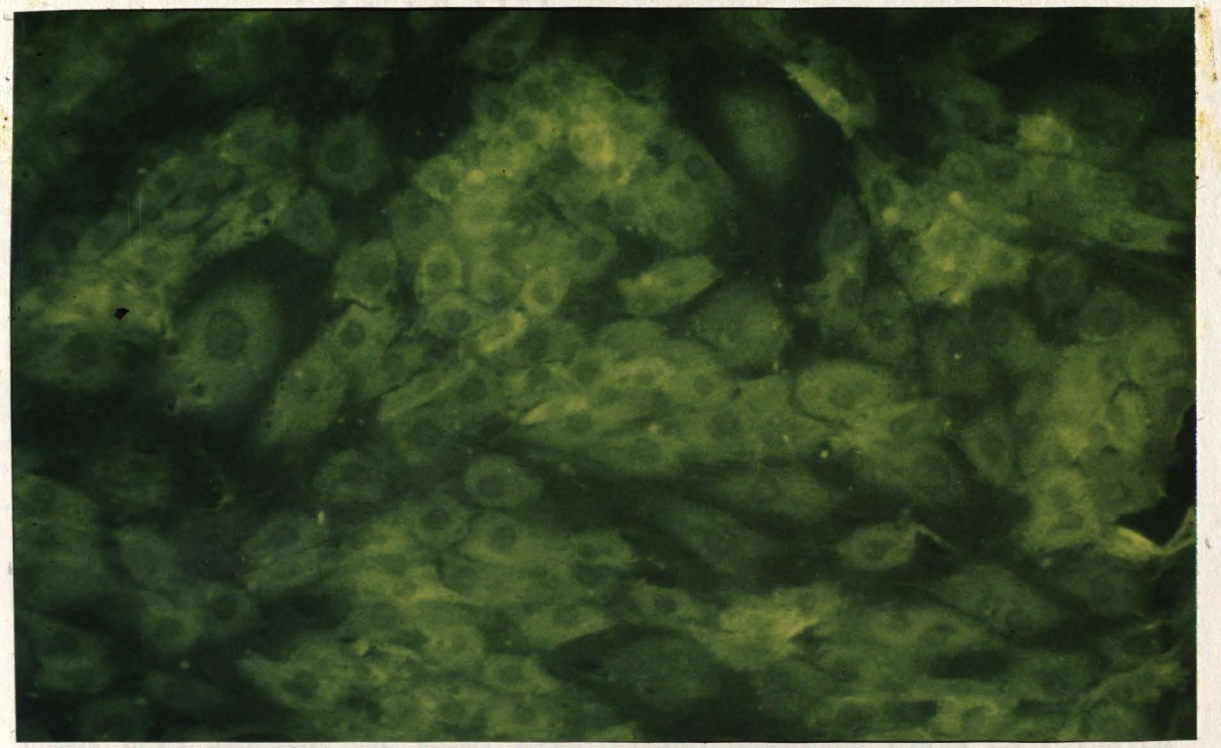
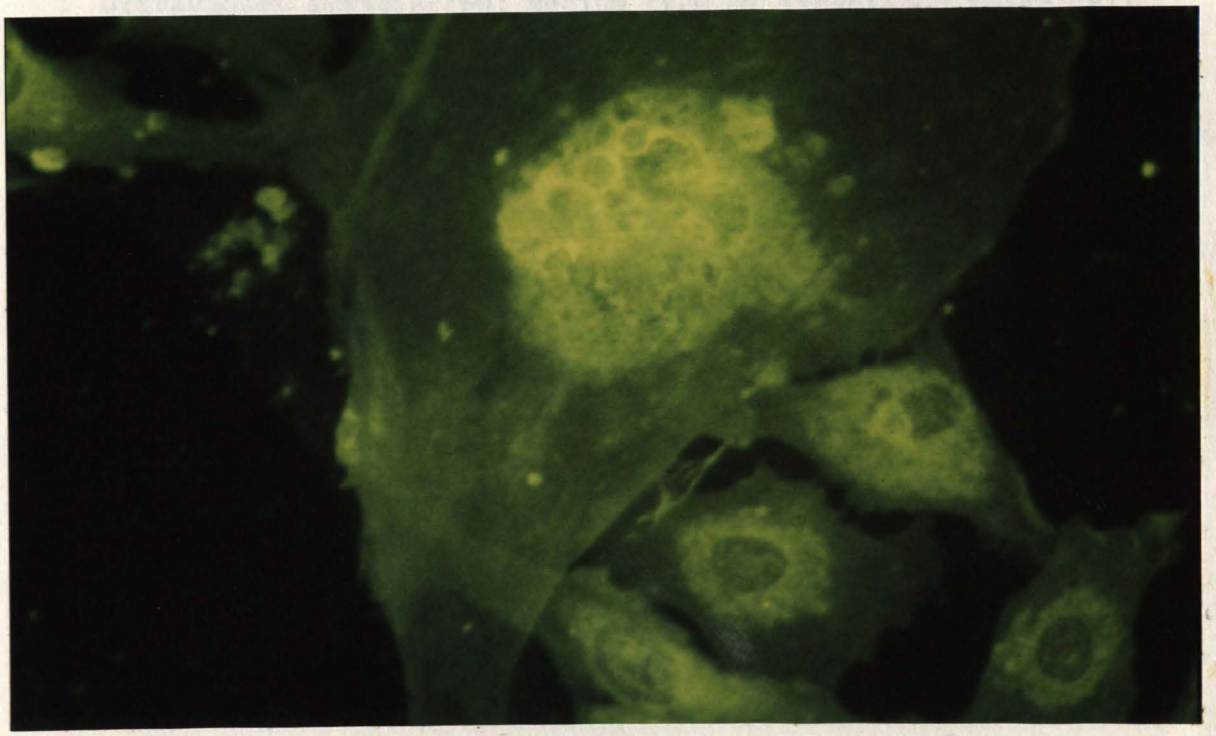


Figure 17. Bovine ocular tumor cell culture #1A exhibiting enhanced perinuclear fluorescence with herpesvirus DN599 antisera five days subsequent to 20 sec UV irradiation. Indirect immunofluorescent stain. X 800.





cell cultures.

pH 8.4 medium: Tumor cell cultures of pH 8.4 medium maintained their usual growth characteristics with individual cultures degenerating at 10 to 150 days. No distinct cytopathic effects were noted in the cultures. Normal BCJ and BCS cells degenerated after 5 to 8 days in culture.

Subinoculation of medium from all high pH tumor cell cultures onto rabbit kidney cells and rabbit spleen cells caused cellular changes. Extensive cytoplasmic vacuolization was noted in RK cells inoculated with pH 8.4 medium from #1A, #3, #5, #7, and #32 (Fig 18). Syncytia were observed in RS cells infected with pH 8.4 medium from tumor cell culture #32 (Fig. 19). Direct negative stain EM revealed no distinct viral particles.

Ultraviolet irradiation: Medium from UV irradiated slide cell cultures were subinoculated onto RK and RS cells. Cellular changes were similar to those mentioned in the pH 8.4 experiment. In addition, foci of rounded cells were noted in cells infected with medium from cultures #2 and 5. No viral particles were noted on direct negative stain EM.

Serology

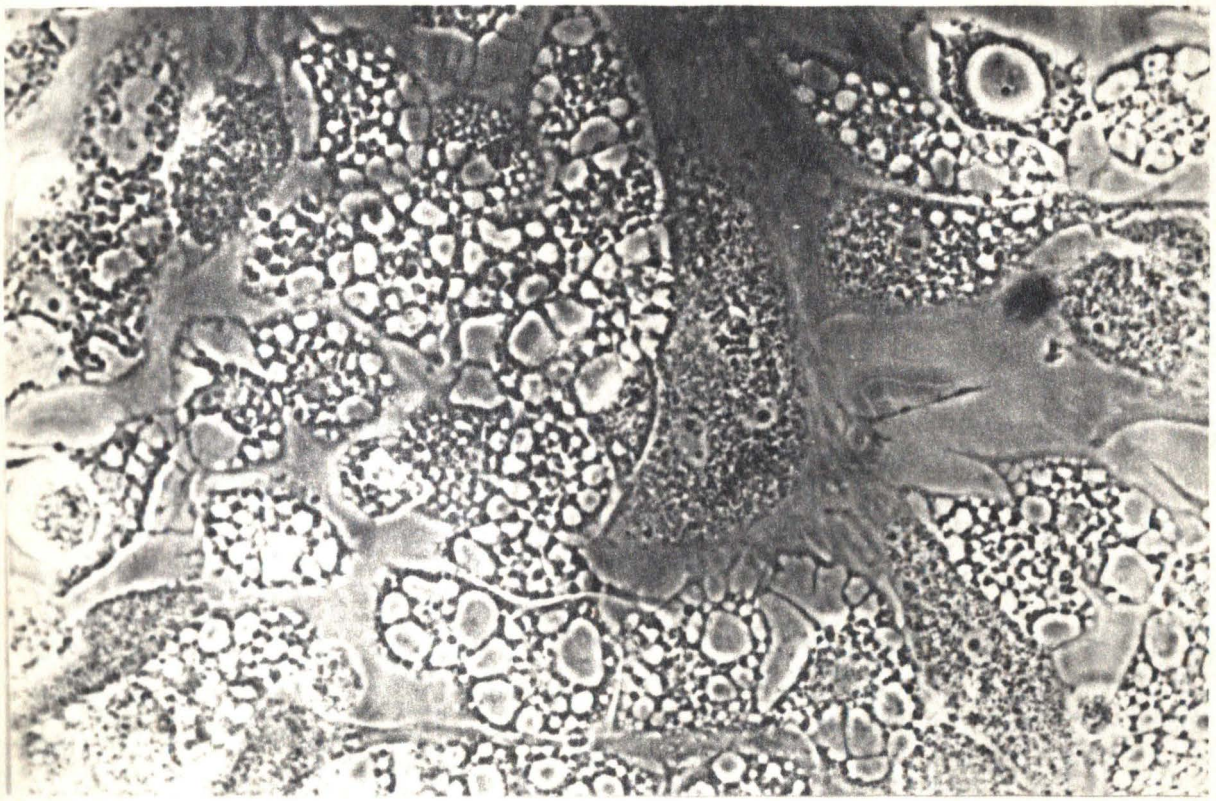
Serum Neutralizaiton Test: The IBR virus antibody titers of control cows from a herd with no evidence of ocular tumors (SDSU), cows with cancer eye lesions (Dvorak), and cows from a known herpesvirus DN599, positive herd (Winter) are listed in Table II.

Indirect Immunofluorescence Test: Table II lists serum antibody

levels of the same three groups of cows to herpesvirus DN599. Negative control bovine fetal serum on herpesvirus DN599 infected cells gave no fluorescence (Fig. 20). Nuclear and cytoplasmic fluorescence was noted with positive sera (Fig. 21).

Figure 18. Rabbit kidney monolayer inoculated with pH 8.4 medium from bovine ocular tumor cell culture #1A. Extensive cytoplasmic vacuolization was evident. X 550.

Figure 19. A syncytium observed in rabbit spleen monolayer infected with pH 8.4 medium from bovine ocular tumor cell culture #32. X 550.



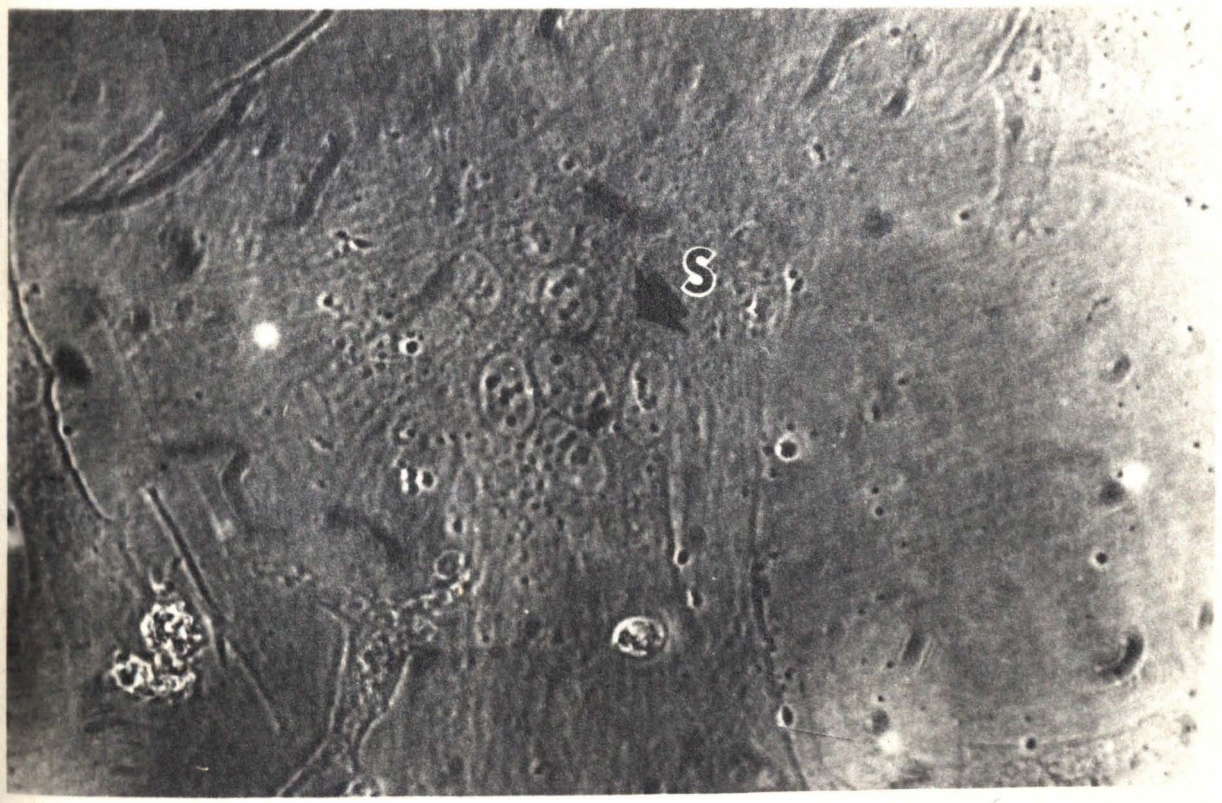
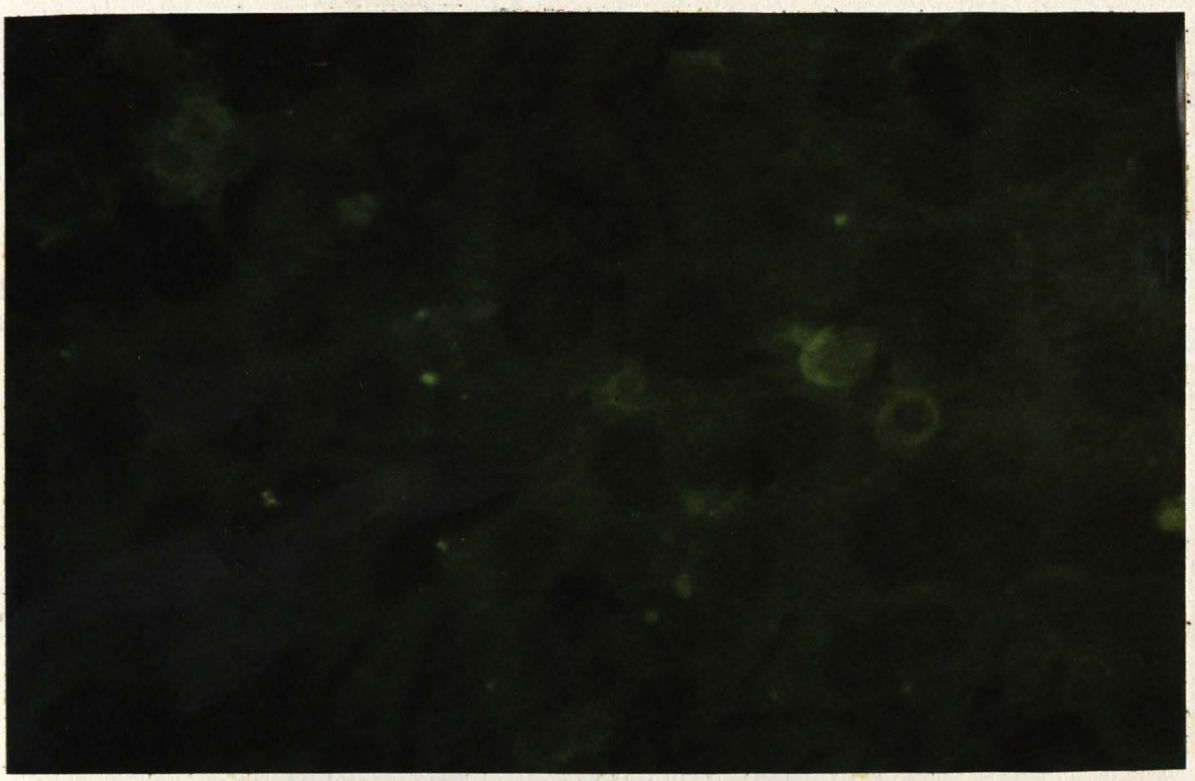

S

Figure 20. Herpesvirus DN599 infected normal bovine corneoscleral cells. No fluorescence was noted with negative control bovine fetal serum. Indirect immunofluorescent stain. X 530.

Figure 21. Herpesvirus DN599 infected normal bovine corneoscleral cells. Intense nuclear and cytoplasmic fluorescence was observed at a 1:256 dilution of serum from Dvorak cow #133. Indirect immunofluroescent stain. X 215.

Table II. Serum Antibody Titers - Herpesvirus DN599 and IBR Virus

	ID. #	Age	DN599 Titer	IBR Titer	
Part A- SDSU Cows	501	10	16	32	
	504	10	8	NT	
	506	10	2	NT	
	660	9	4	NT	
	655	9	8	32	
	716	8	32	32	
	827	7	64	16	
	815	7	8	NT	
	806	7	32	64	
	911	6	64	32	
	919	6	32	16	
	033	5	32	8	
	047	5	64	64	
	011	5	16	64	
	012	5	32	8	
	105	4	8	16	
	104	4	16	16	
	101	4	16	32	
					Lesions
Part B- Dvorak Cows	117	11	256	128	papilloma
	106	11	64	32	papilloma
	68	11	256	32	papilloma
	9	11	512	128	papilloma
	113	10	512	8	carcinoma
	70	9	512	8	no lesion- heavy lacrimation
	32	9	512	128	papilloma
	4	9	64	16	papilloma
	107	8	128	4	plaque & pap.
	5	8	256	64	papilloma
	99	7	256	64	papilloma
	80	7	128	64	carcinoma
	44	7	64	256	pinpoint plaque- heavy lacrimation



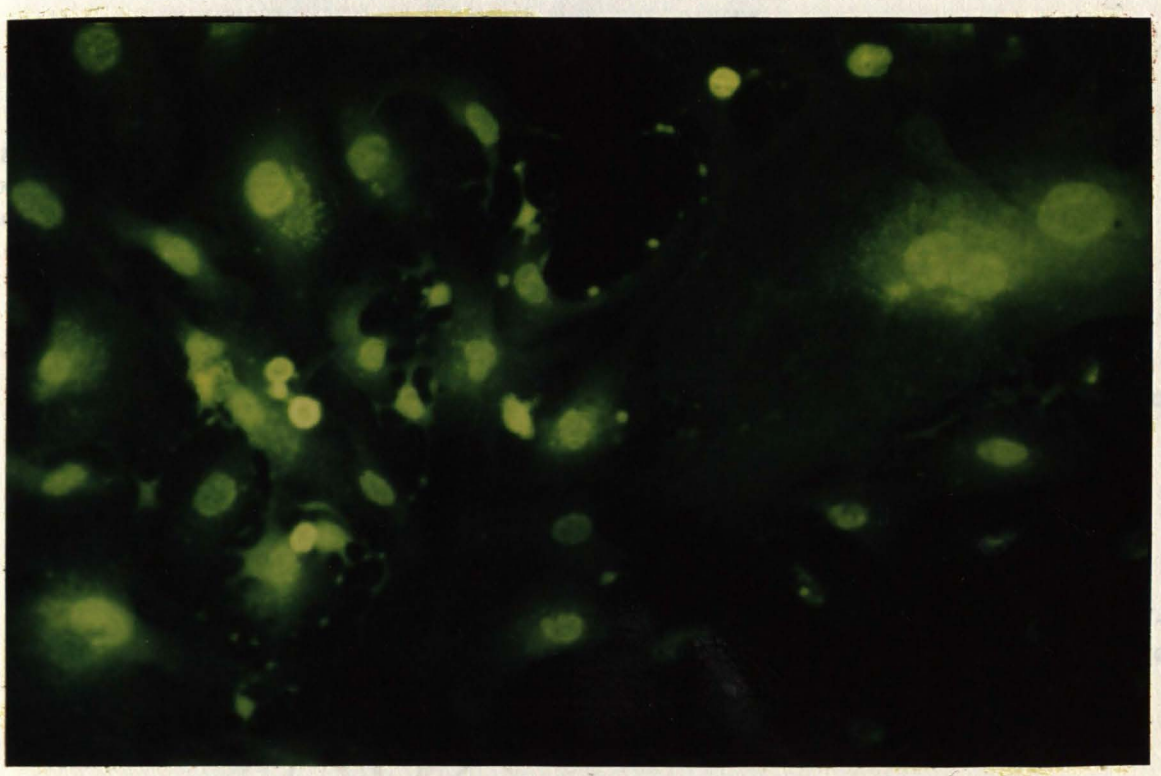


Table II. Serum Antibody Titers - Herpesvirus DN599 and IBR Virus (cont.)

	ID. #	Age	DN599 Titer	IBR Titer
Part-C Winters	2	4-6	8	32
	18	4-6	256	64
	20	4-6	4056	64
	28	4-6	512	8
	45	4-6	2048	128
	51	4-6	32	64
	62	4-6	8112	64
	64	4-6	1024	32

DISCUSSION

Heck et al. (27), Sykes et al. (53), and Van Kampen et al. (57) reported their inability to isolate viruses from bovine ocular squamous cell carcinomas. Similarly no viruses were isolated from the tissue homogenates of the 31 bovine ocular tumors processed in the present study. These results were at variance with Taylor and Hanks' isolation of IBR virus from 15 of 32 bovine eye tumors, but from none of 20 normal eyes (55). IBR virus may have a predilection for the eye tumor epithelial tissue or cattle with cancer eye may be more susceptible to IBR virus infection. Taylor and Hanks, who obtained their samples from a single abbatoir, did not indicate if the specimens were taken on a single day, and did not mention the possibility of an IBR outbreak in a herd coincidently affected with ocular squamous cell carcinoma. These factors may explain the high incidence of IBR virus in the bovine eye tumors examined in their study.

Cells derived from cancer eye lesions were previously reported as slow in adhering to culture flask surfaces and generally resistant to trypsinization (53). The ocular tumor cells cultured in this study exhibited primary outgrowths in 3 to 8 days and trypsinization was successful as a subculturing method. These cell cultures are morphologically similar to those cultured by Sykes et al. (53). Ocular tumor cells cultured in this study exhibited consistent palisading cells, a peculiarity not previously reported in cell cultures derived from similar tissues. Cellular transformation

by oncogenic viruses such as herpesviruses is characterized by a continuous proliferation in vitro of cells forming a piled up, dense layer (13,29).

Cell cultures obtained from bovine ocular tumors were morphologically similar to mammalian cells transformed by HSV (22, 23, 24, 43) and to cervical tumor cell cultures from which HSV-2 was subsequently isolated (10, 11). Cell cultures exhibited atypical cells with large lobulated nuclei, multiple nucleoli, several nuclei, cytoplasmic vacuoles, cytoplasmic inclusions, aberrant growth, and piling. These characteristiecs suggested the possibility of herpesvirus involvement in bovine ocular squamous cell carcinoma.

Electron microscopy of the normal BCJ cell cultures revealed no unusual features. The 45 nm diameter microtubules noted in the nucleus of tumor cell culture #1A (Fig. 11) were similar in size to those observed by Strandberg and Aurelian in the early stages of canine herpesvirus infection of dog kidney cells (51). Microtubules 35 nm in diameter have been reported in the Lucke herpesvirus renal adenocarcinoma of the frog (49). The nature of nuclear microtubules, whether cellular in origin or unusual viral structures is unknown at this time. It has been suggested that microtubules provide pathways for virus egress from the infected cell (51).

Virus-like particles approximately 20 nm and 68 nm in diameter were noted in the second passage of #1A (Fig. 12). Since no cytopathic effects were observed in the culture, the particles may have been defective or immature viruses. The 24 nm particles are also in the

size range of rhinoviruses. These RNA viruses require 32-34 C temperatures for growth, thus at 37 C incubation this virus may not produce detectable CPE.

The 18 nm diameter cytoplasmic fibrils noted in tumor cell cultures #1A and #5 (Fig. 13) are similar to the nuclear and cytoplasmic filaments seen by Couch and Nahimias in HSV-2 infected chorioallantoic membranes (19). The cancer eye cell cultures also exhibited concentrations of filamentous material which are somewhat reminiscent of the "aggregates of endoplasmic reticulum which appear to consist of rough-surfaced profiles" seen by Arhelger et al. (6).

Unidentified 45 nm diameter particles were also seen in #1A and #5. The diameter of these particles is consistent with both the diameter of the intranuclear microtubules and the core diameter of the 68 nm particles. The nucleoid of herpesviruses has been shown to measure between 50 and 75 nm in diameter in infected cells (58). Sydiskis, in a series of experiments, found 45 to 79 nm viral particles to be DNA and structural protein containing precursors to pseudorabies virions (52). Possibly, the 45 nm and 68 nm particles observed in ocular tumor cell cultures #1A and #5 may be herpesvirus precursors.

Presently, an ocular explant of cow #32 remains in culture. Explant cultures, which resemble the in vivo condition more than do cell monolayers, were used to investigate the possibility of virus release later in tumor development. Although no virus was isolated from the medium periodically removed from tumor explant cultures,

cytoplasmic vacuolization and cytoplasmic inclusions were observed in BFS monolayers inoculated with the culture fluids. These vacuoles and inclusions were similar to those observed in bovine ocular tumor monolayers. No cellular changes were displayed by control BFS monolayers. The cytoplasmic inclusions were revealed as double stranded viral DNA by DNase and AO staining of inoculated BFS cell cultures. Apparently the EC released viral DNA or some viral component into the culture fluid which, when inoculated onto BFS cell monolayers, produced cytoplasmic inclusions and vacuoles. The actual identity of this transferred entity requires further study.

Electron microscopic evidence of herpesvirus infection, such as irregular nuclei with clumping and margination of chromatin, widening of the perinuclear space, intracisternal particles, membrane accumulations, numerous cytoplasmic vesicles of variable size, and accumulation of lipid globules (1, 17, 28, 46, 49). was noted in BFS monolayers inoculated with EC medium (Fig. 14, 15). Although no mature herpesvirus virions were observed, the possibility exists that the virus may have been present in a very low concentration or as a nonproductive infection not detectable by electron microscopy.

It has been well established that cells transformed by DNA tumor viruses contain virus specific antigens (42). The sensitive IIF test was employed to detect possible viral antigens in ocular tumor cell cultures. The IBR virus IIF test was negative in both the tumor cell cultures and the BCJ and BCS cell cultures, indicating

that this herpesvirus was not involved in the inclusion body formation. Granular, perinuclear, cytoplasmic fluorescence was observed in ocular tumor monolayers examined for the presence of herpesvirus DN599 specific antigens. Enhanced fluorescence with DN599 antisera was observed in similar cultures following both UV irradiation and maintenance under pH 8.4 medium. Apparently these stress situations caused an increased expression of DN599 viral antigens within the tumor cells. The fact that no fluorescence was seen in normal BCJ and BCS monolayers under similar conditions indicated that DN599 is involved in the cytoplasmic inclusions and possibly in the etiology of bovine ocular squamous cell carcinoma.

Both herpesvirus latency and viral oncogenicity require the establishment of a stable virus-cell relationship by transferring viral genes to the host cell. Latently infected cells harbor the viral genome in a completely repressed state while a partially transcribed viral genome is associated with virus-induced oncogenic transformation (9). Although mature virions may be detected in some herpesvirus transformed neoplastic cells, virus expression in transformed cells is generally restricted to early functions which do not result in morphologically detectable virions (30).

Since virus replication in recurrent herpetic infections is induced by a variety of emotional and physical conditions of stress, cancer eye cell cultures were subjected to hormone treatment, UV irradiation and pH 8.4 medium. Attempts to induce productive virus infections in these cell cultures have thus far been unsuccessful.

Rapp and Duff similarly reported no induction of virus synthesis following 5-bromodeoxyuridine treatment of hamster embryo fibroblasts transformed by UV irradiated HSV-2 (43). Although no distinct viral cytopathic effect was noted in tumor cell cultures subjected to high pH medium or UV irradiation, inoculation of these culture fluids onto rabbit kidney and spleen monolayers produced cellular changes reminiscent of viral infection.

Since induction methods, while potentially useful, may not elicit production of virus if transformation had been accomplished, negative results cannot be interpreted to indicate absence of inducible virus genetic information. The possibility also exists that the ocular tumor cell cultures are infected with a defective virus whose expression would not be inducible.

Herpesvirus DN599 produces non-neutralizing antibodies which are detectable by the IIF test (45). The results of a limited serological survey of negative and positive control cows and cows with cancer eye lesions indicate that the latter two groups have significantly higher antibody titers to herpesvirus DN599. This herpesvirus, originally isolated from a bovine respiratory tract infection, is serologically identical to an isolate from a lymphosarcomatous cow (40). The significance of these viruses as disease producing entities has not been fully established.

Herpesvirus DN599 has been shown to be antigenically distinct from IBR virus and other bovine herpesviruses (40). It is evident from antibody titers in Table II, that the two herpesviruses do not

serologically cross-react. Although cows with cancer eye lesions had antibody titers to IBR virus, the levels were not unusually high. IBR virus is a common bovine virus and similar antibody titers are often found in clinically normal cattle. The expected incidence of cattle serologically positive to herpesvirus DN599 has not been established.

SUMMARY

No viruses were isolated from tissue homogenates of 31 bovine ocular tumors or from tumor tissue cultures. Cancer eye lesions and normal bovine ocular tissues were successfully cultured as monolayers and as explants. The tumor cell monolayers were morphologically similar to mammalian cells transformed by herpes simplex virus type 2 and to human cervical tumor cell cultures which subsequently yielded the same virus. Medium removed from ocular tumor explant cultures and inoculated onto bovine fetal spleen cell monolayers resulted in cytoplasmic inclusions and cytoplasmic vacuolization similar to those observed in ocular tumor monolayers. Cytoplasmic inclusions were found to consist of double stranded DNA. Electron microscopy revealed cellular changes resembling that observed with herpesvirus infections.

Herpesvirus DN599 specific antigens were detected in cancer eye cell monolayers by in the indirect immunofluorescence test. Enhanced fluorescence was noted following exposure of similar cultures to pH 8.4 medium or to UV irradiation. No IBR virus specific antigens were detected in ocular tumor cell cultures or normal control cell cultures under similar conditions.

Serological results indicated significantly higher antibody titers to herpesvirus DN599 in cattle from which the virus was isolated and in cattle with ocular tumors, as compared to clinically normal control cattle. No unusually high IBR virus antibody titers were found.

In this study, IBR virus could not be incriminated as a causative agent of cancer eye. Results indicated that herpesvirus DN599 or an antigenically similar herpesvirus may be involved in the etiology of bovine ocular squamous cell carcinoma and its precursor lesions.

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