Ultrastructural studies of the bovine syncytial virus

Arlis Dormon Boothe
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Ultrastructural studies of the bovine syncytial virus

by

Arlis Dormon Boothe

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INTRODUCTION

Viruses are widely distributed in nature and hundreds have been described. Many viruses are not associated with any clinical diseases and what role, if any, they execute in the host system remains to be determined.

Diseases of cattle which have no known etiology include many of the neoplasms. In the field of oncology, bovine lymphosarcomas have recently been of interest since some of those in laboratory animals now have a definite relationship with known viruses (33, 35, 36, 37, 43, 56, 73, 77, 82, 92, 111, 112, 113, 129). These viruses are not confined to any particular group or viral family under the present system of classification (71, 130) and include both RNA and DNA viruses.

Few of the DNA viruses are naturally oncogenic. Almost all members of the papovavirus group can cause benign tumors such as warts of many of the animal species. Some, such as rabbit papilloma, are initially benign, but may become malignant. Polyoma and SV-40, which rarely if ever cause malignant tumors in nature, regularly produce neoplasia when inoculated into newborn rodents. Several members of the adenovirus group, of human and animal origin, are oncogenic when inoculated into newborn hamsters. Present data indicate that adenoviruses of human types 12, 18, and 31 have the highest oncogenic potential.
Herpesviruses are associated with Lucke's renal adenocarcinoma of leopard frogs (61, 63, 132), Marek's disease of chickens (13, 79), lymphosarcoma-like conditions of primates (67, 68, 69, 70), Burkitt's lymphomas of children (27, 28), and cervical carcinoma of women (78).

A number of the RNA viruses have been unequivocally associated with neoplastic diseases. In contrast to the DNA viruses, the importance of RNA viruses is heightened by the fact that they are oncogenic under natural conditions. Most are included in the leukovirus group, of which three subgroups have been extensively investigated: the avian leukoviruses, the murine leukoviruses, and the mammary tumor viruses of mice. Recently, a feline leukemia virus (49, 50) and a canine mast cell leukemia virus (90, 93) have been reported and although considered RNA viruses, they have not been classified. Rous sarcoma virus of chickens (102) is included with the avian leukoviruses but a feline fibrosarcoma virus (109) has not been classified.

Many diseases of man have a similar counterpart in animals. Thus, such a disease may first be studied in lower animals since they lend themselves to experimental methods more readily than does man. Lymphosarcoma of animals provides a good model for lymphosarcoma of man. Many research programs are currently very active in lymphosarcomas of animals and include many species. Although much study has
been done, no known etiology of lymphosarcoma has been
determined in large domestic animals such as swine (9, 32,
46, 58), sheep (44, 86, 131), cattle (24, 25, 34, 48, 72,
101, 123), and horses (127). Work on bovine lymphosarcoma
at the National Animal Disease Laboratory revealed that an
agent isolated from normal and lymphosarcomatous cattle
produced massive syncytial formations in cell cultures and
this syncytial producing property could be transferred
from culture to culture by a cell-free filtrate (64). The
accumulated data indicated that the syncytial forming agent
was viral in nature and that further supportive evidence
was needed for confirmation and tentative placement in
viral classification. Consequently, this study was under-
taken to obtain information about the fine structure of the
host cell, the morphology of the syncytial producing agent,
and cytopathologic interrelationship of this agent with
the host cell.
Recently, several viruses have been isolated which produce in its host cell a characteristic reaction characterized by extensive syncytial formation but little or no other cytopathic effect. Such syncytium-forming viruses have been isolated from cattle (64, 83), man (1, 11), monkeys (12, 26, 89), cats (55, 66), rabbits (7, 75), hamsters (52), chickens (17), and sheep (108, 119).

Cattle

Bovine syncytial virus

In 1967, W. A. Malmquist detected a cytopathic effect in the growth of mixed cell cultures derived from bovine embryonic spleen (BESp) cells that had been inoculated with minced lymph nodes, buffy coat cells or cellular elements of milk from lymphosarcomatous and apparently normal cattle. The nature of the cytopathic effect was syncytial formation incorporating anywhere from 2 to 50 nuclei per syncytium and involving up to 80% of the nuclei of a monolayer.

Malmquist et al. (64) in 1969 reported that the gamma-globulin fraction of sera from certain lymphosarcomatous and normal cattle, whose buffy coat or cellular elements of milk contained the syncytial producing agent, when conjugated with fluorescein isothiocyanate, would react with
specific sites of the syncytial formations producing a characteristic fluorescence. It was found that cell cultures containing the syncytial formations, when sonicated, would produce a suitable antigen for reacting with sera of animals from which the cell culture syncytial producing agent could be isolated.

The syncytial producing agent could be passed from one cell culture to another by using whole cells, cell culture supernatant, or cell culture filtrate from 0.22 μ Millipore membranes. It was concluded that the syncytial producing agent was a bovine syncytial virus (BSV).

**Bovine respiratory syncytial virus**

Paccaud and Jacquier (83), in 1970, reported isolation of a syncytial-forming virus in embryonic calf kidney (ECK) and embryonic calf lung (ECL) from 2 herds of cattle during an outbreak of respiratory disease. The characteristic cytopathic effect was syncytial formations of various size which contained homogenous acidophilic cytoplasmic inclusions of various size and shape. These inclusions were not stained by acridine-orange. No nuclear alterations were observed. The isolates were ether and chloroform sensitive, heat sensitive, acid labile, and failed to hemadsorb or hemagglutinate human group "0", calf, guinea pig or chick erythrocytes at 4°C, 20°C, or 37°C. Only about 1/3 of infectivity was recovered.
after passage through 0.45 µ Millipore membranes and it was completely retained by 0.22 µ membranes. No morphological studies were made. Evidence of a relatively close antigenic relationship between the bovine agent and the human respiratory syncytial virus was demonstrated by cross-neutralization and complement fixation test.

Human syncytial virus from nasopharyngeal carcinoma

Achong et al. (1) in 1971 reported finding a syncytial forming virus in cell cultures derived from a human nasopharyngeal carcinoma which was structurally indistinguishable from the bovine syncytial virus and the feline syncytial virus. The virus was found only in about 10% of the intact cells of suspension cultures of lymphoblastoid cells released from the original monolayer after 105 days in vitro. The monolayer consisted solely of lymphoblastoid cells that tended to form syncytia, nuclear projections, annulate lamellae and undulating tubules. The immature virus or nucleocapsids appeared only in the cytoplasm, often arrayed around cytoplasmic vacuoles and adjacent to the plasmalemma. The virions matured by budding across these membranes which were acquired as an outer coat that was in turn covered by evenly spaced spines radiating uniformly over the entire surface. The overall diameter of the virion from spine tip to spine tip was about
100 nm. The diameter of the nucleocapsid was 45 nm and the spines were 13 nm long.

**Respiratory syncytial virus**

Respiratory syncytial (RS) virus was first described under the name of chimpanzee coryza agent by Morris et al. (74) in 1956. It was first isolated from infants with severe respiratory infection by Chanock et al. (11) in 1957 and now is firmly established as a cause of human respiratory illnesses. Respiratory syncytial virus multiplies in cell cultures causing the formation of syncytial masses with eosinophilic cytoplasmic inclusions measuring up to 10 μ across. It resembles more closely the paramyxoviruses than any other group. It lacks the properties of hemadsorption and hemagglutination, fails to multiply in the hen's egg, and shows no serological relationship with known myxoviruses (11). Using sucrose density sedimentation data, Chanock et al. (11) originally estimated RS virus to be 90 to 103 nm in size. Armstrong et al. (4) in 1962, studied ultrasections of infected tissue culture cells and found virions having a 20 nm dense central core and overall diameter of 65 nm. He also found budding filamentous structures 60 to 70 nm wide and up to several microns long. These filaments contained core-like structures 20 nm wide. Bloth and Norrby (6) in 1965 placed extreme limits on the overall
diameter of RS virus to be 50 to 75 nm on negative stained preparations.

Nonhuman Primates

**Simian foamy viruses**

Simian foamy viruses are an ill-defined group of viruses isolated from monkey and ape tissues. These viruses produce syncytia in cell cultures, do not induce demonstrable intracytoplasmic or intranuclear inclusion bodies, and fail to exhibit pathogenicity for day-old mice or hamsters (96). Enders and Peebles (26) in 1954 were first to observe syncytia in cell cultures from monkey kidneys of simian foamy agents while working with measles virus. Rustigian et al. (103) in 1955 made similar observations and since then these viruses have frequently been isolated from monkey kidney cell cultures and are regarded as "common contaminants." Cell cultures of monkey kidney tissue often produce multinucleated cells that later develop a foamy appearance due to the formation of large vacuoles. Many strains of the foamy agents have been described and they have been shown to belong to at least three distinct serological groups, designated as Types I and II by Johnston (53) in 1961 and an additional Type III by Stiles et al. (114) in 1964.

Plummer (89) in 1962 reported foamy virus in 40 to 60% of vervet, rhesus, and cynomolgus monkey kidney cell
cultures, but absent in *Erythrocebus patas*. All 3 types multiplied in primary cell cultures of monkey, rabbit and human kidney tissue. None of the 3 types hemagglutinated or hemadsorbed erythrocytes of monkey, human, horse, sheep, dog, cat, ferret, rat, rabbit, guinea pig, or chicken origin at 4°, 20°, or 37° C. All 3 types had low infectivity titers, were sensitive to ether at 4° C. for 18 hr., and were stable when treated with trypsin (0.25% of 1:250) for 30 minutes at 37° C.

Fluorescence studies by Fleming and Clarke (31) in 1970 first detected the antigen in the nucleus of the cell at 35 hours. Cytoplasmic fluorescence first appeared after 40 hours in the paranuclear region and subsequently spread throughout the cytoplasm giving a diffuse fluorescence which was more intense than that in the nucleus. The nuclear fluorescence diminished and ultimately the cytoplasmic staining became granular. After 70 hours the number of syncytia increased rapidly and the nuclei of the majority of these syncytia showed no significant fluorescence while the cytoplasm showed either diffuse or granular staining.

The nonhuman primate syncytium-forming viruses contained RNA and replicated most efficiently in dividing cells. Parks et al. (85) in 1971 demonstrated presence of an RNA dependent DNA polymerase in purified virions of
Type III, this factor being in common with RNA-containing tumor viruses.

The fine structure of primate syncytium-forming viruses has been studied by Jordan et al. (54) in 1965, Clarke and Attridge (14) in 1968, Clarke et al. (15) and Clarke et al. (16) in 1969. Viral replication involves intracytoplasmic formation of a complete spherical nucleocapsid about 35 nm in diameter. The nucleocapsids mature by budding across the plasmalemma into vacuoles or becoming extracellular. The envelope, acquired from the plasmalemma, possesses projections about 12.5 nm long and has an overall diameter of about 90 nm in ultrathin sections or 110 nm in negative stains.

Simian foamy virus Types IV, V, VI, and VII have been described on basis of serum neutralization test (96). However, no ultrastructural studies were found in the literature.

Mammary tumor virus

Chopra and Mason (12) in 1970 and Jensen et al. (51) in 1970 have described the ultrastructure and development of virus particles in tissue from spontaneous mammary tumor of a rhesus monkey. Large numbers of virus particles were present in intercellular spaces of epithelial cells. Two types of virus particles were described; one was always found intracytoplasmic and the other was extracellular.
The authors suggested that the preformed intracytoplasmic particles matured by budding across the plasma membrane and formed the enveloped extracellular particle. The intracytoplasmic particles measured 60 to 90 nm in diameter and the extracellular enveloped particles ranged from 100 to 120 nm in diameter. The nucleoid of mature particles was 30 to 50 nm in diameter.

Cats

Feline syncytia-forming virus

The feline syncytia-forming virus (FeSFV) was first reported in Ohio by McKissick and Lamont (66) in 1968, Kasza et al. (55) in 1969 and in more detail by McKissick and Lamont (65) in 1970. It was also described in California by Riggs et al. (95) in 1969 and Hackett and Manning (38) in 1971. Scott (107) reported FeSFV in New York in 1971.

The most characteristic biological property of FeSFV was its ability to produce syncytia in cell cultures, especially in young or active dividing cells. It replicates in cell cultures derived from cat, dog, chicken, horse, pig, monkey, and man. Tested isolates have failed to hemagglutinate or hemadsorb cat, chicken, guinea pig, or human "O" erythrocytes. The isolates were sensitive to ether, chloroform, heat, and acid, strongly cell-associated and composed of ribonucleic acid. The nucleocapsids, approximately 45 nm in diameter, appeared
as intact spheres in the cell cytoplasm and they budded through the plasmalemma, acquiring an envelope. The mature virions were found in vacuoles or extracellular. A series of projections developed on the envelope during the budding process. The overall diameter of the mature particle was 100 to 110 nm.

Rabbits

**Rabbit syncytium virus**

Rabbit syncytium virus was first described by Morris et al. (75) in 1965 as an isolate from the tissues of an apparently healthy cottontail rabbit. Since then, Morris isolated a strain of the same virus as a contaminant in a cell culture prepared from kidneys of a laboratory-bred New Zealand rabbit. In 1970, Brown et al. (7) did ultrastructural studies of the rabbit syncytium virus and described it as occurring in the cytoplasm and rarely in the nucleus as a 65 nm nonmembrane-bound sphere and in intracytoplasmic vesicles as a 75 to 80 nm membrane-bound particle. Rabbit syncytium virus was frequently associated with membrane-bound intracytoplasmic and intranuclear rods 75 to 110 nm in diameter. Brown et al. concluded that the rabbit syncytium virus, provisionally classified as a myxovirus, was more nearly like that of the virus of epizootic diarrhea of infant mice.
Hamsters

Hamster syncytial virus

A hamster syncytial virus was isolated by Jensen and Koprowski (52) in 1970 from 14 hamster cell lines transformed by SV-40 virus. The virus was ether and chloroform-sensitive and all infectivity was destroyed after heating at 56° C. for 30 minutes. Hemadsorption and hemagglutination tests were negative. The virus did not react with antisera of commonly known syncytia-forming viruses and did not share GS antigen of hamster sarcoma-leukemia virus complex. Electron microscopy demonstrated particles of 90 to 100 nm in diameter budding from cell membranes. The authors indicated that the virus was vertically transmitted.

Chicken

Chicken syncytial virus

A chicken syncytial virus (CSV) was described by Cook (17) in 1969. The agent was repeatedly isolated from CAL-1, GA, and JM strains of Marek's disease using chick embryo fibroblasts (CEF). The CSV did not produce syncytia in African green monkey kidney, rhesus monkey kidney, human embryonic kidney, mouse embryo, and the Wi-38 line of human embryonic lung. Syncytia were produced in duck embryo tissue culture cells.

All infectivity for CEF was destroyed when treated with ether, chloroform, or sodium desoxycholate. Syncytium
production was not eliminated by maintenance of CSV in CEF in the presence of 5-iododeoxyuridine in the medium, suggesting that the agent belongs to the RNA group of viruses. No immunological relationship was found between CSV and infectious bronchitis virus, infectious laryngotracheitis virus, subgroup A or subgroup B avian leukosis/sarcoma viruses, respiratory syncytial virus, parainfluenza type 1, 2, or 3 viruses, measles virus, herpes simplex virus, Moloney leukemia virus, or AKR mouse leukemia virus.

Electron microscopic examination of ultrathin sections of COPAL-negative\(^1\), syncytium-positive CEF inoculated with either CAL-1 or JM strain of Marek's disease revealed many C-type particles budding from the cell membrane and in intercellular spaces. These virus particles were approximately 100 nm in diameter.

Sheep

Visna-maedi-progressive pneumonia virus complex

Visna virus was first propagated in primary cell cultures of sheep choroid plexus (SCP) by Sigurdsson et al. (108) in 1960. It has since been propagated in ovine kidney and liver cells by Thormar (124) in 1961 and in embryonic bovine trachea (EBTr) cells by Harter et al. (41)

\(^1\)COPAL - complement fixation for avian leukosis.
in 1968. The characteristic cytopathic change in cell cultures of visna virus is the development of large multinucleated cells with long stellate processes. Visna virus antigen accumulates in the cytoplasm of the syncytium where it aggregates in the form of inclusion bodies. Using acridine orange staining, Thorwar (125) in 1966, revealed that RNA increased in the cytoplasm of visna-infected cells and Harter et al. (40) in 1967 showed that the inclusions exhibited the staining properties of RNA. Harter et al. (42) in 1969 revealed that incorporation of radioactive uridine, but not thymidine, into the virus indicated that the viral nucleic acid was RNA. Lin and Thorwar (60) in 1970, Schlom et al. (105) in 1971, and Stone et al. (115) in 1971, demonstrated a visna virus DNA polymerase, a biological characteristic in common with the oncogenic viruses, especially the avian and murine leukemia viruses.

Ultrastructural studies of visna infected cells were first done by Thorwar (124) in 1961. He described double-walled bodies budding at the cell membrane and being released extracellularly. These bodies appeared to later form single membrane particles averaging 85 nm in diameter and contained a centrally located electron-dense core. Coward et al. (18) in 1970 also made ultrastructural studies of visna virus-infected cell cultures and described
2 types of extracellular particles. The smaller was 65 to 110 nm in diameter and contained 20 to 30 nm electron dense core. The larger particle was 100 to 140 nm in diameter, lacked an electron-dense core, contained cytoplasmic-like material, and developed by budding from the cell surface.

Takemoto et al. (119) in 1971 examined ultrastructurally a virus isolated from progressive pneumonia of sheep and demonstrated antigenic and morphologic similarities to visna and maedi viruses of sheep. They described budding particles with an outer membrane having a diameter of 140 to 150 nm and a densely staining inner shell with a diameter of 100 to 115 nm. Extracellular particles were present and variable in size and shape measuring 90 to 110 nm in diameter and each contained a dense eccentric nucleoid measuring about 30 nm in diameter. This viral isolate has also been shown to contain RNA-dependent DNA polymerase (120).

A virus with essentially the same morphological characteristics as visna virus has been repeatedly isolated on BESp cells from cattle with marked lymphocytosis. However, no antigenic relationship to visna virus or other viruses has thus far been demonstrated.

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1Van Der Maaten, M. J., National Animal Disease Laboratory, Ames, Iowa. Data on unclassified viruses of bovine origin that causes syncytial formation in BESp cell cultures. Personal communication. 1971.
Preparation of Bovine Mesothelial Cells

The mesothelium was stripped from the surface of spleens from 5- to 7-month-old bovine fetuses obtained from a commercial slaughterhouse. The mesothelial sheets were finely minced with sharp scissors and placed in Blake bottles containing Eagle's minimal essential medium with antibiotics (penicillin, 100 units/ml; dihydrostreptomycin, 100 μg/ml; kanamycin, 100 μg/ml) and 20% fetal calf serum. The fetal calf serum was tested for the presence of bovine viral diarrhea, parainfluenza-III, and infectious bovine rhinotracheitis viruses. The culture medium was changed after 72 hours and every 7 days thereafter.

Fibroblastic-type cells, often obtained during the early growth phase, were eliminated by permitting the medium to become acid for periods of 3 to 4 days after the original monolayer was formed. The fibroblastic-type cell would selectively slough and the space on the glass surface was reoccupied by the polygonal-shaped mesothelial cells. Several weeks were sometimes required to obtain a uniform monolayer of the polygonal-shaped mesothelial cells. However, with good growth, 10 to 14 days were adequate.

Maintenance of Normal Bovine Mesothelial Cells

After most of the fibroblastic cells were eliminated, stock cells were maintained in Eagle's minimal essential
medium containing the above mentioned antibiotics and 20% fetal calf serum. The medium was changed at weekly intervals and the cultures were expanded at a 1:2 ratio every 2 to 3 weeks. Prior to inoculation, stock cell cultures were split at a 1:2 or 1:3 ratio during 4- or 7-day intervals respectfully for 2 or 3 passages using 75 square cm Falcon plastic tissue culture flasks (Falcon Plastics, Oxnard, California) for growth chambers. Once established, the bovine embryonic mesothelium (BEM) cells could be transferred through 12 to 20 passages over a period of 4 to 6 months before losing their vitality. Once vitality was lost, a new primary cell culture of BEM cells was established.

Source of Viral Material

A Cornell isolate of BSV was the only source that was maintained in BEM cell cultures for this study. All other isolates received, although previously passed several times in BESp cell cultures by the contributors, were processed for electron microscopy without further passage in BEM cell cultures.

The Cornell isolate was provided by P. C. Estes from Cornell University, Ithaca, New York. It was obtained from a one-year-old bull with lymphosarcoma (29). This isolate was a 20th passage lymph node cell culture that had been subsequently passed twice in BESp cell culture.
Other isolates received were supplied by W. A. Malmquist who had isolated them from 4 of 15 cases of clinical bovine lymphosarcoma and 6 normal animals from the National Animal Disease Laboratory herd. Additional isolates were supplied by M. J. Van Der Maaten who had isolated them from private herds and dams and fetuses from a commercial slaughterhouse. The isolates provided by Malmquist and Van Der Maaten were isolated on BESp cells from either cellular elements of milk or buffy coat cells of venous blood.

Maintenance of Infected Bovine Mesothelial Cells

Normal BEM cell cultures were initially inoculated by mixing, during passing, with recently thawed whole cell cultures of the Cornell isolate. This mixture was divided in a 1:2 ratio and placed in 75 square cm Falcon flasks as growth chambers. After a complete monolayer was formed (usually 3 to 4 days), the culture was again expanded at a 1:2 ratio (often being every other day) until syncytia occupied 50 to 80% of the cells of the monolayer.

The peak number of syncytia for any particular passage was usually present by 24 to 48 hours following transfer and as time progressed thereafter, they slowly sloughed and deteriorated as floating cells. When over 50% of nuclei of the cell culture was used in syncytial formations, a new source of BEM cells from the stock cultures
was added, usually at a 1:1 ratio during the centrifugation of the cells in the transfer process. The resulting mixture was divided by placing in two 75 square cm Falcon flasks. The inoculated cell cultures were continually expanded at a 1:2 ratio once a monolayer was formed and normal BEM cells were added during the transfer process when indicated by percentage of syncytial formations. Sixty to 80% syncytia may appear in the subsequent monolayer or it may require 2 to 3 passings before normal BEM cells were added.

Inoculated BEM cell cultures with an excess of 30% of the nuclei incorporated in syncytial formations were needed for electron microscopic examination. Most electron microscopy was done on cell cultures that had over 50% of the nuclei incorporated into syncytial formations.

Light Microscopy

Normal and inoculated BEM cell cultures were planted on coverslips in Leighton tubes. Once monolayer formation was complete, the coverslips were removed and the cells were placed in Zenker's, Bouin's, or formalin fixatives for histochemical studies.

Electron Microscopy

Normal and infected BEM cells were dispersed with 0.02% EDTA in phosphated-buffered saline and sedimented by
centrifugation at 120 x g for 5 minutes. The cells were resuspended in 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.4) for 30 minutes. They were then sedimented by centrifugation, double rinsed by resuspending and centrifugation in 0.2 M sodium cacodylate buffer (10 to 30 minutes each), and left overnight at 4°C. suspended in 0.2 M sodium cacodylate buffer. The tissue culture cells were post-fixed by suspending them in 1% osmium tetroxide in 0.2 M sodium cacodylate buffer (pH 7.4) for 30 minutes, and then double rinsed by resuspending and centrifugation in 0.2 M sodium cacodylate buffer (10 to 30 minutes each).

The post-fixed cells were pelleted in warm agar (60 to 65°C) by centrifugation at 1300 x g for 5 to 10 minutes. The pellet was cooled to 4°C (refrigerated) and cut into 1- to 2-mm cubes. The cubes were left in 0.2 M sodium cacodylate buffer overnight. Then the cubes were dehydrated through a graded series of alcohols (30%, 50%, 70%, 95%, and 100%), and embedded in Epon 812 according to the method of Luft (62).

Thin sections were cut with an Ultratome (LKB Instruments, Inc., Washington, D.C.) equipped with a diamond knife (E. I. DuPont de Nemours and Co., Wilmington, Delaware) and picked up on 200 mesh uncoated copper grids. The sections were double stained with aqueous lead citrate (pH 12.0) and uranyl acetate (pH 4.2)
according to the method of Venable and Coggeshall (128). Sections were examined with a Philips EM-200 electron microscope (Philips Electronic Instruments, Mount Vernon, New York) at 60 Kv.
RESULTS

Morphology of Bovine Embryonic Mesothelial Cells from Normal Cell Cultures

**Light microscopy**

Light microscopy of the normal control monolayer revealed cells with regular size nuclei and polygonal shape (Figure 1). The cytoplasm was relatively uniform in staining intensity and in distribution around nuclei. It contained few small vacuoles.

**Electron microscopy**

Fine structure studies revealed that the typical cell contained a centrally located nucleus and one or more prominent nucleoli (Figure 2). Cells from older cultures contained many autophagosomes (Figure 3). Small cytoplasmic vacuoles were a prominent feature of all cells and several cells contained an extensive fibrillar network, usually in a paranuclear position (Figures 4 and 5).

The chromatin was in small clumps that were uniformly distributed throughout the nucleoplasm of most cells (Figure 4) but occasionally was more concentrated at the nuclear periphery (Figures 2 and 3). The nucleoli were composed of very fine granules tightly packed into a network of dense bands with interwoven lighter zones. The dark bands of the nucleoli were often extensive and formed a nucleolocentema, often observable as a filamentous
Figure 1. Light micrograph of a normal control monolayer of BEM cell culture. Note uniform size and shape of both nuclei and whole cell. Giemsa stain. X 60
Figure 2. Electron micrograph of a BEM cell from a normal cell culture. This was the most typical cell found and contained several small and medium size cytoplasmic vacuoles (V). Note round-shaped nucleus (N) with its prominent nucleolus (Nu) and small clumps of chromatin (Ch) dispersed evenly throughout the nucleoplasm. The cytoplasm (C) is limited by an irregular border, the plasmalemma (Pm), and contains electron-dense mitochondria (M), elements of both smooth endoplasmic reticulum (Ser) in the area of the Golgi complex (G) and rough endoplasmic reticulum (Rer), polyribosomes (Pr), clumps of fibrils (F), multivesicular body (Mv), and autophagosomes (A). X 13,300

A - autophagosome
C - cytoplasm
Ch - chromatin
F - Fibril
G - Golgi complex
M - mitochondrion
Mv - multivesicular body
N - nucleus
Nu - nucleolus
Pm - plasmalemma
Pr - polyribosome
Ser - smooth endoplasmic reticulum
Rer - rough endoplasmic reticulum
V - vacuole
Figure 3. Electron micrograph of a BEM cell in an aged normal control cell culture with many electron-dense autophagosomes (A) in its cytoplasm. Note round nucleus (N) with prominent nucleolus (Nu) and nuclear membrane (Nm). The mitochondria (M) are very electron-dense and the cytoplasmic border very irregular in shape. X 8,100

A - autophagosome
M - mitochondria
N - nucleus
Nm - nuclear membrane
Nu - nucleolus
Figure 4. Electron micrograph of a BEM cell from same cell culture as that in Figure 2. Note extensive fibrillar network (F) in the paranuclear position, nuclear bodies (Nb) in the nucleus (N), electron-dense mitochondria (M), and lack of organelles in the periphery of the cytoplasm. Inset enlarged in Figure 5. X 10,400

F - fibril
M - mitochondria
N - nucleus
Nb - nuclear body
Figure 5. Enlargement of area outlined in Figure 4. Note fibril (F) in the paranuclear fibrillary network, the dilated rough endoplasmic reticulum (Rer) and its extension to close proximity of the perinuclear cisterna (Pc) between the two nuclear membranes (Nm). X 64,560

F - fibril
N - nucleus
Nm - nuclear membrane
Pc - perinuclear cisterna
R - ribosome
Rer - rough endoplasmic reticulum
zone by light microscopy.

In a few cells the nuclear border was markedly irregular with deep invaginations into the nucleus (Figure 6). Cross sections of these invaginations were occasionally observed and gave the false impression of nuclear inclusions (Figure 7). The inner and outer nuclear membranes were prominent and nuclear pores were observed to be traversing the space between the 2 membranes (Figures 8 and 9). A fibrillar network of chromatin granules was occasionally observed that appeared to extend into nuclear pores giving the impression that nuclear pores may be permeable for transfer of molecules from the nucleus to the cytoplasm or vice versa (Figure 10). When seen in tangential section, nuclear pores were more numerous than anticipated from transverse sections of nuclear membranes (Figures 11 and 12). In sections tangential to the nuclear surface, the pore was outlined by a circle which represents the line of fusion between the inner and outer nuclear membranes (Figure 11).

The Golgi complex appeared as several layers of interrupted sacs that were stacked one upon the other and generally located in a juxtanuclear position (Figures 13 and 14). Commonly associated with the Golgi complex were small dense vesicles and elements of the smooth endoplasmic reticulum. There was marked variation in
Figure 6. Electron micrograph of part of a nucleus (N) in a BEM cell from a normal cell culture. Note deep cytoplasmic invagination of nuclear membrane (Nm) with inclusions of mitochondria (M). X 31,200

C - cytoplasm
Ch - chromatin
M - mitochondrion
N - nucleus
Nm - nuclear membrane

Figure 7. Electron micrograph of transverse section of cytoplasmic invagination in a nucleus (N) of a BEM cell from a normal cell culture. Note the multivesicular bodies (Mv) within the inclusion. X 49,200

C - cytoplasm
Ch - chromatin
Mv - multivesicular body
N - nucleus
Nm - nuclear membrane
Figure 8. Electron micrograph of nucleus in BEM cell of a noninfected cell culture. Note nucleus with many nuclear pores (arrows) traversing the perinuclear cisterna (Pc). X 21,900

A - autophagosome
Ch - chromatin
M - mitochondrion
Nu - nucleolus
Pc - perinuclear cisterna
Figure 9. Electron micrograph of the paranuclear region of a BEM cell from a noninfected cell culture. Note transverse section of the nuclear membrane (Nm) with dilated perinuclear cisterna (Pc) between the two nuclear membranes and the nuclear pores (Np) traversing it. X 49,200

C - cytoplasm
N - nucleus
Nm - nuclear membrane
Np - nuclear pore
Pc - perinuclear cisterna
V - vacuole

Figure 10. Electron micrograph of the paranuclear region in the same cell as in Figure 9. Note detail of a longitudinal section through a nuclear pore (Np). The inner nuclear membrane (Inm) is reflected as the wall of the nuclear pore and the outer nuclear membrane (Onm), enclosing the perinuclear cisterna (Pc). The chromatin (Ch) of the nucleus appears to extend through the nuclear pore. X 148,800

C - cytoplasm
Ch - chromatin
Inm - inner nuclear membrane
N - nucleus
Np - nuclear pore
Onm - outer nuclear membrane
Pc - perinuclear cisterna
Figure 11. Electron micrograph of cytoplasm from a non-infected EEM cell on a plane that is tangential to surface of nucleus. Note circular nuclear pores (Np) having dense centers (arrows) and scattered throughout the chromatin (Ch). Cytoplasmic microtubules (Mt) appear to be associated with nuclear pores. X 64,600

C - cytoplasm
Ch - chromatin
M - mitochondrion
Mt - microtubule
Np - nuclear pore
Pr - polyribosome
V - vacuole
Figure 12. Electron micrograph of nucleus of noninfected BEM cell in a sagittal plane medial to that shown in Figure 11. Note nuclear pores (Np) where the plane of section is tangential to the nuclear membrane (Nm). Compare with Figure 11. X 64,600

C - cytoplasm
Ch - chromatin
M - mitochondrion
N - nucleus
Nm - nuclear membrane
Np - nuclear pore
Figure 13. Electron micrograph with a panoramic view of a noninoculated BEM cell. Note juxtanuclear position of the membranous Golgi complex (G) and continuity of endoplasmic reticulum with plasmalemma (arrows). Inset enlarged in Figure 14. X 16,800

F - fibril
G - Golgi complex
M - mitochondrion
N - nucleus
Nm - nuclear membrane
Pm - plasmalemma
Rer - rough endoplasmic reticulum
V - vacuole
Figure 14. Enlargement of enclosed area in Figure 13. Note detail of membranous sacs of the Golgi complex (G), the electron dense transport vesicles (Vt), the paranuclear fibriles (F), and electron dense mitochondria (M). X 37,500

C - cytoplasm
F - fibril
G - Golgi complex
M - mitochondrium
N - nucleus
Nm - nuclear membrane
Pm - plasma membrane
Vt - transport vesicle
the amount of rough endoplasmic reticulum from one cell to the next with virtually none in some to large quantities in others. The endoplasmic reticulum was frequently demonstrated to be continuous with the plasma membrane (Figures 13 and 15). The free ribosomes were also very variable in number from a few individually scattered throughout the cytoplasm of some cells to clusters of polyribosomes in other cells.

Mitochondria, some with transversely and others with longitudinally oriented crests or cristae, were scattered throughout the cytoplasm. Mitochondrial cristae were demonstrated to be infoldings of the inner mitochondrial membrane. Some of the cristae terminated as a closed apex in the finely granular mitochondrial matrix and others formed complete transverse bridges with the inner mitochondrial membrane of the opposite side. The membranes of cristae in some of the mitochondria were thin and distinct in an electron-lucent mitochondrial matrix (non-energized form) while other membranes of cristae were indistinct and the mitochondrial matrix was filled with very fine electron-dense granules (energized form) (Figures 16 and 17). Energized and nonenergized mitochondria were never found in the same cell.

Centrioles were a frequent finding and usually were present in a juxtanuclear region adjacent to the Golgi
Figure 15. Electron micrograph of periphery of cytoplasm of BEM cell from noninfected cell culture. Note extension of smooth endoplasmic reticulum (Ser) to the plasmalemma (Pm). X 31,200

M - mitochondrion
Mv - multivesicular body
Pm - plasmalemma
Ser - smooth endoplasmic reticulum
Figure 16. Electron micrograph of nonenergized mitochondrion. Note distinct membranes of the mitochondrial cristae (Mc), the continuation of inner mitochondrial membrane as an infolding forming the cristae (arrows), and the ribosome "studded" rough endoplasmic reticulum (Rer) adjacent to the mitochondrion. X 88,800

C - cytoplasm
Mc - mitochondrial cristae
Mm - mitochondrial matrix
Rer - rough endoplasmic reticulum

Figure 17. Electron micrograph of an energized mitochondrion. Note electron dense mitochondrial matrix (Mn), lack of distinct membranes in the mitochondrial cristae, the dilation of the cisterna (Mcc) of mitochondrial cristae, and its continuation with the space between membranes (arrows) of the double membrane wall of the mitochondrion. X 153,800

C - cytoplasm
F - fibril
Mcc - cisterna of mitochondrial cristae
Mm - mitochondrial matrix
R - ribosome
complex (Figure 18). When both centrioles of a cell were sectioned longitudinally, they were oriented at right angles to one another. A series of parallel lines were visible in the long axis of each centriole. A transverse section of a centriole demonstrated that the parallel lines were longitudinally oriented structures consisting of 27 microtubules arranged in 9 triplets (Figure 19). Each microtubule of the triplet shared its wall with the adjacent tubule. Cytoplasmic microtubules with a diameter of 15 to 20 nm were present among the vesicular profiles in the vicinity of the centrioles. Occasionally, paired parent-daughter centrioles were present in the same area of the cell cytoplasm (Figure 20). Whenever the number of centrioles exceeded more than 4 in number, their orientation in relation to each other was much more haphazard than when only 2 were present in the same cell (Figures 21 and 22). On several occasions, when the centrioles were close to the plasma membranes, 1 of the centrioles would be observed as a basal body of a cilium while the other centriole still maintained its perpendicular arrangement to the basal body (Figures 19 and 23). Although external fibrous appendages were observed in both centrioles and basal bodies, no rootlets were observed to be associated with the basal bodies of BEM cells. Occasionally 2 to 4 cilia were observed on the
Figure 18. Electron micrograph of cytoplasm in a BEM cell from noninfected cell culture. Note paired centrioles, the parallel lines in the wall of centrioles, the central vesicle (Cv) and external fibrous appendage (Efa) radiating outward from the centriole. X 148,800

Cv - central vesicle of centriole
Efa - external fibrous appendage

Figure 19. Electron micrograph of cytoplasm of a BEM cell from noninfected cell culture. Note paired centrioles, one as a basal body of a cilium (Ci). The 27 microtubules of the centriole are arranged into 9 fused triplets with blades whose angle with the axis of the centriole form a "pinwheel". Note the microtubule (Mt) arrangement with the external fibrous appendages (Efa) and invagination of plasmalemma (Pm) around the cilium and attaching near the basal body. X 64,600

Ci - cilium
Efa - external fibrous appendage
Mt - microtubule
Pm - plasmalemma
Figure 20. Electron micrograph of cytoplasm in a BEM cell of noninfected cell culture. Note pair of parent-daughter centrioles located near plasmalemma (Pm), the presence of two central vesicles (Cv) in the two centrioles closest to the plasmalemma, and the stage of coated vesicle (Vc) formation by an invagination of plasmalemma into the cytoplasm. X 64,600

Cv - central vesicle of centriole
Pm - plasmalemma
Vc - coated vesicle
Figure 21. Electron micrograph of a BEM cell from non-infected cell culture. Note irregular shaped nucleus, the 6 centrioles clustered in its cytoplasm (enclosed area), the cytoplasmic inclusions in the nucleus (N), even distribution of chromatin (Ch), prominent nucleolus (Nu) and many mitochondria (M) in the cytoplasm. Enclosed area enlarged in Figure 22. X 10,400

- C - cytoplasm
- Ch - chromatin
- M - mitochondrion
- N - nucleus
- Nu - nucleolus

Figure 22. Enlargement of enclosed area in Figure 21. Note parallel lines in the oblique section of the centrioles. One centriole is incorporated in a basal body of a cilium which extends into a vacuole. Note microtubules (Mt) in cytoplasm surrounding centrioles. Tips of external fibrous appendage of basal body can be seen (arrows). X 37,500

- Mt - microtubule
Figure 23. Electron micrograph of periphery of cytoplasm in a BEM cell from noninfected cell culture. Note longitudinal view of a cilium that extends from a deep invagination of cytoplasm, the microtubular subfibrils (Fs) in the cilium and the many free ribosomes (R) in the cytoplasm. X 84,800

Fs - subfibril
R - ribosome
surface of a cell (Figure 24).

Light and electron microscopic examination of the noninfected BEM cell cultures for syncytia and virus particles consistently revealed that the virus under study and other known bovine syncytial forming viruses were not introduced with the primary BEM cell cultures or fetal calf serum used in the culture medium.

Morphology of Bovine Embryonic Mesothelial Cells from Inoculated Cell Cultures

**Light microscopy**

Inoculated BEM cell cultures examined by light microscopy contained syncytia of variable sizes and were easily differentiated from the polygonal-shaped normal cells (Figure 25). A syncytium contained up to 40 or more nuclei and over 80% of the nuclei of cells included in the monolayer were often included in syncytial formations. The arrangement of nuclei within the syncytium was variable and often would surround large inclusion bodies.

Sera that gave positive immunodiffusion test, when conjugated with fluorescein, also intensely stained the cytoplasm of many cells in the inoculated cell cultures. This fluorescent antibody stained material was in the form of small solid granules and circles (Figure 26). Large granular amorphous structures were also observed adjacent to or among the cluster of nuclei and stained intensely.
Figure 24. Electron micrograph of cytoplasm of BEM cell from a noninfected cell culture. Note longitudinal section of 2 cilia and tangential section of tips of 5 additional cilia, all in the same cytoplasmic invagination. Many microtubules (Mt) are present in the cytoplasm around basal bodies and centriole. X 64,600

C - cytoplasm
Ci - cillum
Cv - central vesicle of centriole
Mt - microtubule
Pm - plasmalemma
Figure 25. Light micrograph of monolayer of BEM cell culture inoculated with BSV. Note the many syncytia dispersed between the mononuclear BEM cells. Giemsa stain. Compare with Figure 1. X 60
Figure 26. Light micrograph of syncytium from BESp cell culture inoculated with BSV and stained with fluorescent antibody. Note the fluorescent bodies, some round and others forming circles. X 520

Figure 27. Light micrograph of syncytium from BESp cell culture inoculated with BSV and stained with fluorescent antibody. Note the large linear inclusions between the nuclei. X 520
with fluorescein conjugated sera and morphologically resembled similar inclusions observed with histochemical reactions (Figures 27, 28, and 29).

**Electron microscopy**

Electron microscopy of the inoculated cell cultures also demonstrated large syncytium among mononuclear cells. Although the nuclei could be found dispersed throughout the plane of section of the cell, they were more frequently observed in eccentric clusters of 2 to 5 nuclei. As many as 15 nuclei were observed in a cell in 1 section (Figures 30 and 32). The cells were more vacuolated than the controls and contained variable quantities of dense debris within variable size membrane lined vacuoles analogous to the large inclusions demonstrated with histochemical and fluorescent antibody stains.

Occasionally, the plasmalemmas of 2 mononuclear cells or a mononuclear cell and a syncytium were observed to be in close apposition to each other as a tight junction. In some such tight junctions, portions of the membranous barrier between the 2 cells appeared to be lacking, thus continuity of the cytoplasrn of the 2 cells (Figure 31).

**Plasmalemma Associated Bovine Syncytial Virus**

Viral nucleocapsids were found in budding forms contiguous with the membranes of vacuoles or plasmalemma
Figure 28. Light micrograph of a syncytium from a BEM cell culture inoculated with BSV and stained by Giemsa method. Note the small granular foci in the cytoplasm and compare with Figure 26. X 600

Figure 29. Light micrograph of a syncytium from a BEM cell culture inoculated with BSV and stained by the Giemsa method. Note the large inclusions between the nuclei and compare with Figure 27. X 600
Figure 30. An electron micrograph of a syncytium from a BEM cell culture inoculated with BSV obtained from a lymph node cell culture of a 1-year-old lymphosarcomatous bull. Note a syncytium with several nuclei (N), many mitochondria (M) and vacuoles (V) in the cytoplasm, the prominent nucleoli (Nu), and the margination of the chromatin (Ch). X 3,350

Ch - chromatin
M - mitochondrion
N - nuclei
Nu - nucleoli
Pm - plasmalemma
V - vacuole
Figure 31A. Electron micrograph of the cytoplasm of 2 BEM cells from a cell culture inoculated with BSV. Note the close apposition of the 2 plasmalemmas that appear to be attached to each other. X 64,600

Pm - plasmalemma

Figure 31B. Electron micrograph of the cytoplasm of 2 BEM cells from a cell culture inoculated with BSV. Note the close apposition and interlocking position of the 2 plasmalemmas. In areas (arrows) there appears to be continuity between the cytoplasm of the 2 cells. X 49,200

Pm - plasmalemma
Figure 32. Electron micrograph of a syncytium from BEM cell culture inoculated with BSV obtained from a lymph node cell culture of a 1-year-old lymphosarcomatous bull. The section represents a plane through about one-fourth of the syncytium and contains 4 nuclei (N), and many small cytoplasmic vacuoles. Note the prominent nucleoli (Nu), margination of the chromatin (Ch), and irregular border represented by the plasmalemma (Pm). Enclosed area enlarged in Figure 33. X 5,800

Ch - chromatin
N - nucleus
Nu - nucleolus
Pm - plasmalemma
of syncytia and mononuclear cells (Figures 33, 34, and 35). It was not determined if the viral particles were more prevalent in syncytia or mononuclear cells since the 3rd dimension of the cell was not examined. A higher percentage of syncytia did contain the budding viral nucleocapsids when compared with the mononuclear cells.

The nucleocapsids, with one exception (Figure 36), were first observed just beneath the plasmalemma of cytoplasmonic vacuoles or cell exterior (Figures 37 and 38). No immature stages of development of these nucleocapsids were observed. The outer membrane of the viral particle was derived from the plasmalemma during the process of budding (Figure 39). The nucleocapsids were spherical and their centers varied from electron-dense to electron-lucent. Projections, which were prominent on many of the budding viral particles, were first observed where the nucleocapsid attached to the cell membrane. The projections appeared to be retained on the surface of the outer membrane of the mature viral particles found in vacuoles of the cytoplasm of cells, but they were lacking on those that were extracellular (Figures 40 and 41).

In infected BEM cell cultures with high percentages of syncytia, viral particles were observed in 50% or more of the cells examined and were found in budding forms at the plasmalemma of the cells with irregular borders or along
Figure 33. Enlargement of enclosed area in Figure 32. Note the 35 to 45 nm nucleocapsids (Nc) associated with the plasmalemma. X 64,600

Nc - nucleocapsids
Figure 34. Electron micrograph of cytoplasm from a BESp cell of a cell culture that was inoculated with buffy coat cells from an apparently normal cow. Note nucleocapsids in process of being enveloped by the plasmalemma (Pm), and nonenveloped nucleocapsids (Nc) in the cytoplasm. Compare the size of nucleocapsids with the ribosomes in the polyribosome (Pr) clusters. X 49,200

Nc  - nucleocapsid
Pm  - plasmalemma
Pr  - polyribosome
Rer - rough endoplasmic reticulum
V   - vacuole
Figure 35. Electron micrograph of cytoplasm of BESp cell from a cell culture inoculated with cellular elements of milk from a lymphosarcomatous cow. Enveloped virions (Vi) are present in vacuoles and nucleocapsids are found associated with membranes of small vacuoles (arrows). Note the free ribosomes (R) and nonenergized mitochondria (M). X 64,600

M - mitochondrion
R - ribosomes
Vi - virion
Figure 36. Electron micrograph of cytoplasm of BESp cell from a cell culture inoculated with buffy coat from fetus of a clinically normal cow harboring BSV. Note free viral nucleocapsids (Nc) dispersed in cytoplasm between ribosomes (R) and electron-dense granules (Gr). Note the 2 nucleocapsids (arrows) in top right of figure in position to bud into vacuole (V). X 64,600

Gr - electron-dense granules
Nc - nucleocapsid
R - ribosomes
V - vacuole
Figure 37. Low power electron micrograph of cytoplasm of a syncytium in a BEM cell culture inoculated with BSV obtained from lymph node cell culture of 1-year-old lymphosarcomatous bull. Many viral nucleocapsids are present beneath the plasmalemma (arrows). Enclosed area enlarged in Figure 38. X 8,100

M - mitochondria
V - vacuole
Pm - plasmalemma
Figure 38. Higher magnification of the enclosed area in Figure 37. Note the many nucleocapsids (arrows) associated with the plasmalemma. X 64,600
Figure 39. An electron micrograph of periphery of cytoplasm of a BESp cell from a cell culture inoculated with cellular elements of milk from a lymphosarcomatous cow. Note viral nucleocapsids (Nc) associated with the plasmalemma (Pm). Note the many spikes on the virus bud. Enclosed area enlarged in inset. Figure 39, X 148,800. Inset, X 295,000.

Nc - nucleocapsid
Pm - plasmalemma
Figure 40. Electron micrograph of cytoplasm and cytoplasmic projections of a BEM cell from a cell culture inoculated with buffy coat cells of blood from apparently normal cow. Note many immature virions associated with the plasmalemma (Pm). Enclosed area includes enveloped virions and is enlarged in Figure 41. X 37,500

Pm - plasmalemma
Figure 41. Enlargement of outlined area in Figure 40 with 4 extracellular enveloped virions. Note trilaminar or unit membrane structure of viral envelope and compare with trilaminar or unit membrane structure of plasmalemma of host cell (arrows). X 247,000
membranes of cytoplasmic vacuoles in cells having a prominent Golgi complex. Occasionally, a degenerate cell was found and it usually contained virions (Figure 42).

The plasmalemma associated viral particles had the following range of dimensions: 35 to 45 nm as the diameter of the nucleocapsids; 14 to 16 nm from the nucleocapsid through the outer membrane; 14 to 18 nm as the length of the projections; and an overall diameter of 90 to 115 nm.

**Endoplasmic Reticulum Associated Bovine Syncytial Virus**

After further serial passage of the inoculated BEM cell cultures, nucleocapsids similar to those of viral particles associated with the plasmalemma were observed in the cell cytoplasm and associated with double membrane or tubular-like structures (Figure 43). These nucleocapsids were in various stages of being enveloped by double membranes of variable lengths (Figures 44, 45, and 46). Most of the nucleocapsids were enclosed individually but occasionally 2 or more were enclosed within a common envelope. In some instances, several nucleocapsids were lined up along a double membrane and were budding across it—often in opposite directions (Figures 47 and 48). These membranes were often flared at one end and were continuous with the rough endoplasmic reticulum (Figures 49, 50, 51, 52, and 53).

The nucleocapsids were 35 to 45 nm in diameter. The
Figure 42. Electron micrograph of the cytoplasm from a BSV infected BESp cell which has degenerated. The cell culture was inoculated with buffy coat cells of an apparently normal cow. Note presence of a dense halo around nucleocapsids (arrows) and absence of the unit membrane structure of the viral envelope as observed in Figure 41. X 191,300
Figure 43. Electron micrograph of an area in the cytoplasm of a BESp cell from cell culture inoculated with buffy coat cells of an apparently normal cow. Note the nucleocapsids (Nc) associated with the membrane of a large cytoplasmic vacuole (V) and double membrane structures deep in the cytoplasm. Note aggregate of electron-dense material thought to be viroplasm (Vp). X 84,800

Nc - nucleocapsid
Rer - rough endoplasmic reticulum
V - vacuole
Vp - viroplasm-like material
Figure 44. Electron micrograph in an area from a syncytium in a BESp cell culture inoculated with buffy coat cells of an apparently normal cow. Note large dense granular inclusion (I), viroplasm-like material (Vp), and intracytoplasmic clusters of endoplasmic reticulum associated (ERA) viral particles. Compare large inclusions with areas that reacted with fluorescent antibody stain in Figure 27 and the large inclusions in Figure 29. Enclosed areas enlarged in: A - Figure 45, B - Figure 46, and C - Figure 47. X 5,800

ERA - endoplasmic reticulum associated viral particle
I  - granular inclusion
N  - nucleus
Vp - viroplasm-like material
Figure 45. Enlargement of area "A" in Figure 44. Note parts of large inclusions (I), the many endoplasmic reticulum associated viral particles (ERA) and some viral nucleocapsids that are misshaped (arrows). X 64,600

ERA - endoplasmic reticulum associated viral particle
I - granular inclusion
Figure 46. Enlargement of area "B" in Figure 44. Note the large granular inclusions (I) that appear to be composed of membranes and cellular debris and probably represent large autophagosomes. Three or more viral nucleocapsids of endoplasmic reticulum associated viral particles appear to be enveloped within a common envelope (ERA) X 64,600

ERA - endoplasmic reticulum associated viral particle
I - granular inclusion
Figure 47. Enlargement of area "C" in Figure 44. Note extensive entwining of tubular-like structures between the nucleocapsids and the various stages of envelopment of nucleocapsids by this membranous structure. The nucleocapsids appear to be budding across these double-walled membranes, often in opposite directions (arrows). X 64,600

ERA - endoplasmic reticulum associated viral particle
N  - nucleus
Nm  - nuclear membrane
Figure 48. Electron micrograph of cytoplasm of BSV infected BESp cell from a cell culture inoculated with buffy coat cells from an apparently normal cow. Note 2 areas of extensive tubular-like structures that are entwined between the many viral nucleocapsids, the branched tubular structure (arrow), the nearby rough endoplasmic reticulum (Rer), and nonenergized mitochondrium (M). X 64,600

Rer - rough endoplasmic reticulum
M - mitochondrium
Figure 49. Low power electron micrograph of cytoplasm from a BSV infected BESp cell from a cell culture inoculated with buffy coat cells from an apparently normal cow. Note large membrane bound vacuoles filled with debris (I), viroplasm-like material (Vp), and intracytoplasmic viral nucleocapsids associated with endoplasmic reticulum (ERA). Enclosed area enlarged in Figure 50. X 16,800

ERA - endoplasmic reticulum associated viral particle
I - granular inclusion
Pm - plasmalemma
Rer - rough endoplasmic reticulum
Vp - viroplasma-like material
Figure 50. Enlargement of area outlined in Figure 49. Note association of the tubular-like structure between viral nucleocapsids and its continuation with the rough endoplasmic reticulum (arrow). Inset is that of a viral nucleocapsid forming a viral bud in a tubular-like structure. Figure 50, X 112,000. Inset, X 135,000

ERA - endoplasmic reticulum associated viral particle
Nc - nucleocapsid
M - mitochondrion
Rer - rough endoplasmic reticulum
Figure 51. Electron micrograph of cytoplasm from a BSV infected BESp cell from a cell culture inoculated with buffy coat cells from an apparently normal cow. Note rough endoplasmic reticulum (Rer) as being continuous with (arrow) and providing the source of material for the double walled envelopes of viral nucleocapsids (Nc). X 64,600

Nc - nucleocapsid
M  - mitochondrion
N  - nucleus
Rer - rough endoplasmic reticulum
Figure 52. Electron micrograph of cytoplasm of a BSV infected BESp cell from a cell culture inoculated with buffy coat cells from an apparently normal cow. Note viral nucleocapsids (Nc) being enveloped by tubular-like structures that are continuous with the rough endoplasmic reticulum (arrows). X 84,800

M - mitochondrion
Nc - nucleocapsid
Rer - rough endoplasmic reticulum
Figure 53. Electron micrograph of an area in the cytoplasm of a BSV infected BESp cell from a cell culture inoculated with buffy coat cells from an apparently normal cow. Note large quantity of rough endoplasmic reticulum (Rer) that is continuous (arrows) with the tubular-like structures of the viral envelopes. X 64,600

Nc - nucleocapsid
Rer - rough endoplasmic reticulum
Vp - viralplasm-like material
inner membrane of the double membrane envelope was 10 to 16 nm from the outer surface of the nucleocapsid and the outer membrane was 27 to 37 nm from the inner membrane. The overall diameter of the double membrane enveloped virion was 110 to 150 nm. Projections, similar to those on budding viral particles associated with the plasmalemma, were occasionally found on the inner membrane of the envelope and extended into the space between the 2 membranes (Figure 50).

Often, both the endoplasmic reticulum associated (ERA) viral nucleocapsids and plasmalemma associated (PA) viral nucleocapsids occurred in the same cell (Figure 43). Although the ERA viral particles were easier to find in some isolates than in others, in no instance were ERA viral particles found in a cell culture without also finding PA viral particles. However, the ERA viral particles could not be demonstrated in all isolates where the PA viral particles were observed. Nucleocapsids that were not associated with either the plasmalemma or the endoplasmic reticulum were found in only one cell of one isolate (Figure 36).

Comparison of Fluorescent Bodies and Electron-dense Granular Bodies

Electron-dense aggregates of viroplasm-like material were frequently found in the cytoplasm of cells from infected
cell cultures that contained either or both of the PA viral particles or ERA viral particles. Such dense aggregates varied widely in number, size and shape. They were never observed in the normal cell cultures, and often cells of infected cell cultures that demonstrated viral particles did not include such aggregates. However, they were present in at least some cells of all of the different isolates examined. These dense aggregates consisted of nonmembrane bound deposits of dense granules that were smaller than ribosomes and appeared about the same size as chromatin material of the nucleus. The aggregate size varied from a few granules to large clumps up to 2 microns in diameter (Figure 54). These aggregates usually were solid, round masses but vacuolated forms did occur (Figure 55). The aggregates were similar in size, shape, and location in the cytoplasm to that of the fluorescent antibody granules observed in the cytoplasm of fluorescent antibody stains of infected cell cultures of BSV. Although the nucleus of many of the syncytial formations did exhibit immunofluorescence, no viral particles were found in the nucleus of any of the BEM cells examined.
Figure 5. Electron micrograph of cytoplasm of a BEM cell from cell culture inoculated with BSV obtained from lymph node cell culture of a 1-year-old lymphosarcomatous bull. Note many aggregates of electron-dense granules that are thought to react with fluorescent antibody and represent a viroplasm-like material (Vp). Note nucleocapsids being enveloped by the tubular-like structures (arrows) and compare viroplasm-like aggregates with the areas that stained with fluorescent antibody in Figure 26. X 31,200

Vp - viroplasm-like material
F - fibril
Figure 55. Electron micrograph of large vacuolated mass of an electron-dense granule of viroplasm-like material from cytoplasm of a BSV infected cell. Note fine granularity of electron-dense material. X 84,800
DISCUSSION

Plasmalemma Associated Bovine Syncytial Virus

Immature virions of BSV associated with the plasmalemma and membranes around cytoplasmic vacuoles were noted by electron microscopic examination of sections from BESp and BEM cell cultures containing syncytia formations. The nucleocapsids were uniform in size and shape (Figures 33, 34, 35, 36, 37, 38, and 40). The virions were enveloped by budding at the plasmalemma, thus the term "plasmalemma associated bovine syncytial virus" (PA-BSV). The trilaminar or unit membrane structure of the plasmalemma was retained in the envelope of the extracellular virions (Figure 41).

Detectable differences in ultrastructure of virions were not found in the different isolates of PA-BSV. Furthermore, BEM and BESp cell cultures were comparable as cell systems for PA-BSV production. Syncytia were produced in both and no structural differences were observed between the virions of the 2 cell systems.

Comparison with C-type RNA tumor viruses

Plasmalemma associated bovine syncytial virus does not fit the morphological criteria of the C-type RNA tumor viruses that have been demonstrated to actually cause leukemia and certain solid tumors in mice (37, 43), chickens (102), cats (49, 50, 94), hamsters (36, 56), and
observed in lymphosarcomas and other tumors of rats (129),
guinea pigs (77, 82), swine (46), or in human (22, 76),
simian (12, 51), and reptilian (133) tumor cells. The
nucleocapsids of the C-type RNA tumor viruses are larger
(60 to 70 nm) and mature at the plasmalemma during the
process of budding; whereas, PA-BSV nucleocapsids are
smaller (35 to 45 nm) and appear as an already mature form
at the plasmalemma. Furthermore, free nonmembrane
associated BSV nucleocapsids were found in the cytoplasm
of the cell (Figure 36). The C-type RNA tumor viruses
are enveloped by the host cell plasmalemma resulting in a
smooth surface envelope giving an overall diameter of 90 to
100 nm to the extracellular virion. The PA-BSV particle
was also enveloped by the plasmalemma but possessed
prominent projections on its surface resulting in an
overall diameter of 90 to 115 nm from tip to tip of pro­
jections. Sometimes extracellular PA-BSV did not retain
its projections and thus resulted in a smooth enveloped
virion measuring 80 to 90 nm (Figure 41). An intermediate
membrane often described as being located between
nucleocapsid and envelope in C-type RNA tumor viruses
was not found in PA-BSV. Although PA-BSV particles differ
from C-type RNA tumor viruses in location of maturation
of the nucleocapsids and possessing projections on its
envelope, they did have the following in common with C-type
viruses: the nucleocapsids were assembled in the cytoplasm, 
the nucleocapsids were enveloped at the plasmalemma, virions 
were ether sensitive, and the overall diameter of the 
virions were in a size range of 90 to 115 nm.

Comparison with other syncytial forming viruses

Ultrastructural studies have not been done on bovine 
respiratory syncytial virus, but it was reported to be 
antigenically related to human RS virus (83) which is more 
pleomorphic and smaller than PA-BSV. Rabbit syncytium 
virus is smaller than PA-BSV and possesses no projections 
on its envelope (7). Although hamster syncytial virus does 
approach the size of PA-BSV, no detailed morphology has 
been described for comparison with PA-BSV (52).

The viruses of visna-media-progressive pneumonia 
complex are much more variable in size than PA-BSV, ranging 
from 65 to 150 nm (18, 119, 124). Three distinctly dif­
ferent viral particles have been described in budding and 
extracellular forms of viruses of this complex compared 
with only 1 form for PA-BSV. Furthermore, visna 
nucleocapsids were formed during the budding process at 
the plasmalemma and thus more closely resembled that of 
the C-type RNA tumor viruses than PA-BSV.

Plasmalemma associated bovine syncytial virus closely 
resembled FeSFV, simian foamy viruses, and a virus isolated 
from human nasopharyngeal carcinoma (Table 1). These 4
Table 1. Comparison of PA-BSV,\(^a\) ERA-BSV,\(^b\) FeSFV,\(^c\) simian foamy viruses, and virus from human nasopharyngeal carcinoma

<table>
<thead>
<tr>
<th>Virus</th>
<th>Nucleic acid composition</th>
<th>Nucleocapsid size</th>
<th>Site of envelopment</th>
<th>Total diameter of enveloped virion</th>
<th>Projections on surface of envelope</th>
<th>Ether sensitivity</th>
<th>Hemagglutination</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA-BSV</td>
<td>RNA(^d)</td>
<td>35-45</td>
<td>Plasmalemma</td>
<td>90-115</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>14-18 nm</td>
<td></td>
<td>0(^e)</td>
</tr>
<tr>
<td>FeSFV</td>
<td>RNA</td>
<td>45</td>
<td>Plasmalemma</td>
<td>100-110</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td></td>
<td>No</td>
</tr>
<tr>
<td>Simian foamy viruses</td>
<td>RNA</td>
<td>35</td>
<td>Plasmalemma</td>
<td>90-110</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>12.5 nm</td>
<td></td>
<td>No</td>
</tr>
<tr>
<td>Virus from human nasopharyngeal carcinoma</td>
<td>0</td>
<td>45</td>
<td>Plasmalemma</td>
<td>About 100</td>
<td>Yes</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>13 nm</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) PA-BSV - plasmalemma associated bovine syncytial virus.
\(^b\) ERA-BSV - endoplasmic reticulum associated bovine syncytial virus.
\(^c\) FeSFV - feline syncytial forming virus.
\(^d\) RNA-dependent DNA polymerase has been demonstrated for BSV, but no RNA analysis has been determined.
\(^e\) 0 - not determined.
viruses have nucleocapsids which were preformed before the budding process. Their nucleocapsids were of similar size and were enveloped at the plasmalemma. The enveloped virions possessed prominent projections on surface of their envelopes and a total diameter of 90 to 115 nm. Thus, the viruses probably should be grouped together as a class. Spumaviruses has been suggested for such a group of viruses.¹

Endoplasmic Reticulum Associated Bovine Syncytial Virus

It seems plausible that BSV, which buds at the plasmalemma, could also bud through the rough endoplasmic reticulum (RER). This study revealed that the double membrane envelope of the virions in the cytoplasm had its origin from the RER (Figures 49, 50, 51, 52, and 53). The morphology of the nucleocapsid of ERA-BSV particle was similar to that of PA-BSV, and the distance from the nucleocapsid to the envelope of PA-BSV was approximately the same as the distance from the nucleocapsid to the first membrane of the ERA-BSV particle. The 2 virions differed in that the envelope of the ERA-BSV particle had a 2nd membrane with no projections which added 20 to 35 nm

¹Parks, W. P., Viral Carcinogenesis Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland. Foamy viruses from several species: a distinct animal virus group. Personal communication. 1971.
to its overall diameter.

The ERA-BSV particles were considered to be an aberrant form of PA-BSV for the following reasons: the nucleocapsids were the same size, they were associated with PA-BSV immunofluorescence, they were found in the same cells as PA-BSV, and they were not seen in the absence of typical PA-BSV particles. A diagrammatic comparison of PA-BSV and ERA-BSV particles is illustrated in Figure 56.

A possible sequence of events in the development of the ERA-BSV particle (Figure 57) began with the appearance of free nucleocapsids in the cytoplasm. These nucleocapsids became associated with the membranes of the vacuolar system of the cell. In the case of the RER, the width of the cisterna was reduced and the nucleocapsids budded through both membranes of the RER instead of into the cisterna. Altered membranes apparently underwent further fragmentation and fusion producing various intermediate stages before ultimately forming double membrane enveloped virions.

Other known viruses described as associated with the endoplasmic reticulum include simian foamy viruses (15, 16), murine leukemia viruses (10, 20), mammary tumor virus of mice (23), and a virus associated with feline infectious peritonitis (134). All of these viruses were reported to have a stage of maturation by budding into the
Figure 56. An illustration comparing the dimensions of the single membrane-enveloped PA-BSV particle (top) with the double membrane-enveloped ERA-BSV particle (bottom).

Figure 57. An illustration of the origin of the ERA-BSV envelopes. Nucleocapsids are free in the cytoplasm (la), associated with rough endoplasmic reticulum (lb), and budding in opposite directions across altered endoplasmic reticulum (lc). Various intermediate stages (2, 3, 4) of the double membrane-enveloped virions (5) are illustrated.
cisterna of endoplasmic reticulum of the host cell. These viruses differed from ERA-BSV particles by budding across only one membrane and thus not acquiring a double membrane envelope as described for ERA-BSV.

The ERA-BSV particles were not contaminants since they could be isolated repeatedly from the same animal and control cell cultures remained negative.

**Fluorescent Antibody Particles and Electron-dense Granular Bodies**

It was previously suggested (64) that the intense cytoplasmic immunofluorescence indicated maturing virions at the membranes surrounding cytoplasmic vacuoles. The particulate, solid, and circular cytoplasmic bodies observed in immunofluorescent studies of BSV inoculated cell cultures corresponded in shape, size, and location to electron-dense granular masses and vacuolated viroplasm-like bodies instead of virions. These electron-dense bodies were considered to be viral-associated proteins. In all cultures that had immunofluorescence, PA-BSV particles were found. Endoplasmic reticulum associated bovine syncytial virus particles were not found in all cultures that had PA-BSV immunofluorescence; however, ERA-BSV particles were not found in the absence of PA-BSV immunofluorescence.
Syncytial Formation

Difference between cell membranes

Syncytial formation (polykaryocytosis) is a well-known characteristic of some ether-sensitive herpes- and paramyxoviruses (57, 98, 118). These viruses are known to mature at the host-cell membrane, and this is the part of the cell where changes induced by viruses lead to confluence of cells. Plasmalemma associated bovine syncytial virus matures at the host cell membrane and is ether sensitive\(^1\); thus, having characteristics in common with other known syncytial producing viruses.

Roizman (97) has presented evidence that syncytia are formed by fusion of functioning cells differing in some phenotypic characteristic. Thus, syncytial forming viruses induces alteration of cell membranes. These membranes fuse with membranes of uninfected cells giving rise to a syncytium. It has been reported that a viral infected cell actively "recruits" the uninfected cell and that this recruitment is discriminatory since mitotic cells are never taken into a syncytium (99).

In this study of BSV, not only were large syncytial formations produced, but often up to 80% of the cells in the monolayer were involved. It has been shown that BSV

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is not only ether sensitive but also strongly cell associated and was detectable only in the undiluted cell-free filtrate.\textsuperscript{1} Thus, once a few cells of an inoculated cell culture became infected and since few viruses appeared to be released, this would allow many uninfected cells to remain available for incorporation into and formation of large syncytia.

Varying stages of cell fusion were recognized in light and electron microscopic studies of BEM cells (Figure 31). Cell fusion started with the formation of small cytoplasmic bridges between adjacent cells at points where the plasmalemmas of the 2 cells were in direct contact. The exact nature of the structural changes occurring in the plasmalemma which permitted connecting bridges to be formed between cells could not be established with electron microscopic studies. Although it has been reported that cells with numerous microvilli on their surface fused more readily than cells with fewer microvilli (100, 106), the mechanism by which the virus induces cell fusion remains obscure.

There is a general trend for malignant cells and cells from established cell lines to have a higher fusion capacity than primary and secondary diploid cells (91).

\textsuperscript{1}Malmquist, W. A., National Animal Disease Laboratory, Ames, Iowa. Data on bovine syncytial virus. Personal communication. 1971.
The BEM cells used in this study were nonmalignant and often were only in the 2nd or 3rd passage.

**Effect of virus on syncytial formation**

Under certain conditions, a syncytial producing virus may express itself in 2 different ways; one which can cause syncytial formation and the other some different cytopathic effect (19). The only cytopathic effect of BSV in BEM and BESp cell cultures was syncytial formation. However, since no other cytopathic effect, other than syncytial formation, has been observed with BSV, it does not rule out that BSV may exist in cell cultures without producing any cytopathic effect.

**Effect of environment on syncytial formation**

Syncytia have been reported to be more numerous in viral infected cell cultures maintained in medium with more than 5% serum supplement (104). This may have contributed to the success of syncytial production in BEM cell cultures infected with BSV since 20% fetal calf serum was used in the culture medium.

A specified virus may cause syncytial formation in some types of cell cultures but not in others (97). In addition to BEM cell cultures, BSV caused syncytial formations in a rabbit cornea cell line (64), in BESp cell
cultures, and in BHK-21 cell cultures. However, its replication to equivalent infectivity titers in these cell cultures was not attempted due to its close cell association. The BESp cell cultures were also a good host cell culture in which other viruses could induce syncytial formations. However, BEM and BESp cell cultures were the most optimal host for syncytial production by BSV. Thus, BEM cell cultures infected with BSV would provide a good model system for study of polykaryocyte formation.

Age of cell and time of syncytial formation

Evidence has been presented that young cells are more capable to fuse and form syncytia than aged cells (126). "Age" refers to the time for which cells have been cultured at any given passage level. Often, virus-induced syncytia may be more extensive in cultures composed of cells in the logarithmic growth phase than in stationary growth phase. Also, a subculture of infected "aged" cells with minimal nonprogressive syncytial formations may develop extensive syncytia when subcultured without further introduction of virus (88). Peak syncytial formation was achieved by 24 to

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48 hours in BEM cells infected with BSV and usually corresponded with completion of the monolayer. Thereafter, the syncytia remained fairly static in number and size for 2 to 3 days and then sloughed. If the number of syncytia was low and if the monolayer was divided, syncytial formation was again active and usually would involve more of the monolayer than in the previous passage.

Development of syncytia in young and aged cell cultures probably reflects difference in the cellular metabolism in the 2 "age" conditions. Cellular DNA, RNA, and protein synthesis decreases considerably when cells become confluent and enter the stationary phase (59). Changes may occur in the lipid composition of the plasmalemma during "aging" of the cell, thus reducing the ability of old cells to fuse (47). Also, interferon production has been shown to increase in aged cells of certain cell cultures (8).

"Early" syncytial formation involves rapid fusion of cells, usually within 3 hours of infection, and is considered essentially a laboratory artifact since it is nontransmissible and only occurs following inoculations at extremely high virus multiplicities (91). "Late" syncytial formations occur a number of hours after inoculation with moderate or low virus multiplicities and coincide with the most intensive phase of virus multiplication. The late syncytial formation was the most
characteristic of BSV in BEM cell cultures and the highest number of viral particles was usually observed in cell cultures examined by electron microscopy when they contained many syncytia that had formed within a 48-hour period (Figures 32 and 33). However, at this point, no association between virus production and syncytial formation can be correlated since syncytial production has been shown to occur when cell cultures were inoculated with inactivated virus (122).

Tests for specific proteins in syncytial formation

Mitomycin C, cytosine arabinoside, hydroxyurea, the halogenated pyrimidines (BUDR, IUDR, FUdR), 5-fluorouracil, and aminopterin, which are used to inhibit DNA synthesis and thus DNA virus production, failed to inhibit syncytial formations (2, 80, 126). Actinomycin C, actinomycin S, actinomycin D, and proflavine, which are inhibitors of RNA dependent DNA synthesis, inhibited syncytial formation in DNA viral infected cell cultures, but had no inhibitory effect on syncytial formation by RNA viruses (42, 81). Agents inhibiting protein synthesis (puromycin, cycloheximide, p-fluorophenyl, and chloramphenicol) were variable in their prevention of syncytial formations in virus infected cell cultures (45, 81). Thus, there is yet no proof of specific virus-induced proteins which are capable of causing cell fusion. Also, the ability of
chemical and physical agents to cause syncytial formations makes it difficult to acknowledge the need for synthesis of specific virus-induced proteins for syncytial formations. No study has been made on the effect of chemical inhibitors of the various proteins that may be associated with syncytial formations in BSV infected BEM cells.

Normal Bovine Embryonic Mesothelium

**Nuclear pores**

Nuclear pores were prevalent in the BEM cells used in this study, especially when observed in sections that were tangential to the nuclear membrane (Figures 11 and 12). Considerable speculations as to the function of the nuclear pores have been made but no definite conclusions have been stated. However, the nucleus does have communication with the cytoplasm and the nuclear pore would appear to be the route of "least resistance". Indeed, chromatin was observed to extend into a nuclear pore of the nucleus in one cell in this study (Figure 10).

Since the morphology and cytopathology of many unknown viruses are first studied in cell cultures, the nuclear pores in a tangential section of the nuclear membrane (Figure 11) may be confused with viral particles in an area of viroplasm.
Cytoplasmic fibrils

The cytoplasmic fibrils observed in the paranuclear region of many of the noninoculated BEM cells (Figures 2, 4, 5, 13, and 14) were also present in BSV infected BEM cells (Figure 54). These fibrils were frequently in contact with the outer nuclear membrane. Similar fibrils have been described in several different cell types, both epithelial and mesothelial in origin, including the cells of the reticuloendothelial system (5, 21, 121). They have been reported to be more prevalent in activated cells than in dormant cells (84). It has been suggested that this type of fibril is a normal cellular component serving as part of the cytoskeletal system which is probably exaggerated in both reactive and neoplastic processes (5, 121). The chemical nature of the fibrils and their function in the cells containing them is as yet unknown.

Mitochondria

The nonenergized and energized mitochondria (Figures 16 and 17) were present in both normal and BSV infected BEM cells of cell cultures. Both forms were never found in the same cell. The inner mitochondrial membrane has been described as composed of tripartite repeating units (30) and changes in shape of these repeating units are constant within a given mitochondrion and independent of the arrangement of the cristae (87). The energized state can
be produced by transfer of electrons from cytoplasmic matrix to the basepieces of the repeating units or by hydrolysis of ATP to ADP and the transfer of the released energy to the headpiece-stalk sectors of the repeating units (39). The discharge of the energized state to the nonenergized state can be linked to the mitochondrial work performances or reagents which discharge the appropriate form of the energized state (ADP, Ca\(^{2+}\), K\(^{+}\), or TPN\(^{+}\)).

**Centrioles and cilia**

The centrioles observed in this study were a common finding. However, the various stages of differentiation of the centrioles into basal bodies of cilia were initially thought to be unusual since mesothelial cells were considered to be nonciliated. A stage of ciliary development was an alignment of a centriole perpendicular to the membrane of a vesicle similar to those of the Golgi complex (Figures 21 and 22). Then the centriole expanded into the vesicle and formed a ciliary sheath. The ciliary sheath elongated with growth of the cilium and eventually fused with the plasmalemma, forming a deep cytoplasmic invagination around the shaft of the cilium (Figures 19, 23, and 24).

The mesothelial cell is an undifferentiated cell and mesenchymal in origin. Therefore, it is considered to be a pleuropotential cell. In this study, the mesothelium
was from a developing fetus and thus may have retained much of its embryonic status of nondifferentiation. Therefore, it is conceivable that the BEM cells used in this study could develop into ciliated cells. The factors that initiate the differentiation of the centrioles into basal bodies are unknown. Cilia formations have been reported in fibroblasts and smooth muscle cells of organ cultures of chick and rat embryonic tissues (110) and in hamster fibroblasts where the incidence of cilia could be increased with Colcemid treatment (117). These cilia have no rootlets and it has been reported that they have no apparent motility, but a few have been observed to beat erratically (116). It is not known if BEM cells that form cilia are capable of further division.

Comparison of Bovine Embryonic Mesothelial Cells with Bovine Embryonic Spleen Cells

Malmquist et al. (64) initially grew explants of bovine embryonic spleen as a source of cells for cell culture work with BSV. After letting stock cultures remain stable in medium that had become acid, he noticed foci of polygonal-shaped cells among the fibroblastic elements. If the acid medium was maintained on these cultures, the foci of polygonal-shaped cells became larger and the fibroblastic elements sloughed. Soon a complete monolayer of polygonal-shaped cells was obtained.
Preliminary work in this study was made to determine what cell was the origin of the polygonal-shaped cell of the monolayer. It was initially thought that the reticuloendothelial cells of the sinusoids were the source. Tissue explants of the splenic pulp were made and the splenic capsule and as many as possible of the trabeculae were intentionally excluded to avoid as many fibroblasts as possible. These explants gave rise to only fibroblastic-type cells. Since Malmquist had used whole spleen, the next approach was using explants of the surface of the spleen including the capsule. The monolayer of cells obtained was of mixed cell type including, after permitting the medium to become acid for 3 to 4 days, foci of polygonal-shaped cells.

The conclusion was made that the mesothelium of the spleen was the source of the polygonal-shaped cell type. Further explants made only of the mesothelium that was stripped from the surface of the splenic capsule, resulted in foci of polygonal-shaped cells around each explant with minimal numbers of fibroblasts. The foci merged forming a monolayer of polygonal-shaped cells and could be expanded in a 1:2 ratio by 10 to 14 days.

This research indicated that the polygonal-shaped cells in BESp cell cultures were morphologically indistinguishable
from the polygonal-shaped cells obtained from bovine embryonic mesothelium and called BEM cell cultures in this study.
SUMMARY AND CONCLUSIONS

1. Bovine embryonic mesothelial cell cultures were prepared from the mesothelium of the spleen from 5- to 7-month-old bovine fetuses. These cell cultures could be maintained for 4 to 6 months before cessation of cell growth.

2. Light microscopy of stained monolayers of non-inoculated BEM cell cultures revealed that the cell population was made up of polygonal-shaped cells.

3. Electron microscopic examinations of sections from noninoculated BEM cell cultures revealed the following:

   The typical cell contained a centrally located nucleus with 1 or more prominent nucleoli.

   Small cytoplasmic vacuoles were a prominent feature of all cells.

   Several cells contained an extensive paranuclear fibrillar network. This fibrillar network was also prominent in many of the BSV infected cells.

   Cells from older cultures contained many autophagosomes.

   Nuclear pores were numerous when seen in tangential sections of the nucleus and they could easily be mistaken for nonpreviously described viral particles.

   Nonenergized and energized mitochondria were observed but both were never found in the same cell.
Both forms of the mitochondria were also observed in cell cultures infected with BSV. However, again, both forms were not found in the same cell.

Centrioles were a frequent finding and on several occasions one of them would be observed as a basal body of a cilium. Occasionally, 2 to 5 cilia may be observed in a single cell.

4. Light microscopic examination of monolayers of inoculated BEM cell cultures revealed syncytia that may have 40 or more nuclei per syncytium and over 80% of the nuclei of the monolayer may be included in syncytial formations.

5. Sera that gave a positive immunodiffusion test to an antigen made of cell cultures inoculated with BSV, when conjugated with fluorescein, also intensely stained particulate granules and foci in the cytoplasm of many cells of the cell cultures inoculated with BSV.

6. The particulate, solid, and circular cytoplasmic bodies observed in fluorescent antibody stains of BSV inoculated cell cultures corresponded in shape, size, and location to electron-dense granular masses and vacuolated viroplasm-like bodies instead of virions. These electron-dense bodies were considered to represent viral-associated proteins.

7. Electron microscopic examinations were made of
sections from BEM cell cultures inoculated with a syncytial-forming agent isolated from a 1-year-old bull with lymphosarcoma and of sections from BESP cell cultures inoculated with a syncytial forming agent isolated from normal and lymphosarcomatous cattle.

8. The following were noted in electron microscopic examination of sections from inoculated BEM and BESP cell cultures:

Viral nucleocapsids were present in budding forms contiguous with the membranes of vacuoles and the plasmalemma of syncytia and mononuclear cells. These single membrane enveloped virions were called plasmalemma associated bovine syncytial virus (PA-BSV).

The dimensions of PA-BSV particles were as follows: Diameter of nucleocapsids, 35 to 45 nm; distance from the nucleocapsid through the envelope, 14 to 16 nm; length of projections on the envelope, 14 to 18 nm; total diameter of virions, 90 to 115 nm.

Viral nucleocapsids similar to those of PA-BSV were in various stages of budding across the rough endoplasmic reticulum. These double membrane enveloped virions were called endoplasmic reticulum associated bovine syncytial virus (ERA-BSV).

The dimensions of ERA-BSV particles were as follows: Diameter of nucleocapsids, 35 to 45 nm; distance from
nucleocapsids through inner membranes, 10 to 16 nm; distance from inner membranes through outer membranes, 27 to 37 nm; total diameter of virions, 110 to 150 nm.

9. The PA-BSV and ERA-BSV particles differed only in that the envelope of the ERA-BSV particle had a 2nd membrane with no projections which added 20 to 35 nm to its overall diameter. Thus, ERA-BSV particles were considered to be an aberrant form of PA-BSV.

10. When compared with noninoculated cell cultures, BSV was considered to be the cause of the syncytial formations in BEM and BESp cell cultures.

11. Plasmalemma associated bovine syncytial virus differed from the C-type RNA tumor viruses as follows: The PA-BSV nucleocapsids were smaller; the PA-BSV nucleocapsids appeared in a mature form before becoming contiguous with the plasmalemma; the PA-BSV particle contained no intermediate membrane between the nucleocapsid and the envelope; and the PA-BSV particle possessed prominent projections on its envelope.

12. Plasmalemma associated bovine syncytial virus closely resembled FeSPV, simian foamy viruses, and a virus isolated from human nasopharyngeal carcinoma in the following morphological characteristics: Nucleocapsids were similar in shape and size; nucleocapsids appeared as an already matured form at the plasmalemma; nucleocapsids
were enveloped at the plasmalemma; projections were present on the surface of the envelope; and the total diameter of the virions was in the range of 90 to 115 nm.

13. Bovine embryonic mesothelial cell cultures infected with BSV would provide a good model system for the study of polykaryocyte formation.

14. The polygonal-shaped BEM cell was morphologically indistinguishable from the polygonal-shaped BESp cell.
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