JOURNAL OF VIROLOGY, Jan. 1974, p. 197-204
Vol. 13, No. 1
Printed in U.S.A.
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Ultrastructural Studies of a Visna-Like Syncytia-Producing Virus from Cattle with Lymphocytosis

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Received for publication 17 August 1973

A virus structurally similar to viruses associated with maedi, progressive pneumonia, and visna of sheep has been isolated from buffy coat cells of cattle with chronic lymphocytosis. Electron microscope studies revealed three variants of the virion: (i) an intracytoplasmic form 98 to 116 nm in diameter when occurring in a nonlaminated form, (ii) a budding form 120 to 130 nm in diameter, and (iii) an extracellular form 80 to 130 nm in diameter and containing a 30 to 43 nm eccentrically located electron-dense core.

Previous studies on bovine lymphosarcoma resulted in isolation of two viruses which produced syncytia in cell culture (2, 13, 24). The first of these has been called bovine syncytial virus (BSV). It has a morphological relationship to the foamy virus group and has tentatively been classified with them (6, 15). The second syncytia producing agent isolated from cattle was an uncharacterized herpesvirus that was isolated from histologically confirmed cases of bovine lymphosarcoma (24). Any association of these viruses with the etiology of bovine lymphosarcoma remains unknown.

Recently, a third syncytia-producing agent was isolated from three cases of bovine lymphocytosis (25). No antibody has been found to this agent and syncytial formation is the only identifying characteristic in cell culture. Since no serologic or immunologic approach to the study of the agent appeared feasible, an electron microscope examination was initiated. The purpose of this paper is to report the results of the investigation on the morphological features of this third syncytia-producing agent of bovine origin and compare its similarities with other structurally similar viruses.

MATERIALS AND METHODS

Buffy coat cells were inoculated on incomplete monolayers of bovine embryonic spleen (BESp) cells that were prepared as described by Malmquist et al. (13). The inoculated BESp cells were serially propagated in plastic tissue culture flasks until maximum syncytia formation was obtained, usually 20 to 60% of the cells of the monolayer being involved. For light microscopy, inoculated BESp cells were grown on cover slips in Leighton tubes and stained by May-Grünwald-Giemsa technique.

Normal and infected BESp cells used for electron microscope studies were dispersed with 0.02% EDTA in phosphate-buffered saline and sedimented by centrifugation at $120 \times g$ for 5 min. The cells were then resuspended in 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.4) for 30 min, sedimented, double rinsed by resuspending and sedimenting in 0.2 M sodium cacodylate buffer (30 min each), and left overnight at 4 C suspended in 0.2 M sodium cacodylate buffer. They were postfixed by suspending in 1% osmium tetroxide (pH 7.4) in 0.2 M sodium cacodylate buffer for 30 min, and then double rinsed by resuspending and sedimentating in 0.2 M sodium cacodylate buffer (10 to 30 min each). Cells were then pelleted in warm agar (60 to 65 C) by centrifugation at $1,300 \times g$ for 5 to 10 min. The pellet was cooled to 4 C, cut into 1- to 2-mm cubes, and left in 0.2 M sodium cacodylate buffer overnight. The cubes were then dehydrated through a graded series of alcohols (30, 50, 70, and 100%), and embedded in Epon 812 according to the method of Luft (12). Thin sections were cut with an Ultrotome equipped with a diamond knife and picked up on 200-mesh uncoated copper grids. The sections were double stained with lead citrate and uranyl acetate according to the method of Venable and Coggeshall (26). Sections were examined with a Philips EM-200 electron microscope at 60 kV.

RESULTS

Uninfected cell cultures. Examination by light microscopy of the cell cultures revealed a uniform monolayer of polygonal mononuclear cells. The nucleus was centrally located and contained prominent nucleoli.

Electron microscope observations showed polygonal cells with large, prominent nuclei and well-defined chromatin. Cytoplasmic elements consisted of normal mitochondria with intact cristae, prominent Golgi complex, sparse rough

endoplasmic reticulum, many free ribosomes, and polyribosomes often oriented in a helical arrangement. No virus-like particles were encountered.

Virus-infected cell cultures. Light microscope examination demonstrated syncytia involving a few cells to approximately 60% of the cells of the monolayer (Fig. 1).

Electron microscope observations of the syncytia revealed crescent-shaped, budding virions 120 to 130 nm in diameter arising from the plasma membrane (Fig. 2). These virions consisted of a single thin outer electron-dense unit membrane with a wider electron-dense crescent underlying it (Fig. 3). The dense inner membrane was approximately 20 to 25 nm from the outer membrane. Although the budding particles were prominent, some were detached and had an electron-lucent center (Fig. 4). These detached virions measured 110 to 120 nm in diameter and the inner membrane was 20 to 25 nm from the outer membrane, thus similar in shape and size to the budding form.

A second type of viral particle was observed extracellularly and in large membrane-bound vacuoles of cells (Fig. 5). These particles were closely associated with cells that contained the budding form of the virus and were often located between the long finger-like projections containing the budding virions. These viral particles were variable in size with diameters of 80 to 130 nm. They usually contained a single eccentrically located electron-dense core, 30 to 43 nm in diameter, separated from the external limiting membrane by a less electron-dense zone. Occasionally, bar-shaped structures were present in place of the electron dense cores (Fig. 5). These extracellular particles with electron-dense cores were by far the most frequently observed virions and often they were the only form of viral particle that could be found in some preparations of infected cell cultures.

A third form of the viral particle was occasionally observed in the cytoplasm of syncytia that contained the budding forms. It appeared as a ring-like structure with parallel membranes and had a 98 to 116 nm outer diameter with the inner membrane regularly spaced 17 to 22 nm from the outer membrane (Fig. 6). Infrequently, if the inner ring occurred alone in the cytoplasm, an array of ribosome-like particles was attached to it (Fig. 7).

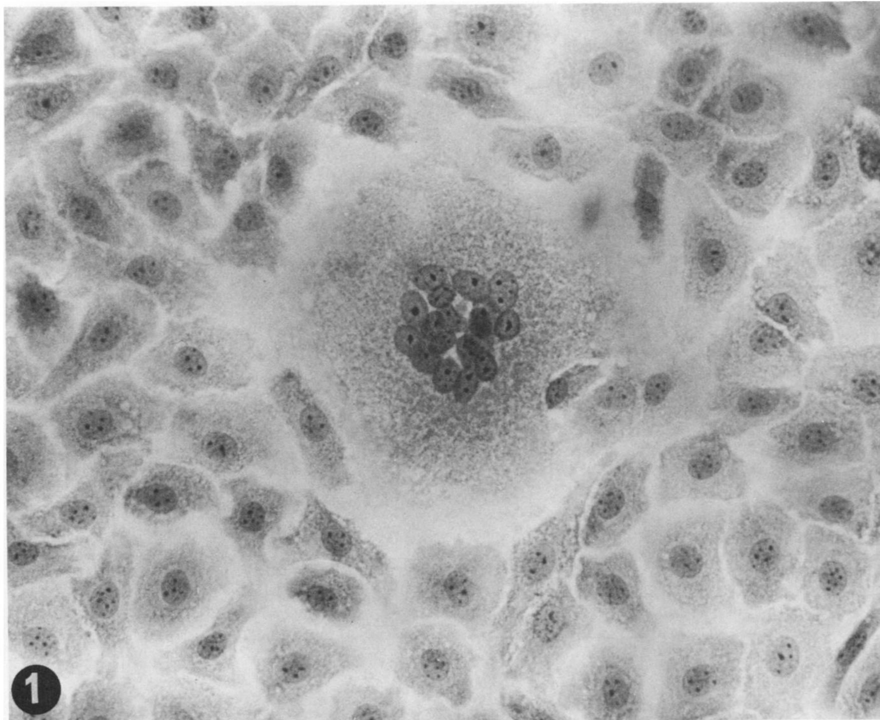
Laminated structures consisting of 1 to 5 partial or complete concentric electron-dense rings spaced at regular intervals of 17 to 22 nm were occasionally observed clustered in the cell cytoplasm, often in the perinuclear region (Fig. 8). The innermost ring formed an incomplete circle 60 to 70 nm in diameter and resembled the innermost shell of the intracytoplasmic and budding forms. It was not determined if the laminated structure consisted of concentric rings or whether it was actually a continuous coil of an electron-dense membrane.

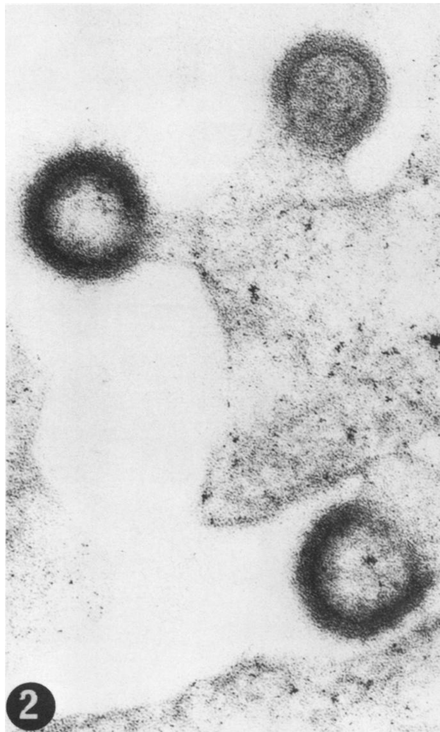
Thin sections prepared from pellets of infected BESp cell culture supernatant contained a large number of enveloped particles containing single eccentric electron-dense cores structurally identical to the eccentric dense core particles observed in infected cell cultures as described above. Several double-walled particles with electron-lucent centers could be demonstrated among these eccentric dense core particles and were structurally similar to the detached budding particles also described above.

DISCUSSION

The structure of this syncytia-producing virus from cattle was like that of the viruses associated with visna, maedi, and progressive pneumonia of sheep (4, 21). These viruses from sheep are antigenically and structurally similar (Table 1). The intracytoplasmic laminated forms were similar although they were never associated with budding forms as has been described for visna virus (3). The antigenic relationship of this virus to the viruses associated with visna, maedi, and progressive pneumonia remains to be determined.

The presence of three distinctly different morphological identities of the virion raises the question as to the role that each plays in the infected cell culture. The intracytoplasmic particles were never observed close to the plasmalemma or budding through any of the cell membranes, indicating that escape or infectivity, or both would rely on the destruction of the host cell. The inner membrane of the intracytoplasmic particles corresponds with the inner membrane of the budding forms. The intracytoplasmic particles were also found in the same cells as the budding forms. Therefore, the intracytoplasmic particles may be considered an aberrant form of the virus. Since the extracellular virions with the eccentric electron-dense core were the most prominent, they may represent budding forms that have become detached with the inner electron-dense membrane collapsed to produce the eccentric electron-dense core in an otherwise electron-lucent budding form. The bar-shaped structure in several virions is probably an intermediate stage of the collapsed electron-dense membrane. It has been proposed that in some of the oncogenic RNA viruses the nucleic acid, in





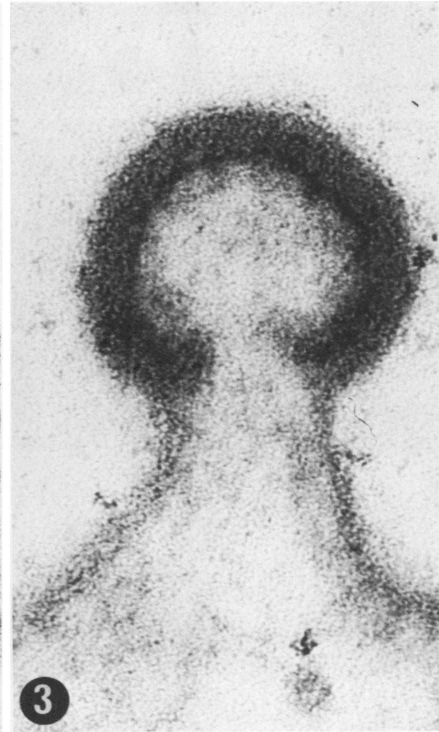
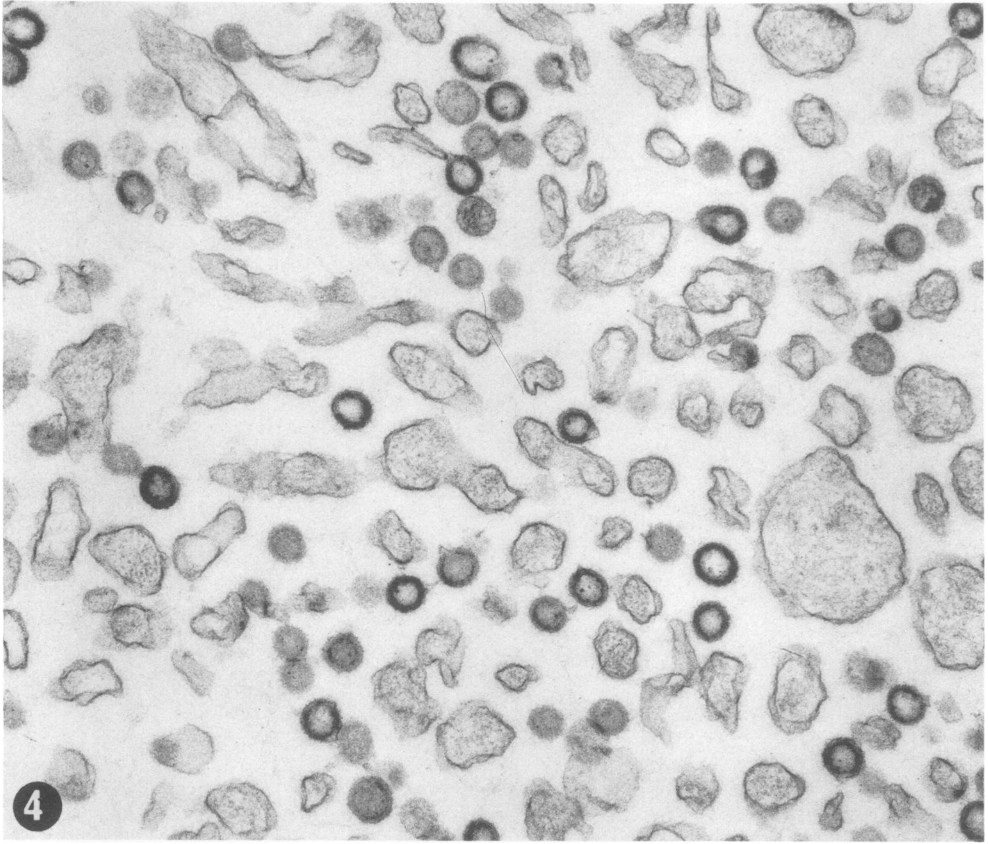


FIG. 1. *Cell culture monolayer of BESp cells showing a syncytial formation associated with the virus in this study.* ×*560.*

FIG. 2. *Electron micrograph of budding viral particles at the plasmalemma illustrating translucent centers of the nucleocapsids.* ×*146,000.*

FIG. 3. *Electron micrograph of budding virion illustrating a thick electron-dense inner membrane beneath the viral envelope.* ×*356,000.*



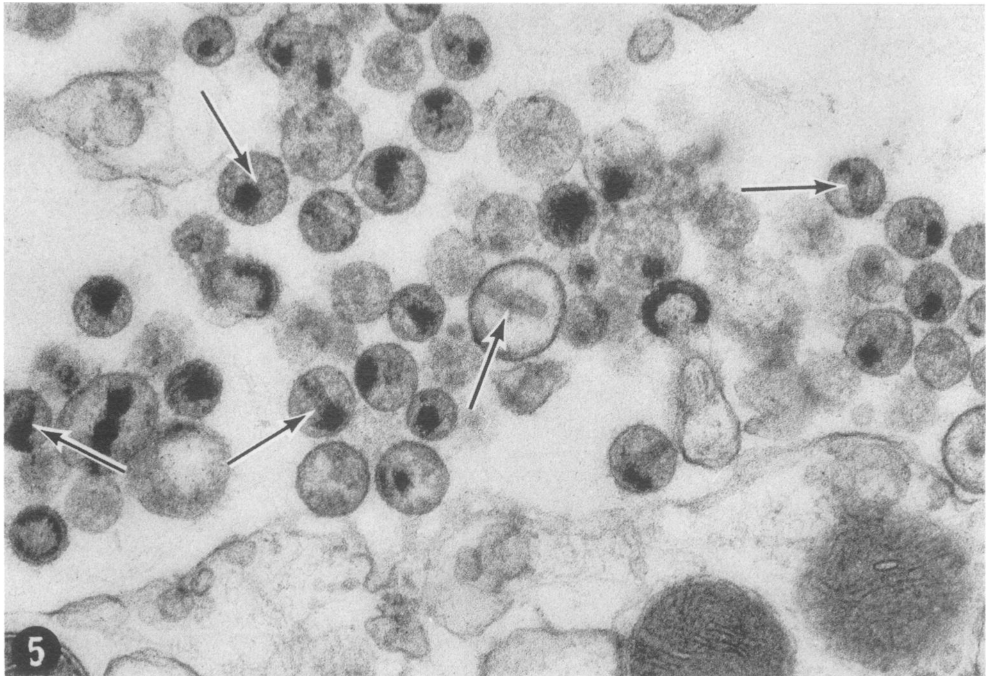
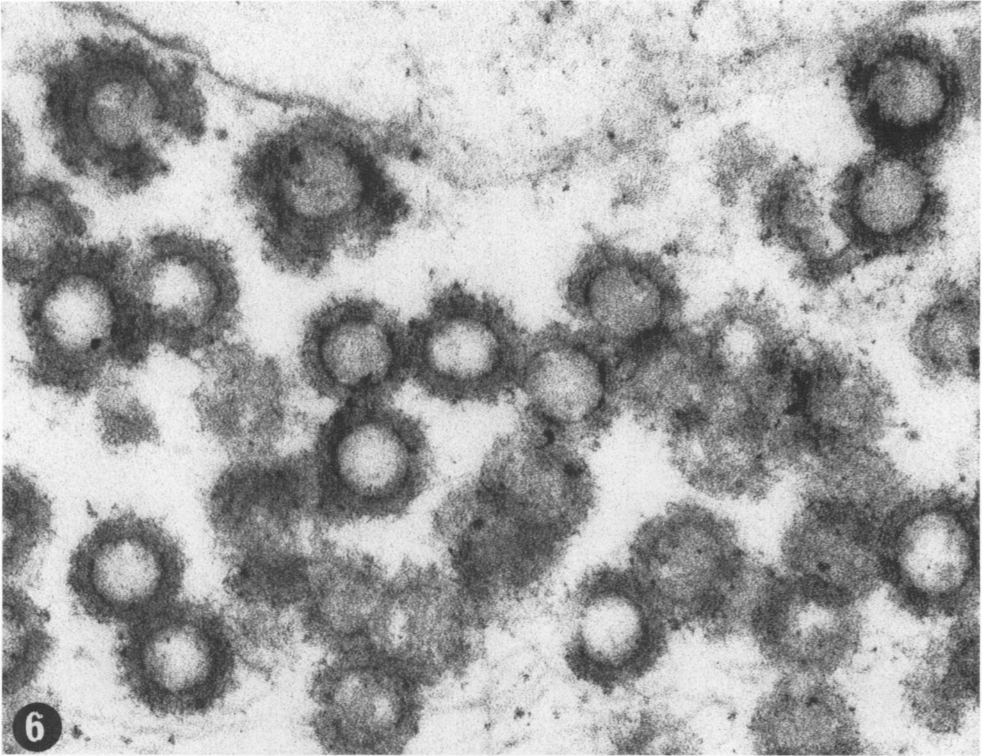


FIG. 4. *Electron micrograph of extracellular virions. Note the translucent centers characteristic of the budding virions.* ×*42,900.*

FIG. 5. *Electron micrograph of extracellular virions characterized by eccentric electron-dense core and bar-shaped structure extending across the virions (arrows).* ×*65,200.*



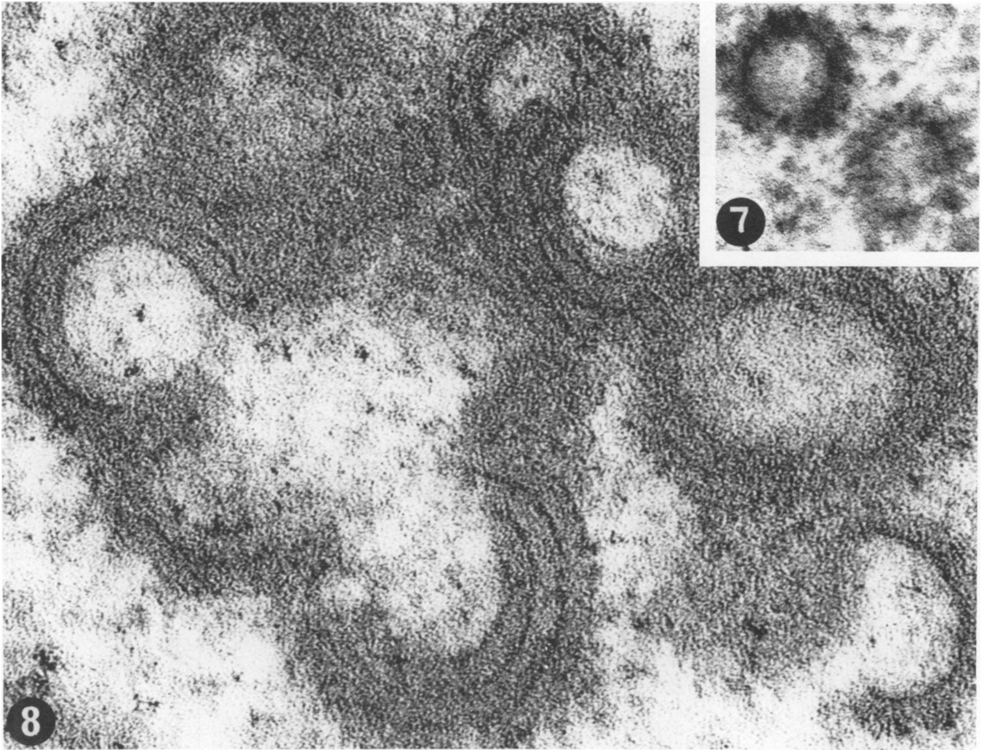


FIG. 6. *Electron micrograph of cluster of intracytoplasmic virions characterized by translucent centers. Note the dense inner membrane.* ×*150,500.*

FIG. 7. *Electron micrograph of intracytoplasmic structures resembling viral particles and consisting of a single dense membrane with ribosome-like particles attached to the periphery. Compare structures with the virions in Fig. 6* ×*182,000.*

FIG. 8. *Electron micrograph of cluster of intracytoplasmic laminated structures illustrating concentric electron-dense rings 17 to 22 nm apart.* ×*290,000.*

TABLE 1. *Comparison of bovine visna-like virus with other structurally similar viruses*

Virus	Size in thin sections (nm)			Comments
	Intracytoplasmic	Budding	Extracellular (nucleocapsid)	
Bovine visna-like virus	98–116 Laminated forms	120–130	80–130 (30–43)	Nucleocapsid formed during budding; nucleocapsid eccentric in extracellular virion
Progressive pneumonia virus	95–105 (21) Laminated forms	140–150 (21)	90–110 (21) (30–40)	Nucleocapsid formed during budding (21); nucleocapsid eccentric in extracellular virion (21); 5- to 10-nm spikes on surface of negative stained virion (20)
Visna virus	Present, size not reported (3, 21) Laminated forms	100–140 (4)	110–120 (20) 70–100 (23) (30–40)	Nucleocapsid formed during budding (23); nucleocapsid centrally located in extracellular virion (20, 23); 5- to 10-nm spikes on surface of budding and negative stained virions (20)
Maedi	Not reported	Present, size not reported (22)	60–90 (22) (30–40)	Nucleocapsid formed during budding (22); nucleocapsid centrally located in extracellular virion (22)
BSV	110–150 (2) Rough endoplasmic reticulum associated (ERA) particles	95–115 (2)	63–77[a] (2) (35–45)	Nucleocapsid preformed in cytoplasm (2); nucleocapsid centrally located in extracellular virion (2); 14- to 18-nm spikes on surface of budding virions (2); spikes not always distinct on extracellular virion (2)
Oncogenic RNA associated viruses	Intracytoplasmic A-particle 75–85 (1, 9, 18, 27)	85–110 (7)	90–120 (14) (60–90)	Nucleocapsid formed during budding (20); nucleocapsid centrally located in extracellular C-type virion (20); nucleocapsid eccentric in extracellular B-type virion (5); 5- to 10-nm spikes on surface of negative stained virion (Rous sarcoma) (20)

[a] Spikes not included.

association with other substances attached to an appropriate site on the developing intermediate membrane of the budding particle at the cellular periphery (11). At this site the nucleic acid is twisted into a coil and forms a larger rope-like super coil within the budding particle resulting in a hollow sphere, the electron-dense shell of the nucleocapsid with its electron-lucent center. Such an arrangement of nucleic acid could explain the structure of the budding particles with the electron-lucent centers found in this study. The collapse of spherical coil of nucleic acid would probably occur rapidly and thus intermediate stages would rarely be observed in electron microscope preparations. A structure produced in this manner may not have the structural regularity of an organized unit, such as a nucleocapsid, thus supporting an explanation as to why the electron-dense centers of virions in this study have an eccentric position and a wide variation in shape and size.

A projected sequence of events in the maturation of the virions may be as illustrated in Fig. 9. The viral nucleocapsid is assembled beneath the plasmalemma concurrent with the budding process. The budded virion is released forming the extracellular virion having an electron-lucent center. The nucleocapsid of the newly formed extracellular virion collapses to form the intermediate bar shape structure and ultimately the round eccentric electron-dense core. Alternate possibilities could be formation of the virion with the bar-shaped structure as the end result, or the round eccentric electron-dense core may be formed without first passing through an intermediate bar-shaped form.

The relationship of the viral particles in this study to RNA oncogenic viruses should be considered since these isolates came from cattle with marked long-term lymphocytosis (Table 1). The virus in this study differed from C-type virus particles as follows: It was slightly larger; there were intracytoplasmic laminated forms compared to no preformed intracytoplasmic forms for C-type virus; and there were various forms of the virions compared with only budding and extracellular forms for C-type virus. The agent in this study was similar to the C-type virus in that its nucleocapsid does mature at the plasmalemma during the process of budding.

The intracytoplasmic forms of the virus in this study were similar in shape but larger than the type A particle of Bittner's agent. This virus, as well as Bittner's immature B particles, became enveloped at the plasmalemma and

FIG. 9. *An illustration of proposed sequence of events in development of bovine visna-like virus in cell culture (see text for discussion).*

proceeded to maturity. In both viruses, maturation involves a change in the internal components which result in the formation of an electron-dense center and dilation of the envelope. The nucleocapsid is surrounded by a dense line with an intervening electron-lucent space in Bittner's agent. This line was absent in the virions of this study.

In addition to BSV (13), a herpesvirus (24) and other known viruses of cattle that may produce syncytia in cell cultures (8, 10, 16), another structurally distinct syncytia producing virus has been found associated with lymphocytosis of cattle. Due to its structural similarities with visna virus, which has been found to have an RNA-dependent DNA polymerase similar to that present in RNA oncogenic viruses, further enzymatic and antigenic investigations of the bovine isolate are indicated (17, 19).

ACKNOWLEDGMENTS

Acknowledgment is given to Ralph M. Glazier and Thomas L. Glasson for illustrations and photographic processing, and Doris I. Buck and Merlin K. Lyon for technical assistance.

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