CHAPTER 1

Arenavirus Ultrastructure and Morphogenesis

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I. INTRODUCTION

Although lymphocytic choriomeningitis (LCM) virus was identified as an infectious agent during the early decades of virology research (Armstrong and Lillie, 1934; Traub, 1935), no information on its structure or classification was obtained until many years had elapsed. The development of cell culture systems for virus growth and improved preparative procedures for electron microscopy enabled the initial identification of characteristic LCMV particles in thin sections of virus-infected cells (Dalton *et al.*, 1968). Soon thereafter, other viruses belonging to the Tacaribe complex were found to have a similar morphology (Murphy *et al.*, 1970), leading to the classification of these agents in a new virus family (Rowe *et al.*, 1970). Subsequently, some additional information has been obtained on the structure of the internal components of arenaviruses. This chapter reviews our current knowledge of the virion structure and the process of viral morphogenesis for this family of viruses.

II. VIRION MORPHOLOGY

The virions of the arenavirus family are lipid-enveloped, pleomorphic particles. When examined in thin sections, LCMV particles were

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found to range from 50 to over 300 nm in size; the small to medium-sized particles were generally spherical, whereas larger particles were pleomorphic (Dalton *et al.*, 1968). A similar morphology was subsequently described for other members of the family (Murphy *et al.*, 1970). Virions possessed an electron-dense unit membrane with external surface projections. In the interior, one or more electron-dense granules, 20–25 nm in diameter, were observed in most particles, although occasional particles appeared to lack such structures. Incubation of sections prepared in a water-miscible embedding medium with ribonuclease resulted in the disappearance of the granules, indicating their content of RNA (Dalton *et al.*, 1968). The granules have subsequently been identified as host-cell-derived ribosomes (see below), and the characteristic granular internal structure was the basis for the name given to the virus family (arena=sandy, Rowe *et al.*, 1970). Some sectioned virions exhibit an apparently ordered, circular arrangement of ribosomes, which sometimes appeared to be connected by linear electron-dense structures (Murphy and Whitfield, 1975). It has been reported that under certain preparative conditions, LCM virus particles exhibit a homogeneous electron-dense core (Muller *et al.*, 1983), but the basis of these apparently different morphological features remains to be determined.

In negatively stained preparations, the surfaces of arenavirus particles were found to be covered with distinct spikes, which appeared to vary in spacing on the envelope (Murphy *et al.*, 1970). Examples of virus particles visualized by negative staining or thin-section electron microscopy are shown in Fig. 1. Some of the surface projections appear triangular when observed end-on (Fig. 1D); however, this is not uniformly observed. At high magnification, the surface spikes on Pichinde virions appear club-shaped (Murphy and Whitfield, 1975; Vezza *et al.*, 1977; Fig. 1B). The negatively stained virus particles are also generally spherical (Fig. 1C), although some pleomorphism is evident, particularly in the larger particles in virus preparations (Fig. 1D). No long filamentous virions of the type often seen with orthomyxoviruses and paramyxoviruses have been observed. Internal structural details have been difficult to resolve in intact virions; however, after disruption of the viral envelope, strand-like nucleoprotein complexes are observed (see below).

III. NUCLEOCAPSID STRUCTURE

Although the arrangement of viral internal components within intact arenavirus particles has not been determined, several studies have been carried out on the structure of nucleoprotein components isolated from disrupted virions. Elongated, strand-like structures, 9–15 nm in diameter were observed to be released from spontaneously disrupted Pichinde virions (Vezza *et al.*, 1977). The nucleocapsids of Tacaribe and Tamiami virions have been isolated by detergent disruption of virions

and isopycnic centrifugation in CsCl, in which they band at a buoyant density of 1.31–1.36 g/cc (Palmer *et al.*, 1977; Gard *et al.*, 1977). When examined by staining with uranyl acetate (Palmer *et al.*, 1977), the nucleocapsids were found to be filamentous structures 5–10 nm in diameter. Most of the nucleocapsids appeared to be closed, circular structures (Fig. 2). Length distribution measurements revealed two predominant size classes of about 640 nm and 1300 nm length, presumably corresponding to the two size classes of viral genome RNAS. Two viral ribonucleoprotein size classes were also reported when LCM virus was disrupted with a detergent and analyzed in sucrose density gradients (Pedersen and Konigshofer, 1976). Host-cell-derived ribosomes were not found to be associated with these nucleocapsid structures, although they are present in virions (see below). By negative staining with sodium phosphotungstate (Gard *et al.*, 1977) the nucleocapsids were found to be 3–4 nm in diameter and had a beaded appearance indicating a series of globular subunits spaced at a periodicity of about 5 nm. The viral RNA in the nucleoprotein complexes is single-stranded and sensitive to digestion with ribonuclease (Gard *et al.*, 1977). The major polypeptide in the nucleoprotein is the 63-kDa polypeptide designated NP, which may represent the individual globular units; in addition, the L polypeptide (250 kDa), which is believed to possess transcriptase activity, has also been associated with nucleoprotein complexes (Buchmeier and Parekh, 1987). Evidence has been obtained as well for association of RNA polymerase enzyme activity with the nucleoprotein complexes (Leung *et al.*, 1977).

In preparations of Pichinde virus disrupted by osmotic shock and negatively stained with phosphotungstic acid, released nucleocapsids appeared as convoluted strands with a diameter of 12 nm (Young and Howard, 1983). Some examples of apparently helical structures were identified; these were 12–15 nm in diameter and were composed of 4–5 nm globular subunits. Uncoiling of these structures occurred after purification in a urograffin gradient revealing linear strands consisting of globular nucleosome-like structures, similar in appearance to the CsCl-purified nucleocapsids described above. Under conditions of high ionic strength, the nucleocapsids were found as more condensed, globular structures about 15 nm in diameter. Frequent appearance of circular forms of nucleocapsids was attributed to putative complementary sequences at the 5′- and 3′-ends of genomic RNA species (Young and Howard, 1983), which have subsequently been demonstrated by sequence analyses (Bishop and Auperin, 1987).

IV. INCORPORATION OF RIBOSOMES INTO VIRIONS

The characteristic electron-dense granules found in thin sections of arenavirus particles were subsequently shown to correspond to ribo-

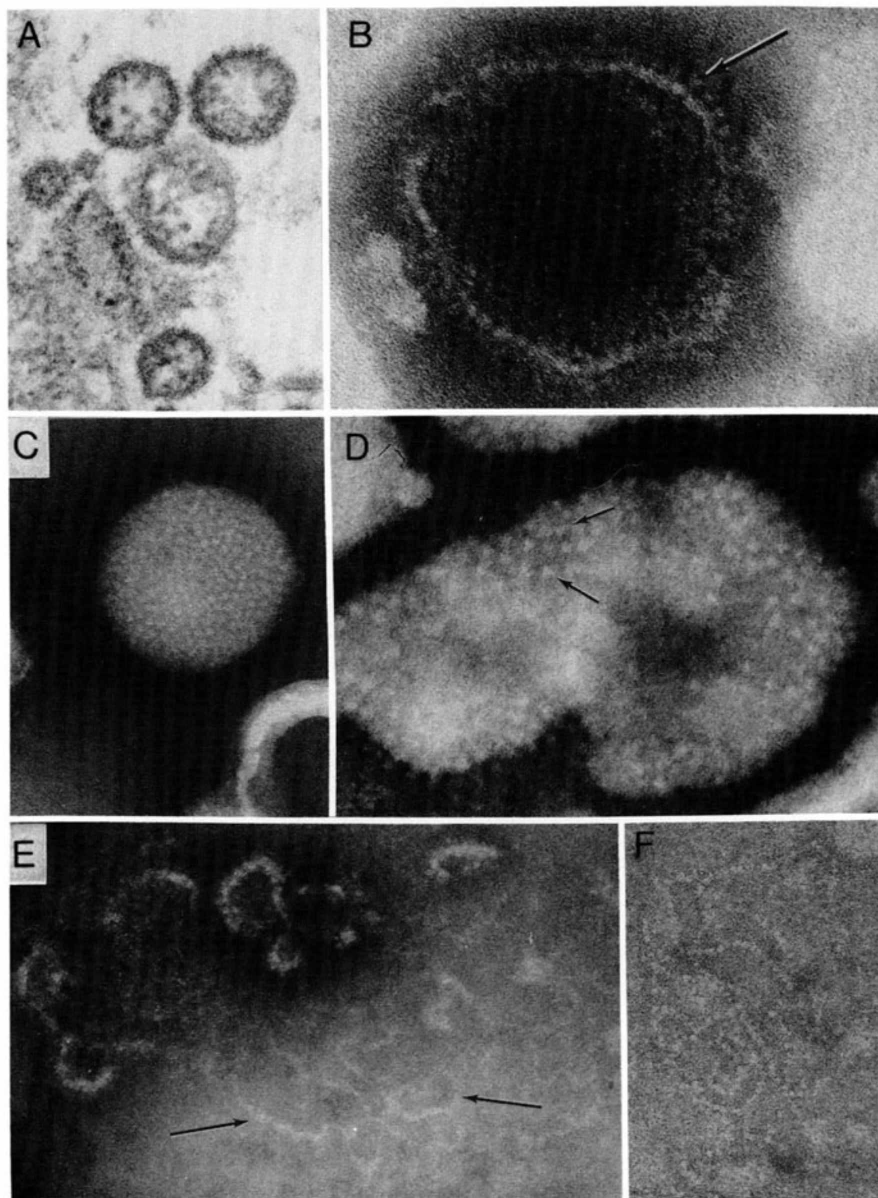

FIGURE 1 (A–F). Electron micrographs of arenavirus particles and subviral components. (A) Thin section of Pichinde virus particles released from infected BHK-21 cells. Many ribosomes are evident in the interior. (B) Single Pichinde virus particle penetrated by stain, revealing a distinct electron-lucent membrane. Individual surface spikes, 5 nm in length, can be resolved (arrow). (From Vezza *et al.*, 1977.) (C) Spherical Tacaribe virion covered with closely spaced surface spikes. (D) Higher magnification of a pleomorphic Tacaribe virion; some of the surface spikes (arrows) appear triangular. (E) Spontaneously disrupted Tacaribe virions, revealing fragments of the viral envelope and strand-like components believed to be the internal ribonucleoproteins (arrows). (F) Preparation of Tamiami ribonu-

somes, which are apparently indistinguishable from those found in normal uninfected cells. Ribosomal 60S and 40S subunits have been isolated from disrupted virions and were shown to have properties similar to those of cell ribosomes, including the presence of 28S and 18S ribosomal RNA species as demonstrated by sedimentation analyses, oligonucleotide fingerprinting, and the presence of methylated bases (Farber and Rawls, 1975; Pedersen and Konigshoffer, 1976; Vezza *et al.*, 1978). The ribosomes are capable of directing protein synthesis in vitro, when supplied with mRNA and the appropriate factors to promote translation (Chinault *et al.*, 1981).

The significance of ribosomes in arenavirus particles is not known. However, infectious virus particles have been prepared lacking detectable ribosomes (Vezza *et al.*, 1978), and studies employing cells with ts ribosomes (Leung and Rawls, 1977) showed that virus propagated in these cells grew essentially equally well at either high or low temperatures. It is therefore likely that ribosomes are not essential components of arenaviruses, and that they may be incorporated into virions during budding because of the lack of specificity in the assembly process.

V. FINE STRUCTURE AND TRANSMEMBRANE TOPOLOGY OF THE ENVELOPE GLYCOPROTEINS

In most arenaviruses, two surface glycoproteins designated GPl and GP2 have been identified, and are synthesized from a common precursor, whereas in Tacaribe, Tamiami, and Junin viruses, only a single glycoprotein similar in size to GPl has been resolved. Several additional glycoproteins have been reported in LCM virus by one group (Bruns *et al.*, 1983), but their identity remains uncertain. The amino acid sequences of the envelope glycoproteins of several arenaviruses have been deduced from the nucleotide sequences of cloned cDNA copies of viral RNAs (see Chapter 10). The sequence analyses reveal two long hydrophobic domains, which may be involved in anchorage of the glycoproteins to the viral membrane. A hydrophobic stretch of 36 amino acids is apparent near the N-terminus of the glycoprotein precursors, which is slightly longer than the signal-anchor domains involved in N-terminal anchorage of glycoproteins such as the influenza neuraminidase, and paramyxovirus hemagglutinin-neuraminidase glycoproteins (Nayak and Jabbar, 1989). A single arginine residue characteristically interrupts this hydrophobic domain in arenavirus glycoproteins, but it has been demonstrated that introduction of such a residue in the influenza NA

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cleoproteins purified in a CsCl equilibrium gradient, showing beaded strand-like components. (C, D) Negative staining with potassium phosphotungstate. (C, E, and F are from Gard *et al.*, 1977.) (A) ×100,000; (B) ×400,000; (C) ×180,000; (D) ×340,000; (E) ×180,000; (F) ×220,000.

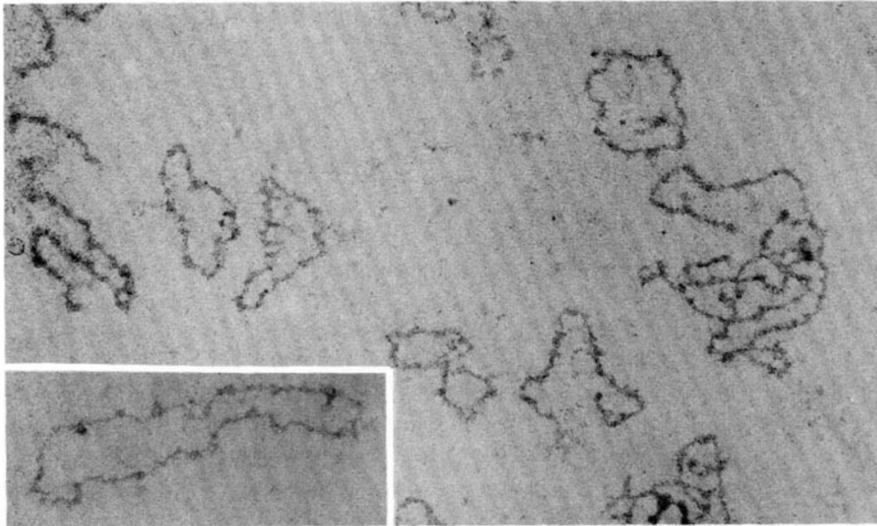

FIGURE 2. Tacaribe nucleocapsid structures from a CsCl gradient, stained with uranyl acetate, indicating a closed, circular structure. (Courtesy of Dr. Erskine Palmer; see Palmer et al., 1977.) Magnification: ×110,000.

signal-anchor domain had no effect on its function (Sivasubramanian and Nayak, 1987). Recent work by Burns *et al.* (1990) indicates that this N-terminal hydrophobic domain is absent in the mature virion form of the LCMV glycoprotein.

A second hydrophobic domain of 23 residues is present near the C-terminus of the glycoprotein precursor. Recent evidence from Burns and Buchmeier (1991) indicates that LCMV GP2 but not GP1 is an integral membrane protein, thereby implicating only the C-terminal hydrophobic domain in membrane anchorage. Thus only one of the two final glycoprotein cleavage products (GP2) is anchored to the membrane, whereas GP1 is associated to the virion by its noncovalent interactions with GP2. It has not been clearly established whether the two cleavage products remain associated as a single complex glycoprotein molecule, nor is it known whether the individual glycoprotein molecules are present as oligomeric structures in the virion. Some evidence suggesting the presence of glycoprotein oligomers has been obtained by analysis of LCMV glycoproteins using gel electrophoresis under nonreducing conditions (Wright *et al.,* 1989), in that monoclonal antibodies against the GPl (44 kDa) glycoprotein were found to react in Western blots not only with GPl monomers, but also with glycoprotein species of higher molecular weight found under these conditions. Evidence for oligomeric glycoprotein complexes has also been reported using a cross-linking procedure (Bruns and Lehmann-Grube, 1983) although these investigators observed several additional higher-molecular-weight glycoprotein species that have not been reported in studies from other laboratories.

Cross-linking studies by Burns and Buchmeier (1991) reveal homopolymeric forms of LCMV GP1 and GP2, but no mixed oligomers containing both GP1 and GP2.

The glycoproteins of arenaviruses were shown to be spike-like projections exposed on the external surface of the viral envelope by proteolytic digestion with pronase, bromelain, or chymotrypsin (Vezza *et al.*, 1977; Gard *et al.*, 1977; Buchmeier *et al.*, 1978). Such treatment yielded smooth-surfaced, spikeless particles, which were noninfectious and lacked the viral glycoproteins, whereas the NP protein was protected from digestion indicating its internal location.

Evidence has been obtained that the GP1 glycoprotein of LCMV is present in a more peripheral location in the viral envelope than is GP2. Only GP1 was found to be labeled by surface iodination procedures (Buchmeier *et al.*, 1981; Bruns *et al.*, 1983), and the GPl molecule also contains more highly processed oligosaccharides than GP2 (Buchmeier and Oldstone, 1979). Studies with monoclonal antibodies also have demonstrated that GP1 contains the major epitopes that interact with neutralizing antibodies (Buchmeier *et al.*, 1981).

The morphology of the glycoprotein appears to be club-shaped in some negatively stained images (see Fig. 1B), and in end-on views, a hollow structure has been reported (Murphy and Whitfield, 1975; Vezza *et al.*, 1977). In partially disrupted virions penetrated by negative stain, differences in electron density are sometimes apparent at positions in the envelope where surface spikes are localized, suggesting that the spike structure is embedded in, and traverses through the lipid bilayer (Fig. 1B).

VI. ASSEMBLY AND RELEASE

A. Maturation at the Cell Surface

The only stage in virus assembly that has been visualized using electron microscopy is the final process of budding at the plasma membrane (Dalton *et al.*, 1968; Murphy *et al.*, 1970). Presumably, viral nucleocapsids are assembled in the cytoplasm, since the nucleoprotein antigen can be identified throughout the cytoplasm using immunofluorescence (Zeller *et al.*, 1988). By analogy with other enveloped viruses (Stephens and Compans, 1988), it is likely that the viral glycoproteins are synthesized on membrane-bound polyribosomes in the rough endoplasmic reticulum and are transported through the Golgi complex, where simple, high-mannose oligosaccharides are processed into complex carbohydrate chains. The final movement of glycoproteins to the plasma membrane is presumably mediated by a vesicular transport process, as has been observed with other enveloped viruses.

Membrane changes seen at the sites of virus budding include an

increase in density of both membrane lamellae in discrete areas large enough to form a viral envelope (Murphy *et al.*, 1970). Surface projections were observed on the exterior of emerging virus particles. In some cells observed at late stages of infection, extensive regions of the plasma membrane were involved in virus assembly. Virions are released after completion of budding by pinching off at the plasma membrane (Fig. 3).

In addition to demonstrating the assembly of virions, electron microscopic studies of arenavirus-infected Vero cells have revealed distinctive intracytoplasmic inclusions consisting of aggregations of electron-dense granules with the appearance of ribosomes (Murphy *et al.*, 1970; Murphy and Whitfield, 1975). The inclusions were variable in size and shape and appeared to become progressively more dense during the course of infection. Immunolabeling of infected cells indicated that these inclusions contain virus-specific antigens (Abelson *et al.*, 1969). The functional significance of such inclusions has not been determined.

No information has been obtained, as yet, on the molecular interactions between viral components that lead to the budding process at the plasma membrane. In contrast to most other enveloped viruses, arenaviruses do not appear to possess an internal protein that corresponds to a matrix (M) protein, which for other viruses is likely to play an important role in the organization of viral components during assembly. Thus, it is likely that the molecular interactions involved in virus assembly may be unusual. The following are some possible mechanisms that could be involved in the assembly process.

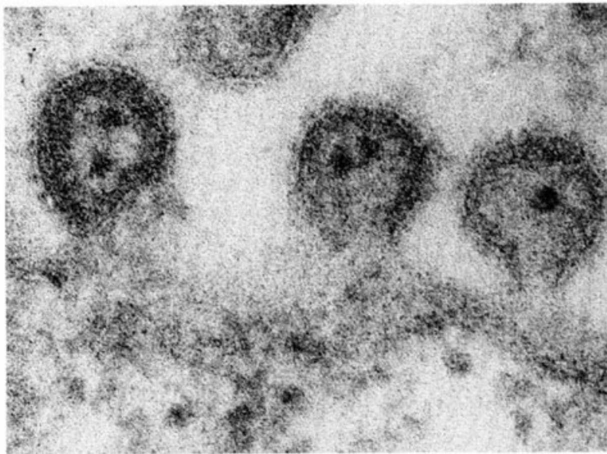

FIGURE 3. Tacaribe virions, containing one or two ribosomes, in the process of budding from an infected Vero cell. A diffuse layer of electron-dense material, which may represent the viral nucleoprotein, is localized beneath the envelope. (From Compans and Bishop, 1985.) Magnification: ×180,000.

B. Interaction between Nucleocapsids and Envelope Proteins

Since the only major virus-specified structural polypeptides that have been identified as components common to all the arenaviruses studied to date are the glycoproteins and the nucleoproteins, it seems most likely that assembly of virions may occur by direct interaction between the nucleoprotein subunits in the nucleocapsids and a cytoplasmic tail on the glycoproteins, which would be exposed on the internal surface of the plasma membrane. As shown in Fig. 3, thin sections of budding virions show a layer of electron-dense material that underlies the viral membranes and could correspond to an extended form of the nucleocapsid. Such a model for virus assembly would predict that the nucleocapsid is organized beneath the lipid bilayer in a regular arrangement, which has not, as yet, been demonstrated.

The NP proteins of LCM and Tacaribe virions have been found to a be heterogeneous with respect to their isoelectric points, which appears to be due to differences in extent of phosphorylation (Bruns *et al.*, 1986; Boersma and Compans, 1985). In the case of LCMV, evidence has been obtained that a subpopulation of NP molecules is associated with the envelope fraction of virions (Bruns *et al.*, 1986) and is exposed on surfaces of infected cells (Zeller *et al.*, 1988). Certain monoclonal antibodies directed against the NP protein were found to react with antigens expressed on the surfaces of infected cells as shown by immunofluorescence, and it was suggested that a relatively hydrophobic stretch of amino acids from residues 441–459 in the sequence of NP might serve as a membrane-spanning region (Zeller *et al.*, 1988). The precise transmembrane topology, as well as the possible functional significance of such membrane-associated NP molecules, remains to be determined. The surface expression of NP was reported to occur somewhat later in the viral replication cycle than the onset of virus release (Zeller *et al.*, 1988), and it remains to be determined whether the surface-associated NP molecules are involved in the assembly and release of budding virions.

C. Lateral Interactions between Viral Glycoproteins

An alternate possibility for organization of the budding virion could involve lateral interactions among the viral glycoproteins to form a domain on the plasma membrane, from which host cell member proteins are excluded. The nucleocapsids would then recognize the cytoplasmic domains of the viral glycoproteins, leading to the budding process. If a significant portion of the envelope protein were located on the cytoplasmic surface of the viral envelope, such a domain could play a role in assembly analogous to that of the matrix protein, in a manner similar to

the E1 glycoprotein of coronaviruses (Sturman, 1977). However, neither the deduced amino acid sequences of the viral glycoproteins (Bishop and Auperin, 1987) nor analyses of protease-treated virions have yielded any suggestion that large protease-resistant cytoplasmic domains may exist (Vezza *et al.*, 1977; Gard *et al.*, 1977). In addition, no evidence has been obtained that viral glycoproteins are packed into viral envelopes in an organized manner, such as that observed, for example, with certain bunyaviruses (von Bonsdorff and Petterson, 1975). Such a regular arrangement of glycoproteins might be expected if lateral interactions among spikes played a major role in organizing the structure of the budding virion.

D. Involvement of Other Viral Proteins

The possibility may also exist that other virion proteins participate in the assembly process, and that they could play a role analogous to that of the M proteins of other enveloped viruses. For example, a polypeptide of 10–14 kDa has been reported in LCMV (Salvato and Shimomaye, 1989) and in Tacaribe (Iapalucci *et al.*, 1989), and a 12-kDa polypeptide has been reported in Pichinde virions (Ramos *et al.*, 1972). Nucleotide sequence analyses indicates that the LCM and Tacaribe gene products share a conserved zinc-binding motif, and immunological evidence has been obtained that the LCMV polypeptide represents a distinct viral gene product (Salvato *et al.*, 1992). Chemical cross-linking studies by this group showed an association of the 12 kDa protein with NP, but the role of this protein is still a mystery.

In the presence of tunicamycin, an inhibitor of N-linked glycosylation, the production of noninfectious Junin virus particles was observed at levels approximately 70% of normal amounts (Padula and de Martinez Segovia, 1984). Incorporation of radiolabeled sugar precursors into such particles was inhibited, and the amount of at least one of the viral glycoproteins (designated gp38) detected in the virus preparation was markedly diminished. It was not determined whether a nonglycosylated molecule corresponding to the polypeptide backbone of gp38 was present in the resulting particles. These results demonstrate that glycosylation of viral glycoproteins is not required for virus particle production, a result similar to that obtained for several other enveloped viruses (Gibson *et al.*, 1979; Basak and Compans, 1983). However, it remains to be established whether nonglycosylated forms of the glycoprotein are involved in virus assembly under these conditions.

Superinfection of Pichinde virus-infected cells with vesicular stomatitis virus (VSV) resulted in the production of pseudotypes that were not neutralized by antiserum to VSV, but were neutralized by antiserum to Pichinde virus (Sengupta and Rawls, 1979). Although these particles have not been characterized biochemically, by analogy to other VSV

pseudotypes they presumably consist of typical VSV particles possessing the surface glycoproteins of Pichinde virus, rather than the VSV-G protein (McSharry *et al.*, 1971). These results suggest that the structural properties of arenavirus glycoproteins are compatible with their incorporation into the envelopes of VSV particles, even though VSV particles are assembled by a process involving the incorporation of an M protein into their envelopes, in apparent contrast to the arenaviruses.

The process of assembly for arenaviruses appears to be less precise than that of most other enveloped viruses. Such lack of precision in virus assembly is indicated by the observed pleomorphism of the virions and the incorporation of host components, such as ribosomes, into virus particles. The incorporation of other RNA species including mRNAs and viral complementary sense RNA species into arenaviruses has also been observed (Bishop, 1990). Nevertheless, the assembly process is sufficiently precise to produce particles containing only a limited number of virus-coded polypeptides as the major structural components of virions.

Some evidence for the incorporation of host-cell-derived antigens into the envelopes of LCM virions has also been obtained, in that the virus was reported to be neutralized by antiserum to host cells (Ofodile *et al.*, 1973). No evidence has been obtained concerning the nature of such host antigens. High-molecular-weight sulfated polysaccharides, presumably of host cell origin, have been detected in LCMV (Bruns *et al.*, 1983) and may represent a host antigen, as has been found for other enveloped viruses (Lee *et al.*, 1969).

VII. ASSEMBLY MECHANISMS AND VIRAL PATHOGENESIS

It is of interest to consider the possible interrelationships between the mechanism of virus release at the cellular level and the pathogenesis of virus infection. A common feature of arenaviruses is their propensity to establish persistent infections, both in cell culture systems and in their natural rodent hosts (Oldstone, 1987). The replication and assembly processes of arenaviruses are clearly compatible with the establishment of a persistent infection, in that synthesis of viral components occurs without any major alterations in biosynthesis of essential host cell macromolecules, and budding of progeny virions at cellular plasma membranes provides a mechanism for virus release without any cytopathic effect.

Initiation of the natural infection process by many viruses occurs at epithelial cell surfaces, such as those that line the respiratory and gastrointestinal tracts. Such body surfaces are lined by polarized epithelial cells, with biochemically distinct apical and basolateral membranes, and it has been demonstrated that entry as well as release of many viruses

from such cells occurs exclusively at either the apical or basolateral membrane domains, depending on the virus type (Stephens and Compans, 1988). As yet, no information has been reported on the mode of entry or release of arenaviruses from such polarized epithelial cells, and it will be of interest to investigate this question in relation to the pathogenesis of virus infection. For example, in rodent hosts, the excretion of large amounts of virus in the saliva as well as in the urine might be consistent with selective release at apical membranes from appropriate cell types.

VIII. CONCLUDING REMARKS

Although substantial progress has been made in studies of the molecular biology of arenaviruses, many questions remain to be answered concerning the ultrastructure of the virion and the process of virus assembly. Determination of the transmembrane topology and quaternary structure of the envelope glycoproteins should be feasible using the same approaches that have been employed successfully with other membrane glycoproteins. Analysis of the precise arrangement of the nucleoprotein components within intact virions, as well as the sequence of events and precise molecular interaction involved in virus assembly, may, however, require novel approaches. Studies of the assembly process could benefit from the use of temperature-sensitive virus mutants, a number of which have been described (Vezza and Bishop, 1977).

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