Arenoviruses in Vero Cells: Ultrastructural Studies

FREDERICK A. MURPHY, PATRICIA A. WEBB, KARL M. JOHNSON, SYLVIA G. WHITFIELD, AND W. ADRIAN CHAPPELL

Health Services and Mental Health Administration, National Communicable Disease Center, Atlanta, Georgia 30333, and Middle America Research Unit, National Institute of Allergy and Infectious Diseases, Balboa Heights, Canal Zone

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Thin-section electron microscopy was carried out on Vero green monkey kidney cell cultures infected with some viruses of the newly constituted arenovirus group. Junin, Machupo, Amapari, Pichinde, Parana, Tamiami, and Latino viruses were morphologically identical and indistinguishable from lymphocytic choriomeningitis virus, the prototype virus of the group. Virus particles were round, oval, or pleomorphic, 60 to 280 nm in diameter, and matured via budding from plasma membranes. Most characteristically, particles contained various amounts of homogeneous, 20- to 25-nm, dense granules; these granules in large masses also formed distinctive intracytoplasmic inclusions. In negative-contrast preparations from infected Vero cell culture supernatant fluids, several of the viruses appeared as pleomorphic membrane-bound forms with rather pronounced surface projections. Most particles were between 90 and 220 nm in diameter, although some reached 350 nm in their longest dimension. Internal structure was not resolved by negative-contrast electron microscopy. All observations supported the current delineation of a distinct arenovirus group.

Morphological similarities between Machupo virus, the etiological agent of Bolivian hemorrhagic fever, Tacaribe virus, and lymphocytic choriomeningitis (LCM) virus led to a proposal that these and immunologically related viruses be placed in a new taxonomic group with LCM virus as the prototype (27, 34). These three viruses in ultrathin section are spherical or pleomorphic in shape and range from 60 to 300 nm in diameter; they have a unit-membrane envelope with projections and contain various amounts of internal electron-dense granules which resemble ribosomes (1, 9, 27). LCM, the recently discovered Lassa virus (7), and viruses of the Tacaribe complex are also related in other ways. Cross-reactivity among members of the Tacaribe complex was demonstrated by complement fixation (38); recently, LCM, Lassa, and some members of the Tacaribe complex were shown to be cross-reactive in an indirect immunofluorescence assay (7, 35). Formal establishment of this virus group was undertaken by a committee; "arenavirus group" was advanced as a name (34).

In the present investigation, morphological and morphogenetical comparisons were expanded to other viruses of this arenavirus group. Thin-section electron microscopy was carried out on Vero cells infected with Junin (28), Amapari (32), Pichinde (Trapido and San Martin, in press), Parana (Webb, Johnson, Hibbs, and Kuns, unpublished data), Tamiami (Calisher, Tzianabos, Lord, and Coleman, Amer. J. Trop. Med. Hyg. 19:520–526, 1970), Latino (Webb, unpublished data), and Machupo viruses (17, 39). Lassa virus was excluded from this study because of hazard. These viruses (except Junin and Machupo), in negative-contrast preparations from Vero cell cultures, were compared with LCM and Tacaribe viruses (10). The morphology of the latter was described by Bergold et al. (3).

MATERIALS AND METHODS

Viruses. The viruses used and their strain designations, origins, passage histories, and infectivity titers are listed in Table 1. Virus stocks were from the collections of the Middle America Research Unit (MARU) and the Arbovirus Reference Laboratory, National Communicable Disease Center (ARL, NCDC) or the Viropathology Laboratory (VPL, NCDC), or both.

Cell cultures. Vero African green monkey cells (ATCC, CCL 81) from different sources and with different passage histories were maintained in the three laboratories (MARU, ARL, VPL). Aspects of these ultrastructural studies were carried out over a period of 3 years. Although most thin-section microscopy was done on cells from MARU and most negative-contrast microscopy of cells from VPL, overlapping included thin-section and negative-contrast microscopic study of infected and control cells from all three laboratories. Cells were grown in monolayers in enriched Eagle's minimal essential.
medium containing 10% newborn calf serum (ARL, VPL) or medium 199 containing 5% serum (MARU). After viral adsorption, cells were maintained on the same medium containing 2% serum (ARL, VPL) or 1% serum (MARU). Cells were harvested at intervals between 3 and 7 days after infection for thin sectioning and at 4 days for negative-contrast preparations. In most cases, parallel cultures harvested at the same times were assayed for infectivity by plaquing in Vero cells or by intracerebral inoculation of mice (LCM virus).

**Electron microscopy.** For thin sectioning, cell monolayers were washed with a phosphate buffer (Millonig's, at 4 C, pH 7.3), scraped from bottles with a rubber spatula, and centrifuged in the buffer at 630 X g for 15 min. Undisturbed packed cells were then fixed with buffered 2.5% glutaraldehyde for 30 min. They were treated with 1% osmium tetroxide, dehydrated in an ethanols series, and embedded in an Araldite-Epon mixture (26). Sections were stained with uranyl acetate and lead citrate. For negative-contrast microscopy, supernatant fluid was diluted 1:1 with water and centrifuged at 100,000 X g for 1 hr. Resultant pellets were dispersed in small volumes of water, mixed with sodium silicotungstate (pH 7.0) and bovine albumin solutions, and sprayed with a nebulizer onto carbon-coated grids.

**Mycoplasma testing.** Virus stocks, un inoculated Vero cells, and harvests of infected cells and supernatant fluids used for electron microscopy were cultured on horse serum-yeast extract-enriched Mycoplasma medium under an anaerobic hydrogen-carbon dioxide atmosphere (11). Uninoculated cells were examined extensively by electron microscopy in parallel with all virus-infected cultures. Vero cells inoculated with Mycoplasma orale 1 and M. pulmonis (Negroni) (kindly provided by John Hierholzer, NCDC) served as controls; these cells were cultured through all electron microscopic and cultural procedures.

**RESULTS**

Thin-section electron microscopy. Each of the arenaviruses reached an infectivity titer of 10⁶ to 10⁷ plaque-forming units (PFU)/ml during the harvest period of 3 to 7 days postinoculation; the minor differences found in time and level of peak titer were not significant in this study. Coincident with the recovery of high titers of infectious virus, large numbers of virus particles were observed in Vero cells infected with all the listed agents. Virus particles were round, oval, or pleomorphic and ranged widely in diameter from 60 to 280 nm (longest dimension). They consisted of a distinctly heavy unit-membrane envelope upon which, in most instances, surface projections (approximately 6 nm in length) could be resolved. Internally, particles contained randomly distributed and widely differing numbers of electron-dense granules (20 to 25 nm in diameter). Junin, Parana, Latino, Pichinde, Machupo, Tamiami, and Amapari viruses were indistinguishable from each other and from Tacaribe and LCM viruses, as previously described (reference 27; Fig. 1-4). Largest accumulations of virus particles occurred at the periphery of cells as a consequence of budding from plasma membranes (Fig. 4-6). Occasionally, virus particles budded from intracytoplasmic membranes and accumulated within vacuoles. Membrane changes at sites of viral budding were characteristic; at each site, a distinct increase in density of both membrane lamellae was restricted to an area large enough to provide an individual viral envelope. As this altered membrane was progressively extruded, surface projections were formed and dense granules appeared to migrate into the

### Table 1. Identification of viruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>Strain</th>
<th>Origin</th>
<th>Passage historya</th>
<th>Infectivity titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Junin</td>
<td>XJ</td>
<td>Parodi</td>
<td>SM₁₁, GP₂, SM₁₄</td>
<td>2 X 10⁶</td>
</tr>
<tr>
<td>Machupo</td>
<td>Carvallo</td>
<td>Johnson</td>
<td>SH₁₂</td>
<td>5 X 10⁵</td>
</tr>
<tr>
<td>Amapari</td>
<td>BeAn 70563</td>
<td>Shope</td>
<td>SM₁₅, (PPF) Vero₂</td>
<td>10³</td>
</tr>
<tr>
<td>Pichinde</td>
<td>An 3739</td>
<td>San Martin</td>
<td>SH₁₄, (PPF) Vero₂</td>
<td>1.6 X 10⁴</td>
</tr>
<tr>
<td>Parana</td>
<td>12056</td>
<td>Webb</td>
<td>SH₁₄, Vero₂</td>
<td>1.5 X 10⁷</td>
</tr>
<tr>
<td>Tamiami</td>
<td>W-10777</td>
<td>Chappell</td>
<td>SH₁₆, Vero₄</td>
<td>4 X 10⁹</td>
</tr>
<tr>
<td>Latino</td>
<td>10924</td>
<td>Webb</td>
<td>SM₂₀, Vero₂</td>
<td>2.5 X 10⁷</td>
</tr>
<tr>
<td>Tacaribe</td>
<td>TRVL 11573</td>
<td>Downs</td>
<td>M &gt; 10⁰</td>
<td>10⁷</td>
</tr>
<tr>
<td>LCM</td>
<td>Armstrong E350</td>
<td>ATCC</td>
<td></td>
<td>10⁶</td>
</tr>
</tbody>
</table>

a All animal passages were via the intracerebral route. Abbreviations: SM, suckling mouse; GP, guinea pig; SH, suckling hamster; M, weanling mouse; PPF, plaque purified; Vero, Vero green monkey kidney cell monolayer culture.

b Not determined.
FIG. 1. Junin virus particles in extracellular space at the periphery of Vero cell. Particles consist of heavy unit-membrane envelope containing various amounts of 20 to 25 nm dense granules. X95,000.

FIG. 2. Pichinde virus particles in Vero cell culture at 7 days postinoculation, at which time the supernatant fluid contained $1.8 \times 10^6$ PFU/ml. X68,000.
FIG. 3. Tamiami virus particles accumulating in extracellular space at 7 days postinoculation. The wide variation in amounts of dense granules was characteristic. ×68,000.

FIG. 4. Amapari virus budding from Vero cell and Amapari virus free in extracellular space (arrow). ×68,000.

FIG. 5. Junin virus particles with surface projections on unit-membrane envelopes (arrows). Envelope structure became more distinct than normal plasma membrane at sites of particle budding. ×110,000.
interior of each bud from the cytoplasm proper (Fig. 5, 6). In some cells observed at the time of peak viral maturation, this functional budding process involved an extraordinary proportion of the plasma membrane (Fig. 6). Late events of infection were marked, in some cells, by alteration of increasingly large or even continuous areas of plasma membrane into the denser lamellae characteristic of viral envelope. Late harvests contained higher proportions of larger virus particles as a consequence of extrusion and pinching off of these larger discrete areas of membrane-envelope with contained dense granules. In its extreme, this alteration involved nearly the whole plasma membrane of some cells (Fig. 7). Such cells contained large numbers of dense granules within the cytoplasm beneath their altered plasma membrane.

In Vero cell cultures infected with each of the arenaviruses, distinctive intracytoplasmic inclusion bodies were found (Fig. 8, 9). Inclusions consisted of a moderately electron-dense, smooth matrix in which masses of the dense granules, identical to those within virus particles, were interspersed (Fig. 10). As illustrated, the granules were very homogeneous in size and indistinguishable from host cell ribosomes. The inclusions varied in their degree of condensation or packing; they did not have structured margins and were widely variable in size and shape. From observations of serial harvests of cells infected with the same virus, it appeared that inclusions became progressively denser and assumed smoother margins during the course of infection preceding development of cytopathic changes (compare Fig. 8 and 9). The location, general appearance, and kinetics of development of these inclusion bodies precisely matched the light microscopic observations made by Buckley and co-workers of Vero cells infected with several of these same viruses (6, 7, 23, 33; Buckley, unpublished data). Ribosomal aggregation was not observed in control cells.

Cytopathic changes in Vero cells infected with each of the listed viruses were asynchronous, but progressively involved destruction of cytoplasmic organization of most cells. Cytoplasmic condensation as a result of retraction or detachment, vacuolation, organelle destruction, and membrane breakdown occurred without specific alteration of nuclear morphology.

Negative-contrast electron microscopy. Conditions of centrifugation and timing of harvests were chosen to yield free virus particles with minimum damage and least contamination with cell debris. When possible, supernatant fluids from the same harvests used for thin sectioning were prepared for negative staining. Attempted variations from the described procedure caused apparent osmotic rupture of particles and an obscuring of surface projections or excessive background debris, or both. In particular, attempts to prepare the especially hazardous viruses, Machupo and Junin, by using glutaraldehyde or osmium tetroxide vapors for inactivation resulted in excessive obscuring of envelope detail.

Tacaribe virus particles occurred in two variations. The smaller particles were round or oval in shape and were covered with closely spaced surface projections (Fig. 11). Surface projections did not have an overall symmetrical pattern. The larger particles were more pleomorphic, and were seen more readily to be constructed of a membranous envelope covered with surface projections. In some cases, projections were separated more widely on larger particles. Internal details were never resolved, although stain usually penetrated larger particles (Fig. 12). The majority of smaller particles with closely spaced projections ranged in diameter from 90 to 140 nm; very few smaller particles were found (minimum diameter, 60 nm). Pleomorphic particles (Fig. 12) were usually less than 220 nm in their largest dimension, but one 350-nm particle was found.

LCM virus, in preparations made from infected Vero cells, was indistinguishable from Tacaribe virus (Fig. 13). Its variability in size and shape, its size range, and the presence of particles in which stain had penetrated paralleled findings with Tacaribe virus. Some apparent variation in the delicacy or fragility of the viral envelope, evident when comparing the illustrations, was probably due to variation in the amount of stain surrounding individual particles. The presence of an osmotically sensitive membrane in the viral envelope was suggested from frequent observations of delicate blebs emanating from the surface of virus particles.

Parana (Fig. 14), Pichinde (Fig. 15), Latino, Tamiami, and Amapari viruses were remarkably similar in morphology to LCM and Tacaribe viruses in negative-contrast microscopy. In general, larger-than-average-size particles are illustrated since their surface projection organization was more clearly delineated. As with Tamiami virus, stain penetration of larger particles failed to reveal internal structures.

Mycoplasma testing. Because virus particle morphology was similar to that of some mycoplasmas (15, 16), an extensive search for contaminants was made. In vitro culturing of virus stocks and of uninoculated and infected Vero cells failed to yield any Mycoplasma. Likewise, repeated thin-section electron microscopy of uninfected Vero cells being used for these studies was unremarkable. No particles with the surface
FIG. 6. Parana virus particles budding from plasma membrane over an extensive portion of cell surface (arrows). The entire surface of some cells was involved in virus production. ×45,000.

FIG. 7. Parana virus infection at 7 days postinoculation. The extreme of plasma membrane alteration involved large areas (arrows), even whole cell surfaces. Dense granules localized beneath such surfaces. ×42,000.
FIG. 8. Tamiami virus infected cell with particles budding from plasma membrane (arrow) and rather uncondensed inclusion material within the cytoplasm. ×34,000.

FIG. 9. Latino virus inclusions at a stage of infection preceding cytopathic changes. An increase in packing of granular elements occurred late in infection, making inclusions appear very dense (arrows). ×10,000.
FIG. 10. High magnification of a Junin virus inclusion of intermediate density. Inclusions consisted of a moderately dense, smooth matrix, in which the same 20- to 25-nm dense granules found within virus particles were interspersed. \( \times 155,000 \).

FIG. 11. Tacaribe virus particle in negative contrast from ultracentrifuged Vero cell culture supernatant; rather closely spaced surface projections cover its surface. \( \times 330,000 \).

FIG. 12. Tacaribe virus particle which has been partially penetrated by negative-contrast medium, revealing the membrane nature of its envelope. Internal detail was not resolved. \( \times 192,000 \).
Fig. 13. LCM virus composite illustrating typical variation in spacing of surface projections and in degree of stain penetration. The lower particle reveals the inner surface of membrane envelope, and the upper-left particle a membranous bleb (arrow). All $\times$230,000.

Fig. 14. Parana virus composite; particles shown are slightly larger than the mean and do not reflect the wide variation in size observed in negative-contrast and thin-section preparations. All $\times$192,000.

Fig. 15. Pichinde virus composite; delicate membranous blebs (arrow) were considered to result from osmotic effects upon envelope membrane. All $\times$192,000.
projections of the described viruses were found in negative-contrast preparations from uninoculated Vero cells. The M. orale I and M. pulmonis (Negroni) controls, studied in thin-section and by negative-contrast microscopy, were distinctly different from the viruses under consideration and identical to published descriptions (2, 15, 16).

**DISCUSSION**

The particles observed in Vero cell cultures infected with each of nine arenaviruses were associated temporally with the development of high-infectivity titers and spatially with inclusion-body formation and cytopathic changes. The consistency of parallel observations with the different viruses maintained in three laboratories over a period of 3 years and the repeated failure to find particles in uninoculated Vero cultures provide presumptive evidence that the described particles were the viruses inoculated. The identical appearance of these viruses and, as previously reported, Machupo virus propagated in human lymphoblastoid cells and lymphoid organs of the rodent, Calomys callosus, further supported the identification of the observed particles (27). Thus, in thin section, the arenaviruses distinctively consisted of a heavy unit-membrane envelope with projections and an unstructured interior containing various amounts of dense granules. They all had a wide size distribution pattern; longest dimensions ranged from 60 to 280 nm. Lascano and Berria independently found similar particles in Junin virus preparations (20) and deTkaczevski and her colleagues studied Junin virus dense granules and inclusions (Septième Congrès International de Microscopie Electronique, Grenoble, France, 1970, *in press*).

In negative-contrast preparations, often from supernatant fluids of the same cells embedded for sectioning, only one type of particle was related consistently to infectivity and the course of Vero cell cytopathology. As illustrated, these virus particles possessed a membrane envelope with distinct surface projections which matched the character of those seen in thin-section preparations. Membrane thickness in negative-contrast microscopy does not correspond to that in thin-section preparations. Virus particle size distribution also was similar to that seen in thin sections, although flattening effects yielded more of the larger particles with negative stain. The character of the particles observed matched some of the known physicochemical properties of these viruses, such as lipid-solvent sensitivity, osmotic fragility, and membrane localization of some specific immunofluorescence (8, 30, 37, 40; C. A. Mims, *personal communication*). Bergold et al. (3) previously reported that Tacaribe virus particles were spherical or pleomorphic and had an envelope with regularly arranged surface structures; their findings were confirmed by observations of this virus in the present study.

The cytoplasmic inclusions, consisting of electron-dense 20- to 25-nm granules packed in a matrix with varying density, clearly were associated with infection. The granules were indistinguishable from host cell ribosomes in size and density. Inclusions corresponded in dimensions and shape to basophilic masses seen by light microscopy in cells infected with these viruses (6, 7, 23, 24; Buckley, *unpublished data*). Likewise, these inclusions correspond to the major sites of localization of viral immunofluorescence. Abelson et al. (1) recently determined by means of immuno-electron microscopic techniques that granular aggregates in LCM-infected cells contain virus-specific antigens. Differences in ribonuclease sensitivity between host cell ribosomes and the granules as they occur within LCM particles supported the hypothesis that they represent multiple viral genome units (9). In the present study, a progression from a rather diffuse distribution of the dense granules in their more lucent matrix to a state of close packing and overall extreme density of inclusions occurred during the course of infection. At sites of viral budding, this progression was manifested by the pinching off of larger particles containing larger numbers of dense granules.

Of particular concern was the similarity in negative-contrast preparations between the viruses under consideration and mycoplasmas; the morphological similarity with M. pulmonis (Negroni) was especially striking (15, 16). Mycoplasmas, in addition to having similarities in size and morphology, are lipid-solvent sensitive, may lack inhibition by antibiotics, and may be the cause of virus-like cytopathic changes in cultured cells. Nevertheless, in the cells and virus stocks used in this study, no evidence of Mycoplasma contamination was found. There was no possible common source of contamination in this series of experiments; overlapping observations included virus-cell combinations, in which field isolations were made directly into Vero cells and limited subsequent passages were made in total isolation from other members of the group. LCM virus was not previously passed in Vero cells. Moreover, consideration that the arenaviruses may actually be obligate intracellular mycoplasmas is untenable. This conclusion is supported by the absence in the specimens examined of mature Mycoplasma bodies, the maturation of the arenaviruses exclusively via budding, the marked resistance of several agents of this virus group to
metabolic inhibitors (under conditions used with other ribonucleic acid viruses; 19, 31, 37), the demonstrated lack of deoxyribonucleic acid in one member virus—Junin (19), and the relationship of these agents to a unique intracytoplasmic inclusion not described in Mycoplasma-infected cultures.

In recognition of the distinctive nature of these viruses, a committee is formalizing the construction of a new taxonomic group, the arenovirus group, with LCM as the type virus (34). In addition to the crucial ultrastructural and serological bases for the grouping, several of the viruses are similar in that they have a limited geographical distribution, an ecological association with a rodent host, and an immunologically mediated mechanism of pathogenesis. Although LCM virus is associated with wild mice in many parts of the world, each of the viruses of the Tacaribe complex is limited to a particular niche in the Western hemisphere, and the majority are associated closely with different cricetine rodents in nonoverlapping locales. The relation of Lassa virus to rodents in Nigeria has not been elucidated. The nature of rodent infection with several of the viruses is similar (4, 5, 14, 18, 24, 25). Such infections may be associated with the development of a chronic virus carrier state in which the host maintains high virus titers in blood and other organs for the duration of life. In LCM and Machupo virus infections, this carrier state is related to viral lymphotropism (25; S. Suzuki and J. Hotchin, Bacteriol. Proc., p. 172, 1969) and ineffective immune elimination of virus (12, 13). The inability to clear virus may be secondary to depression of cell-mediated responsiveness brought about by the unusual nature and tropism of these viruses.

Formation of the arenovirus group focuses attention upon the differing capacity of member viruses to cause human disease. At present four viruses are known pathogens: LCM, Machupo (Bolivian hemorrhagic fever), Junin (Argentine hemorrhagic fever), and Lassa virus (Lassa fever). Others were not proved to be pathogenic for man despite frank exposure, especially via inadvertent laboratory contact. This difference between agents is clearly an area for future study.

The relationship of this new virus group to other, established groups ultimately depends on the weight assigned to particular taxonomic criteria. However, in consideration of their RNA genome, unknown (yet unusual) symmetry, lipid-solvent labile envelope, cytoplasmic development, and maturation upon plasma membranes, these viruses would appear taxonomically closest to avian and murine leukemogenic viruses (9, 21, 22). Similarities in surface structure between Gross leukemia virus (Fig. 7 from reference 29), Visna virus (36), and even some depictions of myxoparamyxoviruses must be set in perspective considering the common mode of maturation of these diverse ribonucleic acid viruses or virus groups. Resolution of the nucleocapsid of leukemogenic viruses as well as the arenoviruses studied here will be necessary for precise placement within classification schema.

LITERATURE CITED


