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Borna Disease Virus: Immunoelectron Microscopic Characterization of Cell-Free Virus and Further Information about the Genome

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The etiological agent of Borna disease, a persistent virus infection of the central nervous system with differently expressed symptomatology, was morphologically unknown. Here we provide the first convincing data on its phenotypic architecture. Salt-released virus comprising the biological parameters of Koch's postulates has an unsegmented single-stranded RNA. A dense band (1.22 g/cm^3) in CsCl contains 90-nm particles which appear to be enveloped and a fraction of 50- to 60-nm particles. Labeling of the virions with neutralizing antisera and colloidal gold conjugates indicates that the 90-nm particles most likely represent the causative agent.

In nature, Borna disease virus (BDV) is the etiological agent of severe neurological symptomatology with exitus in horses, sheep, cats, and ostriches (17, 19, 20, 30). Demonstration of specific viral antigen in people with correlations with affective disorders suggests that BDV or a related virus may be pathogenic in humans (3).

The virus has not been fully classified, and virions (16) as well as intracellular virus-like structural elements were not convincingly visualized (11), although progress in analyzing the agent as a single-stranded RNA virus was made (8, 15, 23, 29). The independent isolation of cDNA clones with RNA from BDV-infected rat brain or infected MDCK cells which were inferred to encode BDV-specific proteins with a molecular mass of 40, 24, and 14.5 kDa, respectively, was the basis of a preliminary characterization of the viral genome (8, 15, 23, 24, 27–29). The large amount of full-length negative-strand RNA in BDV-infected cells and rat brain suggested that BDV is a negative-strand virus (4).

Particle structures isolated by treatment of an infected human oligodendroglia cell line with elevated salt concentrations represent the infectious units (22) and contain at least a negative-polarity 8.5-kb RNA (4). While our paper was in preparation, the isolation of BDV ribonucleoproteins and analysis of the complete sequence of the genome from cell-free virus indicated a negative-strand virus with an unsegmented genome (5–7).

In this study, we present the first data to our knowledge on the morphology of the BDV and confirm recent data on the structure and polarity of the viral genome. The basis of our experiments was an improved purification of released virus by cesium chloride gradient centrifugation. Up to 10 released virus preparations (10 batches), each from 2×10^8 BDV-infected Oligo/TL cells, were layered onto a step gradient (4 ml of CsCl solution, density of 1.3 g/cm^3, and 7 ml of CsCl solution, density of 1.1 g/cm^3) and centrifugated in a Beckman SW40 rotor for 4 h at 34,000 rpm and 4°C. The two opalescent bands with densities of 1.18 and 1.22 g/cm^3 were collected, dialyzed, and layered onto a second step gradient (1 ml each of CsCl solutions with densities of 1.26, 1.22, 1.18, and 1.14 g/cm^3, respectively) and centrifuged in a Beckman SW50.1 rotor additionally for 4 h at 40,000 rpm and 4°C. Recentrifugation of the bands led to considerable sharpening, especially for the band with a density of 1.22 g/cm^3. Resulting bands were collected and were used for RNA extraction, infectivity assays, and electron microscopy.

Biological characterization of the bands. Titration of the two fractions by a cell-enzyme-linked immunosorbent assay (for details, see reference 18) revealed the following: approximately 33% of the virus loaded onto the first gradient was recovered (total of 5×10^6 focus-forming units [FFU] from 15×10^6 FFU). In three independent experiments, the denser band (1.22 g/cm^3) harbored almost all the infectivity (total of 5×10^6 FFU compared with 1×10^4 FFU at 1.18 g/cm^3).

For further biological characterization and proof of Koch's postulates with banded material, in vitro and in vivo experiments were performed: Calibrated samples (100 FFU) of upper and lower bands could be neutralized to 0 FFU with rabbit serum BP-11 (dilution, 1:500) and rat pool serum (dilution, 1:50), whereas species-specific control sera and the monoclonal antibodies Kfu 2, W1, and a herpes simplex virus-specific one had no effect. A similar neutralization effect was obtained with cerebrospinal fluid of a Borna horse (data not shown; see reference 18). Furthermore, five of five Wistar rats died 6 to 8 weeks after infection with the gradient material with a density of 1.22 g/cm^3 (intracerebral inoculation with 0.025 ml) from Borna disease, whereas no infectious material was recovered from five symptomless rats inoculated with material with a density of 1.18 g/cm^3. Three rabbits showed typical signs of BD when inoculated intracerebrally with 0.1 ml of the denser band (1.22 g/cm^3) within 3 to 4 weeks, and no effect was seen with the upper band.

Visualization of the virus. Virus preparations were placed on Formvar-carbon-coated grids and negatively stained with 1% aqueous uranyl acetate. Prior to gradient centrifugation, the negatively stained virus preparations appeared heterogeneous in their composition with large amounts of cellular and membrane debris. Screening of more than 10 individual batches showed low amounts of virus-like particles. The gradient band (1.22 g/cm^3, 5×10^6 FFU/ml), however, contained many such particles and low amounts of debris. The upper band (1.18 g/cm^3) consisted of cellular constituents and mem-

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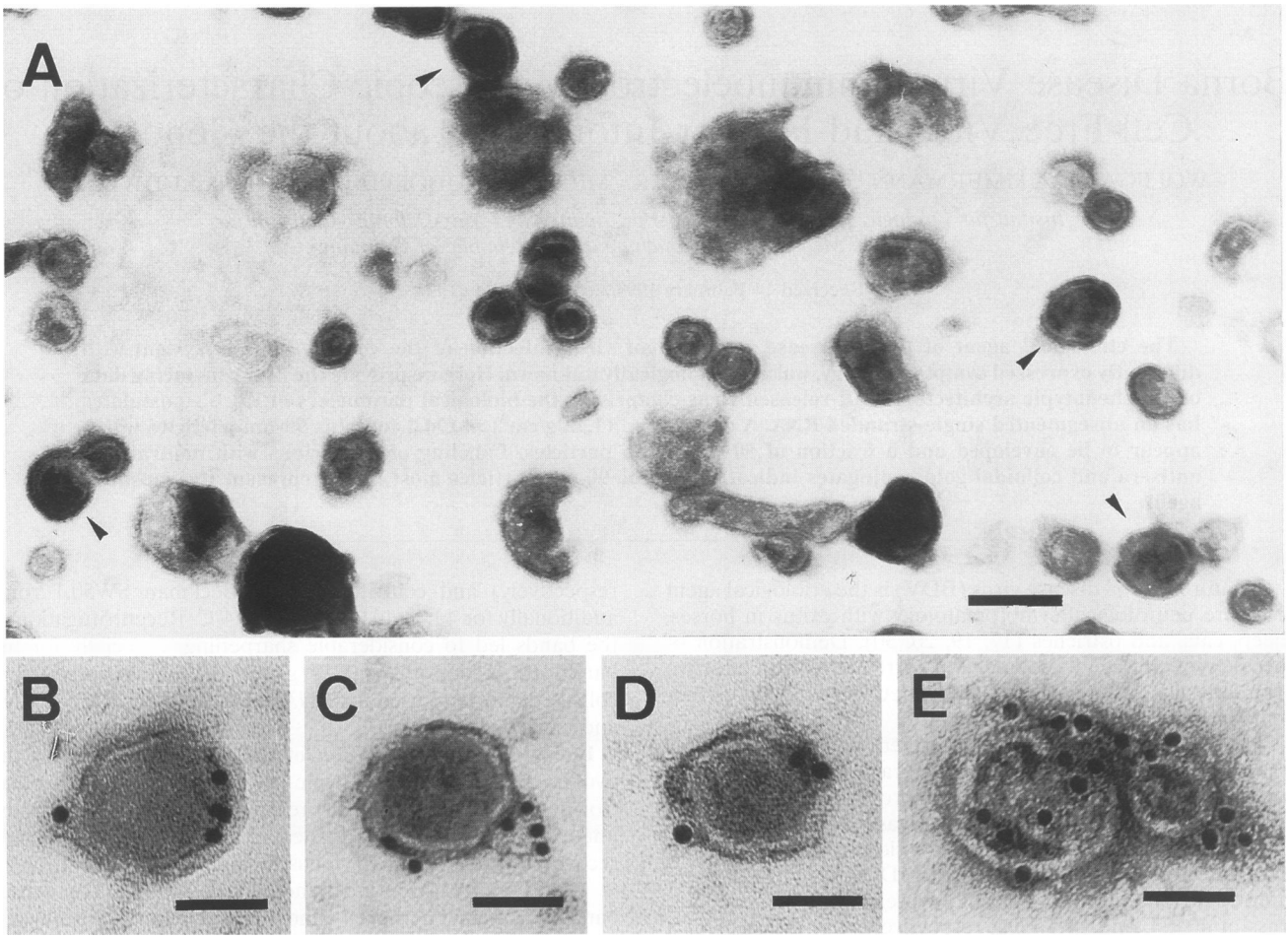

FIG. 1. (A) Representative overview of negatively stained material from the 1.22-g/cm^3 fraction with large 90-nm particles (arrowheads), small 60-nm particles, and cellular debris. Magnification, ×125,000. Bar, 100 nm. (B to E) Shown is immunolabeling of BDV particles obtained from the same gradient fraction with rat pool serum (containing BDV-specific neutralizing antibodies) and anti-rat immunoglobulin G complexed with 10-nm colloidal gold, respectively, followed by negative staining with uranyl acetate. Larger particles are uniformly labeled (B to E). Note also labeling of smaller particles (E). Magnifications, ×245,000. Bars, 50 nm.

brane debris. As shown above, this fraction harbored 0.2% (10^4 FFU) of the infectivity.

Two distinct groups of spherical particles were obvious: one with a diameter of 90 nm (± 10 nm) and the other one with a diameter of approximately 50 to 60 nm. The larger ones appeared to consist of an enveloped electron-dense core (50 to 60 nm). A rough estimate gave an equal ratio of large and small particles with variations in different experiments (Fig. 1A). Typical large particles from this fraction are marked with arrowheads.

Further support to deal with BDV is given by the results of immunonegative staining. The virus preparations were dried onto grids; these were floated, specimen side down, for 1 h on the antibody preparations (diluted 1:50 to 1:250) used in the neutralizing experiments (see above). After incubation, the grids were washed with Tris-buffered saline (20 mM Tris-HCl [pH 7.2], 150 mM NaCl, 1% bovine serum albumin) and incubated for an additional hour on goat anti-rat immunoglobulin G or protein A conjugated with 10-nm colloidal gold particles (BioCell Research Laboratories). Finally, the grids were washed in Tris-buffered saline, fixed with 1% glutaraldehyde in Tris-saline (20 mM Tris-HCl [pH 7.2], 150 mM NaCl), and stained with uranyl acetate.

Marking of the large particles with the neutralizing antisera varied from numerous to one or two gold complexes per virus (Fig. 1B to E). The intensity of labeling depended on the antiserum. Whereas rat pool serum (diluted 1:100) resulted in a rather uniform attachment of gold markers to the majority of particles (three to six gold markers per virus), rabbit serum BP-11 (diluted 1:200) led to a binding of one to three gold complexes per virus. In the 1.22-g/cm^3 gradient fraction, 50-nm particles were also weakly labeled with gold (compare Fig. 1E).

The specificity of labeling was verified with preimmune sera of BP-11 and normal rat serum as well as a monoclonal antibody to herpes simplex virus, which showed no marking of particles.

Further characterization of BDV genome. Morphologically characterized virus which was biologically identified as the etiological agent of Borna disease offered the possibility of clarifying controversial aspects of the genome structure. For this, sense and antisense riboprobes of the plasmid pAF4 were used in Northern (RNA) hybridization (25) to determine the polarity of the genome. The sense RNA probe pAF4-SP6 recognized in RNA preparations from purified virions a band of approximately 9.5 kb, but no viral RNA was detectable with the antisense probe pAF4-T7 (Fig. 2, lanes 1 and 2). The size

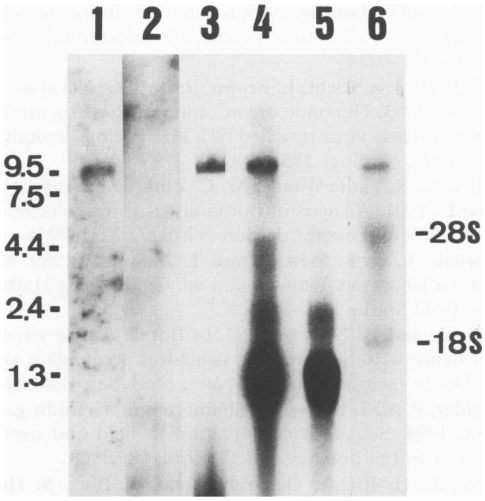

FIG. 2. Characterization of the polarity of BDV-specific RNAs and demonstration of the nonsegmented genome. Shown is RNA extracted from a virus particle preparation analyzed by hybridization with sense (lane 1) and antisense (lane 2) riboprobes of the plasmid pAF4. RNA composition of released virus from Oligo/TL cells labeled with [^{3}H] uridine was characterized by electrophoresis and fluorography (lane 3). Depicted also is the polarity of RNAs extracted from infected rat brain analyzed by hybridization with a DNA probe from the plasmid pAF4 (lane 4) and either antisense (lane 5) or sense (lane 6) riboprobes. Positions of 28S and 18S rRNA are indicated.

of the viral genome could be verified by hybridization of total RNA extracted from BDV-infected rat brain tissue. A DNA probe prepared from the plasmid pAF4 resulted in the demonstration of an approximately 9.5-kb band representing the viral genome and transcripts of 3.6, 2.1, and 0.85 kb (Fig. 2, lane 4). Subsequently, hybridization of the same RNA preparation with both riboprobes pAF4-T7 and pAF4-SP6 revealed the described transcripts (Fig. 2, lane 5) and the viral genome (lane 6), respectively.

To answer the question of genome segmentation, semiconfluent monolayers of Oligo/TL cells were labeled for 48 h with 5 μCi of [5,6-^{3}H]uridine per ml (specific activity, 50 Ci/mmol; Amersham). Metabolically labeled RNA was extracted from purified virus preparations, known to cause typical Borna disease (see above), and electrophoresed in a 1% agarose gel containing formaldehyde. After fluorography and autoradiography, only one band corresponding to a molecular size of approximately 9.5 kb (Fig. 2, lane 3) could be demonstrated. This result excludes genomic segmentation and corroborates data independently obtained with viral genomic RNA isolated from ribonucleoproteins (6).

Conclusions. The increase of virus yields obtained from the human oligodendroglia cell line, refinement of the purification procedure, and the availability of neutralizing antibodies led to the immunoelectron microscopic demonstration of virus particles, in contrast to earlier reports on virus-associated structures (1, 2, 10, 11, 16, 26). From all parameters outlined here, these particles represent the causative agent of Borna disease. At present, our data do not fully explain the existence of the two differently sized particles (90 and 50 to 60 nm). The larger ones seem to consist of an electron-dense inner part which could represent the nucleocapsid and a clearly visible envelope. An envelope of infectious BDV is generally accepted because of its sensitivity to lipid solvents (for a review, see reference 17).

Without further data, we cannot answer whether the smaller particles represent defective or immature ones. However, the way of passaging the virus and its survival in the classic way of persistent infection make the existence of such particles and subviral structures most probable (12). A distinct labeling of the particles with antisera from two species (rabbit and rat) supports the idea that both particles may represent distinct stages in the development of the virus.

An additional hint that the 90-nm particles represent the complete virion comes from preliminary data on ultrathin cryosections of persistently infected Oligo/TL cells prior to the release procedure. Such rarely found particles were located beneath the plasma membrane and had a shape and size (approximately 90 nm) similar to those particles detected in purified virus preparations with a core-like structure (9).

Morphologically, BDV does not fit in any of the known taxonomic groups (21). The involvement of the nucleus during BDV replication, deducible already from the typical inclusion bodies (13), the specific antigen accumulation (17), and recent molecular biological data (4, 8) display biological properties of BDV analogous to those of orthomyxoviruses, which, however, are segmented (14). On the other hand, a relationship to rhabdo- and paramyxoviruses was recently demonstrated by the sequence and the organization of the BDV genome (5, 7). On the basis of our data and the results described above, we propose BDV to be a member of a new virus family or an individual virus type within the order *Mononegavirales*.

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