Med Microbiol Immunol (1993) 182:271–280

Medical Microbiology and Immunology

Analysis of virus-specific RNA species and proteins in Freon-113 preparations of the Borna disease virus

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Received: 6 May 1993

Abstract. Treatment of homogenates from Borna disease virus (BDV)-infected brain tissue or cell cultures with Freon-113 yielded infectious particles with a buoyant density of 1.16–1.22 g/ml. Positive- and negative-stranded BDV-specific RNA species as well as three virus-specific proteins, known to be present in BDV-infected cell extracts, were demonstrated in these Freon-treated fractions. When the Freon-purified virus preparations were treated with RNase A prior to RNA extraction, only negative-stranded, genomic RNA was detected in Northern blot hybridizations using sense and antisense RNA probes. These data substantiate that BDV is a negative-stranded RNA virus.

Introduction

Borna disease virus (BDV) induces a T cell-mediated immunopathological encephalomyelitis in horses, sheep and a broad range of experimentally infected animal species (Richt et al. 1992). BDV has only partially been characterized; in particular, virus-like structures have not been visualized in infected tissues. Purification of BDV has not been achieved, probably because the virus is mainly cell-associated, has a relatively low replication rate, and infectivity is low in all types of tissue and cell cultures. The data available suggest that BDV is enveloped, has a diameter of 80–120 nm (Elford and Galloway 1933; Heinig 1969) and has the physical and replicative properties typical for conventional viruses (Ludwig and Becht 1977; Duchala et al. 1989; Richt et al. 1992). Only recently has it been shown that BDV contains a single-stranded RNA, presumably of 8.5 kilobases (kb) (de la Torre et al. 1990; Lipkin et al. 1990) or 10.5 kb (VandeWoude et al. 1990; Richt et al. 1991; Thierer et al. 1992). The polarity of the genomic RNA has been a matter of controversy. At least three virus-specific proteins were described with molecular mass of 14, 24 and 38/39 kilodalton (kDa) (Schädler et al. 1985; Haas et al. 1986; Thiedemann et al. 1992), respectively.

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In the present study we partially purified BDV from persistently BDV-infected Madin-Darby canine kidney (MDCK) cells or infected rat brain with the organic lipid solvent Freon (Freon-113 synonymous with Frigen; 1,1,2-trichlorotrifluoroethane) and/or CsCl density centrifugation. Virus infectivity, virus-like particles and virus-associated RNAs and proteins were analyzed in these preparations.

Material and methods

Virus

The Giessen strain He/80 of BDV, originally isolated from the brain of a naturally infected horse, was used throughout these studies. It was passaged twice in rabbits and then in newborn Lewis rats by intracerebral (i.c.) inoculation.

Animals

Inbred Lewis rats were provided by the Zentralinstitut für Versuchstierzucht in Hannover, Germany, and infected with BDV i.c. (10^5 $TCID_{50}$) at 4–6 weeks of age.

Infectivity test

Infectivity tests were done on rabbit embryonal brain cells (REB) as previously described (Herzog and Rott 1980). Viral antigen was detected using an indirect immunofluorescence assay employing rat anti-BDV immune sera.

Cell lines

Uninfected (MDCK) and persistently BDV-infected MDCK (BDV-MDCK) cells were used for Freon purification and served as sources of antigen preparations for Western blot analyses (Herzog and Rott 1980). REB cells are primary fetal rabbit brain cells from embryonic day 19 brain tissue (Herzog and Rott 1980).

Freon extraction

BDV-MDCK cells or BDV-infected rat brain were homogenized by ultrasonication (BDV-MDCK cells resuspended in TNE buffer) or with a tissue homogenizer [10% homogenate of rat brain in DMEM with 5% fetal calf serum (FCS)]. The homogenates were treated with Freon (2 vol homogenate and 1 vol Freon) and centrifuged for 20 min at 1000 *g* at 16°C. The aqueous phase I was collected and the remainder was reextracted by homogenization with TNE buffer or DMEM with 5% FCS (25% of original volume) and centrifuged to recover aqueous phase II (1000 *g*, 20 min, 16°C). The aqueous phases were pooled and treated again with 1/3 vol Freon as described above. The final aqueous phase was used for subsequent assays.

Analysis of RNA

RNA was isolated from BDV-infected and uninfected material using the acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomzynski and Sacchi 1987). RNA was electrophoresed at 80 V in a 1.2% agarose-formaldehyde (2.2 M) gel and transferred to

Hybond-N nylon membranes (Amersham, Braunschweig, Germany). The membranes were prehybridized in prehybridization buffer (50% formamide, 6× SSC, 5× Denhardt's, 1% SDS, 1% glycine, 0.05 M Hepes/pH 7.0, 20 µg/ml salmon sperm DNA, 20 µg/ml tRNA, 100 µg/ml heparin) for 4–6 h at 42°C (^{32}P-labeled probes) or 61°C [digoxigenin (DIG)-labeled probes] and then hybridized in hybridization buffer containing 1× Denhardt's with positive- and negative-stranded RNA probes overnight at prehybridization temperatures. The following day the blots containing DIG-labeled RNA probes were washed at room temperature in 2× SSC/0.1% SDS and then twice at 68°C with 0.1× SSC/0.1% SDS. The bound RNA probe was detected using the DIG Luminescent Detection Kit with Lumigen-PPD according to the protocol of the supplier (Boehringer, Mannheim, Germany; Cat. No. 1363514). The blots were exposed to X-ray film at room temperature. ^{32}P-labeled RNA probes (2.5×10^6 cpm/ml) were washed with 6× SSC for 10 min at room temperature and twice for 30 min at 65°C in 0.1× SSC/0.1% SDS before autoradiography at −70°C with intensifying screens.

Buffers and media

1× Denhardt's solution: 0.02% polyvinyl-pyrrolidone/0.02% Ficoll/0.02% bovine serum albumin (Sigma, St. Louis, USA); 1× SSC: 0.15 M sodium chloride/0.015 M sodium citrate (Merck, Darmstadt, Germany); TNE: 10 mM TRIS-HCl, pH 7.2/100 mM NaCl/1 mM EDTA (Merck); DMEM: Dulbecco's MEM medium from Gibco (Eggenstein, Germany).

Western blot analysis

MDCK, BDV-MDCK cells and Freon-treated material was lysed by ultrasonication on ice. The lysates were mixed with an equal volume of sample buffer with 2-mercaptoethanol (Sigma) and boiled. The proteins were separated by electrophoresis in a 12.5% SDS-polyacrylamide gel (Roth, Karlsruhe, Germany) and transferred onto Immobilon (Millipore, Molsheim, France). The nonspecific antibody binding sites were blocked with a solution of 2% BSA (Roth), 0.05% Tween 20 (Sigma) in phosphate-buffered saline (pH 7.4). Polyclonal rat anti-BDV was applied, the blot washed and biotinylated anti-rat immunglobulin (Amersham) was added. Specific binding was detected using 4-chloro-1-naphtol in methanol as substrate (Bio-Rad, München, Germany).

CsCl gradient

CsCl (Roth) was dissolved in TNE buffer and a discontinuous gradient with 1.3 and 1.4 g/ml CsCl (each 2.5 ml) was prepared. The CsCl solution was overlaid with 7 ml of untreated and Freon-treated material. The material was centrifuged for 2.5 h at 4°C and 40000 rpm. After gradient separation, the buoyant density of various fractions was measured and the samples analyzed further by infectivity assays and Northern and Western blot analyses.

Hybridization probes

Plasmids used were cDNA clones B8 and P4, both encoding the 14 and 24 kd protein of BDV (VandeWoude et al. 1990). Clone P4 was constructed by subcloning the entire B8 cDNA insert into the pGEM 3 vector (Promega, Heidelberg, Germany) in such a way to enable transcription of the BDV-specific insert in both directions using polymerase T7 (Promega). T7 polymerase directs transcription of the (+) strand of the P4 cDNA clone and the (−) strand of the B8 cDNA clone. Digoxigenin (DIG)-labeled RNA probes were made from the BDV-specific cDNA clones after linearization of the templates using the DIG RNA Labeling Kit (Boehringer Mannheim; Cat. No. 1175025). ^{32}P-labeled RNA probes for Northern hybridizations (2.5×10^6 cpm/ml) were prepared from the same plasmid DNA templates (P4 and B8) according to protocols from Promega.

Transmission electron microscopy

Freon-treated BDV-infected and uninfected MDCK cells were analyzed after negative staining with 2.5% uranyl acetate. The electron micrographs were prepared using the EM 902 from Zeiss (Oberkochen, Germany). The immunogold-staining experiments were done using polyclonal rat and rabbit anti-BDV antibodies and the respective gold-labeled anti-species antibodies (Plano, Marburg, Germany).

Results

Treatment of BDV-infected material with Freon-113

When homogenates from BDV-infected MDCK cells or BDV-infected rat brain were treated with Freon, infectivity titers dropped by 1 log for BDV-MDCK cells and 2 logs for BDV-rat brain (Table 1). Infection of REB cells with Freon-treated material resulted in a persistent infection and the immunofluorescence staining pattern characteristic for BDV-infected cells. A cytopathic effect was not observed, a finding previously described for the replication of BDV in vitro (Herzog and Rott 1980).

After CsCl gradient centrifugation of Freon-treated BDV-MDCK cells, the peak of infectivity accumulated at a density between 1.16 and 1.22 g/ml; CsCl centrifugation of untreated homogenates of BDV-MDCK cells yielded maximal infectivity titers at similar densities.

Freon-extracted preparations and fractions from CsCl gradients were treated further with RNase A for 1 h at room temperature. This treatment did not alter infectivity titers of the respective preparations (Table 1). This unimpaired infectivity demonstrates that infectious particles resisted the purification procedure in a form where the genomic RNA remained protected from RNase A digestion.

Table 1. Infectivity assay of Borna disease virus (BDV)-infected cell and tissue preparations

Material	Purification with Freon	Virus titer[a] (ID_{50}/ml) before RNase A treatment	Virus titer[a] (ID_{50}/ml) after RNase A treatment[b]
BDV-MDCK	No	10^6	n.d.
	Yes	10^5	10^5
CsCl gradient of BDV-MDCK[c]	Yes	10^4	10^4
BDV rat brain	No	10^6	n.d.
	Yes	10^4	10^4

n.d., Not done

[a] Infectivity assays were performed on fetal rabbit brain cells inoculated with the respective virus dilutions as described previously (Herzog and Rott 1980)

[b] Freon-treated material was incubated with 10 (BDV-MDCK cells) or 20 μg/ml (BDV rat brain) RNase A for 60 min at room temperature

[c] The CsCl-gradient fraction had a density of 1.18 g/ml

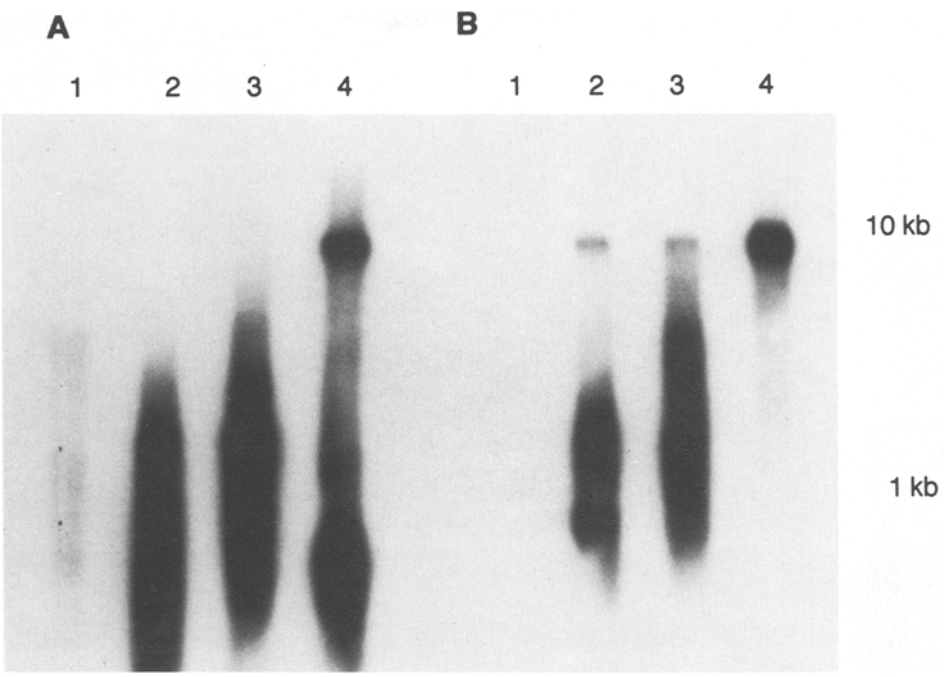

Fig. 1 A, B. Northern blot analysis of Freon-treated material before and after RNase A digestion. Rat brain homogenate was treated with Freon-113; aliquots were used to prepare RNA directly or were treated with RNase A at a concentration of 20 µg/ml or 40 µg/ml for 2 h at room temperature prior to preparation of RNA. **A** and **B** represent transfers from duplicate gels. **A** was hybridized with a riboprobe which detects sense RNA transcripts (B8/T7) and **B** with a riboprobe which detects anti-sense RNA transcripts (P4/T7). Each hybridization contained 2.5×10^6 cpm/ml of probe in the hybridization buffer (see Material and methods). The blots were prehybridized for 4–6 h and hybridized overnight at 42° C. Next day they were washed at room temperature in 6 × SSC and 2 × 30 min with 0.1 × SSC/0.1% SDS at 65° C before exposure. *Lanes: 1,* RNA from uninfected rat brain (10 µg RNA); *2,* RNA prepared from Freon-extracted Borna disease virus (BDV) rat brain treated with 40 µg/ml RNase A prior to RNA preparation (10 µg RNA); *3,* RNA from Freon-extracted BDV rat brain treated with 20 µg/ml RNase A prior to RNA preparation (10 µg RNA); *4,* RNA prepared directly from Freon-extracted BDV rat brain (2.5 µg RNA)

Analysis of BDV-specific RNA species in Freon-treated fractions

To analyze virus-specific RNA species present in Freon-extracted preparations, aliquots of Freon-treated rat brain were either used directly for RNA isolation (Chomczynski and Sacchi 1987), or the specimens were treated further with 20 µg/ml or 40 µg/ml of RNase A for 2 h (Fig. 1) or 5 h (Fig. 2) at room temperature prior to preparation of RNA. Duplicate Northern blots were hybridized with [^{32}P]UTP or DIG-UTP-labeled RNA probes which would detect either positive- or negative-stranded BDV-RNAs. The antisense probe was made from cDNA clone B 8; the sense probe originated from cDNA clone P4 (VandeWoude et al. 1990). Figure 1 A shows that after Freon isolation, positive-stranded BDV-specific RNA species of 0.85, 2.1 and 10.5 kb (lane 4) are still intact as shown with the negative-stranded RNA probe. However, after digestion with 20 µg/ml (Fig. 1 A, lane 3) or

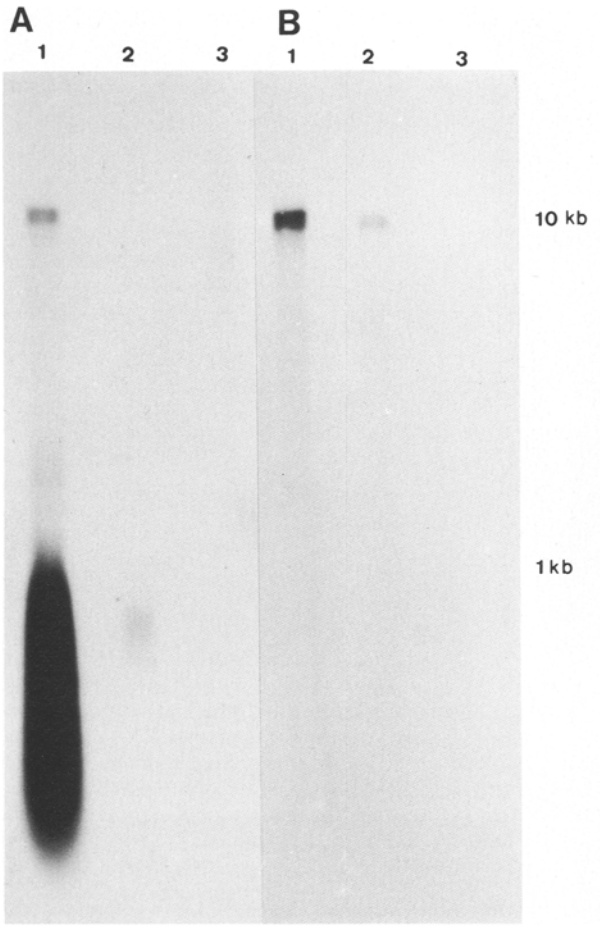

Fig. 2 A, B. Northern analysis of Freon-extracts treated with RNase A for 5 h prior to RNA preparation. Rat brain homogenate was treated with Freon-113 and aliquots were used to prepare RNA directly or treated with 40 μg/ml RNase A for 5 h at room temperature. The RNA was extracted using the method of Chomczynski and Sacchi (1987). After electrophoresis through agarose gels, the RNA was blotted and immobilized onto a nylon membrane. The nylon membrane was prehybridized in prehybridization buffer for 4–6 h and hybridized in hybridization buffer at 61 °C with a negative (**A**) or positive (**B**) RNA probe overnight. The following day filters were washed at 68 °C as described in Material and methods. BDV-specific RNA was detected using the DIG Luminescent Detection Kit. **A** was hybridized with a RNA probe which detects sense RNA transcripts (B8/T7); **B** was hybridized with a RNA probe which detects antisense RNA transcripts (P4/T7). *Lanes: 1,* RNA prepared directly from Freon-extracted BDV rat brain (10 μg RNA); *2,* RNA prepared from Freon-extracted BDV rat brain treated for 5 h with 40 μg/ml RNase A prior to RNA preparation (10 μg RNA); *3,* RNA from uninfected rat brain (10 μg RNA)

40 μg/ml (Fig. 1 A, lane 2) RNase A for 2 h, the largest positive-stranded RNA species of 10.5 kb had disappeared and only a smear of smaller positive-stranded RNA species were found (Fig. 1 A, lanes 2 and 3). The positive-stranded RNA probe hybridized to one RNA of 10.0 kb in Freon-treated material (Fig. 1 B,

lane 4). This means that the only negative-stranded RNA which exists in Freon-treated material has a size of 10.0 kb, which can be considered as the size of the genome. Even after digestion with 20 µg/ml (Fig. 1B, lane 3) or 40 µg/ml (Fig. 1B, lane 2) RNase A, the negative-stranded RNA species of 10.0 kb size was still discernible. A smear of smaller, partially degraded BDV-RNAs of both positive and negative orientations were produced after digestion for 2 h with RNase A concentrations of 20 µg/ml or 40 µg/ml. Since it was not clear whether these subgenomic BDV-specific RNA species were merely associated with virus particles, we digested Freon preparations for an extended period of 5 h with 40 µg/ml RNase A before RNA isolation and Northern blot hybridization. The results of the respective Northern gels are shown in Fig. 2. Here again, positive (Fig. 2A, lane 1)- and negative (Fig. 2B, lane 1)-stranded BDV-specific RNA species were found in the untreated, Freon-extracted RNA preparations. After 5 h of RNase A digestion, virus-specific positive-stranded RNAs were no longer detectable (Fig. 2A, lane 2). In contrast, the positive-stranded RNA probe was bound to a negative-stranded RNA with a size of 10.0 kb (Fig 2B, lane 2); the smear of subgenomic RNA species found in Fig. 1B (lanes 2 and 3) had disappeared after this prolonged treatment (Fig. 2b, lane 2). Comparable results were obtained when Northern blot hybridization of Freon-extracted tissue was carried out with preparations which had been RNAse A digested (40 µg/ml for 2 h) and purified in CsCl gradient centrifugation (data not shown). Therefore, the subgenomic RNA species found in the fractions digested for 2 h with RNase A are apparently not present in the virion particles.

Analysis of virus-specific proteins

To prove whether virus-specific proteins are still detectable after treatment with Freon-113, we analyzed native and Freon-treated fractions of BDV-infected MDCK cells in Western blots. Three BDV-specific antigens of 14, 24 and 38/39 kDa were found in the Freon-treated as well as untreated BDV-MDCK cell fractions (Fig. 3, lanes 3 and 4); all of them had previously been described as BDV-

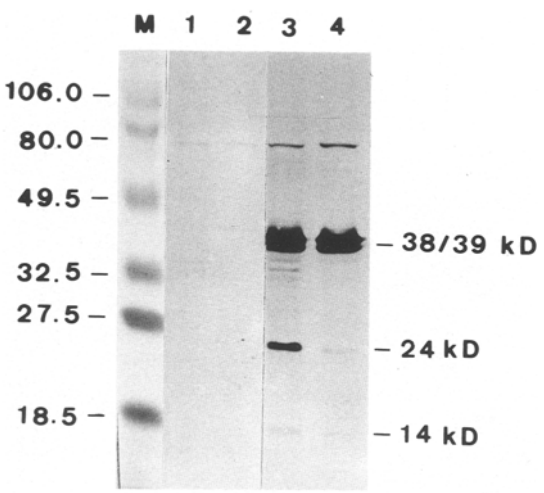

Fig. 3. Western blot analysis of Freon-treated, persistently BDV-infected MDCK cells. *Lanes: 1,* uninfected MDCK cells; *2,* uninfected MDCK cells/Freon-treated; *3,* BDV-infected MDCK cells; *4,* BDV-infected MDCK cells/Freon-treated

specific antigens (Schädler et al. 1985; Haas et al. 1986; Thiedemann et al. 1992). The distinct band of approximately 70 kDa does not represent a BDV-specific protein, since it is also found in the uninfected samples (Fig. 3, lanes 1 and 2). According to the staining intensities, the amounts of 24 and 14 kDa in the Freon-extracted BDV-MDCK cells (Fig. 3, lane 4) were less than in untreated BDV-MDCK cell preparations (Fig. 3, lane 3). Analogous results were obtained after CsCl centrifugation of Freon-treated, BDV-infected MDCK cells. Western blots of CsCl gradient fractions containing maximal infectivity exhibited again the three virus-specific proteins of 14, 24, 38/39 kDa (data not shown).

Examination by transmission electron microscopy

The partially purified virion particles from BDV-MDCK cells were examined by transmission electron microscopy. The only structure which could be identified by electron microscopy were sparse, virion-like particles of 60- to 80-nm diameter (Fig. 4, arrowhead). The presence of a rim-like structure surrounding these particles suggests the presence of an envelope. Similar particles were described previously by Ludwig and Becht (1977). Trials to stain these particles by BDV-specific antibodies employing the immunogold technique were not successful; no label was attached to these particles.

Discussion

In contrast to conventional lipid solvents which inactivate infectivity of BDV completely (Heinig 1969; Danner and Mayr 1979), the lipid solvent Freon-113 has only a limited destructive effect on infectivity; this treatment leaves relatively high

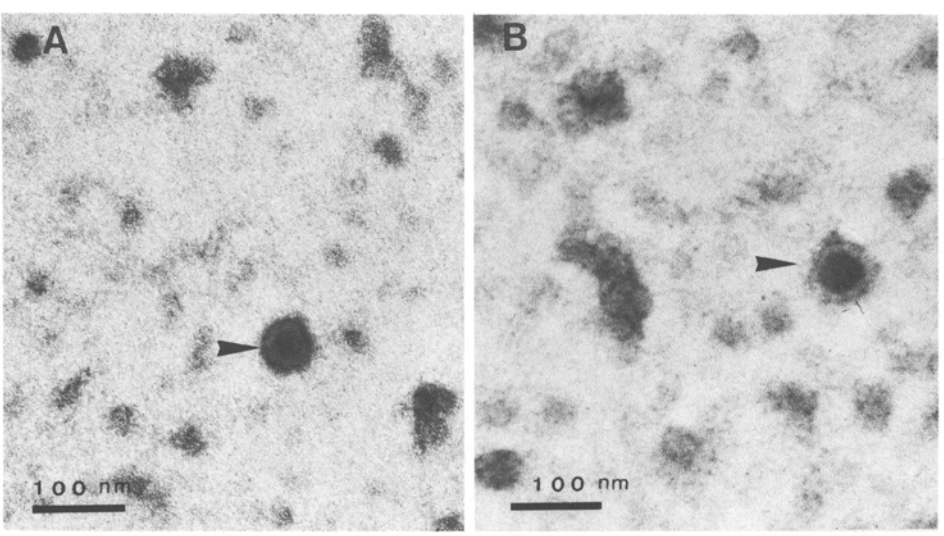

Fig. 4A, B. Transmission electron micrograph of Freon-treated, BDV-infected MDCK cells after negative staining with 2.5% uranyl acetate. *Bars* = 100 nm

infectivity titers. The structural entity carrying BDV infectivity is, therefore, left intact (Table 1). Treatment of homogenates derived from BDV-infected brain tissue or cell cultures yielded infectious particles with a buoyant density of 1.16–1.22 g/ml. Comparable results were obtained when Freon treatment was omitted from the purification protocol. All known viral proteins were still present in the infectious preparations after extraction with Freon. This was also true for a multitude of positive- and negative-stranded RNA species which were found in BDV-infected brain tissue or cell cultures before and after treatment with Freon (Figs. 1, 2). However, after treatment of Freon-purified preparations with RNase A, these samples contained only a negative-stranded RNA species of 10.0 kb (Figs. 1, 2). The fact that infectivity was not destroyed by digestion with RNase A substantiates the assumption that this RNA represents the viral genome which had been incorporated into particles and remains protected from RNase digestion. Freely accessible genomic RNA would have been destroyed by pancreatic RNase as shown previously (de la Torre et al. 1990). From all these findings one can conclude that the particulate structures purified by Freon extraction and density gradient centrifugation carry the elements essential for virus replication, and they are in accordance with the physico-chemical characteristics which had previously been established for BDV (Richt et al. 1992).

It remains uncertain whether the structures depicted in Fig. 4 reflect the virus particles produced by the infected cell, because the virus preparations had undergone treatment with the lipid solvent Freon. Since all three types of viral proteins were present in this Freon-purified material (Fig. 3), they must represent structural proteins of the virus. Even though these proteins must be part of the viral architecture, none of them seems to be exposed at the surface of the virus-like particles, since they could not be labeled with virus-specific antibodies and immunogold. In spite of the absence of a virus-specific marker, these particles may still be considered as virions if one assumes that the outermost layer consists of cellular material which is devoid of virus-specific proteins. In accordance with this interpretation are the findings that convalescent sera collected after natural or experimental infections do not have any neutralizing capacity (Narayan et al. 1983; Carbone et al. 1987).

Analysis of the genome enclosed in these particles favours the concept that BDV is a negative-stranded RNA virus as proposed recently (Briese et al. 1992). The data obtained in our study do not exclude the possibility that additional genomic RNA species exist as part of a segmented genome. It is still puzzling that BDV-specific mRNA exists as overlapping subgenomic RNA species (Richt et al. 1991; Pyper et al. 1993) comparable to the positive-stranded coronaviruses (Holmes 1990), a type of RNA expression which is not characteristic for any negative-stranded RNA virus.

Acknowledgement. We would like to thank Drs. R. Rott and O. Narayan for their continuous interest and support and Dr. B. Boschek for critically reading the manuscript. We also would like to thank H. Will, E. Gottfried and K. Haberzettl for excellent technical assistance. H. B., S. H., J. A. R. and K. W. were supported by the Deutsche Forschungsgemeinschaft (DFG, Forschergruppe "Pathogenitätsmechanismen von Viren"), Bundesministerium für Forschung und Technologie (BMFT) and the Hertie-Stiftung.

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