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JVM 00879

Rapid isolation of morbillivirus nucleocapsid for genomic RNA cDNA cloning and the production of specific core protein antisera

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(Accepted 26 January 1989)

Summary

A procedure is described for the rapid isolation of canine distemper virus nucleocapsid, free from contaminating viral non-core and host cellular proteins. Nucleocapsid isolated in this manner is amenable to ultrastructural evaluation, protein isolation for the production of monospecific hyperimmune serum, and genomic RNA isolation for cDNA cloning.

Nucleocapsid (NC) and a defective NC variant (Df-NC) isolated from 5.5×10^7 Vero cells infected with Ond-CDV is readily visualized on cesium gradients. The calculated density for NC is 1.2976 ± 0.0033 g/ml and 1.2458 ± 0.0056 g/ml for Df-NC. Ultrastructurally, NC appears as long uninterrupted strands, 1.6 ± 0.1 μ m in length, 21.2 ± 1.7 nm in diameter, with well defined capsid subunits. Df-NC are truncated with a uniform length of 85.8 ± 7.1 nm and a 24.5 ± 1.3 nm diameter. A total of 2.1 ± 0.2 μ of NC protein is obtained for every 1×10^6 cells infected; 89.7% of this mass is represented by a 61 kDa protein (N), 8.4% by a 75 kDa protein (P), and 1.9% by a 160-200 kDa protein (L), which is in agreement with the NC constituency of other paramyxoviruses.

Viral N and P proteins, purified by 7.5% SDS-PAGE, were used in the production of hyperimmune serum. Specificity was demonstrated by Western blot analysis. Both antisera were capable of detecting viral antigen in persistently and lytically CDV infected cells by indirect immunofluorescence. A single high molecular weight species of nucleic acid was isolated from purified nucleocapsids com-

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patible with a 14.6 kb morbillivirus genome. Although the efficiency of RNA extraction from purified NC was low (14.2%), sufficient RNA was obtained for gel analysis and the establishment of genomic RNA cDNA clones.

Isolation; Morbillivirus nucleocapsid; cDNA cloning; Specific antiserum

Introduction

RNA viruses with predilection for central nervous tissue are well recognized for their ability to undergo persistence or latency. In the course of such persistence, chronic demyelinating neurological disease may ensue. Such is the case for murine coronavirus (Sorenson et al., 1987; Virelizier et al., 1975), Theiler's murine encephalomyelitis virus (Dal Canto and Lipton, 1979), Semliki Forest virus (Sheahan et al., 1981), measles virus (MV) (ter Meulen and Carter, 1984) and canine distemper virus (CDV) (McCullough et al., 1974). This is well illustrated in the case of the paramyxoviruses by the disease entities subacute sclerosing panencephalitis (SSPE) and old dog encephalitis (ODE), caused by MV and CDV, respectively. An understanding of mechanisms by which persistence is manifested in these systems would provide valuable insights into a fundamental aspect of RNA virus biology and the pathobiology of an important group of neurological diseases.

The evolution of the persistent phenotype from the lytic parental virus is believed to be mediated by virus genomic mutation, which occurs with high frequency in RNA viruses (Holland et al., 1982). These mutations may be manifested as altered patterns of viral gene expression and may be defined by assessing qualitative and quantitative differences in the level of specific viral gene transcription or translocation. To this end, viral gene-specific cDNA probes employed in hybridization assays are used to assess transcriptional activity of a particular gene. This information, in combination with immunocytochemical or immunoadsorption assays employing the appropriate monospecific antibodies, may determine translational levels of the corresponding viral gene transcripts. Recent evidence suggests that it is the initial alteration of major core protein gene expression (N and P in the case of paramyxoviruses) which underlie the establishment of persistence. This is supported by the fundamental role that these proteins play in regulation of viral gene transcription (Kolakofsky et al., 1982; Carlson et al., 1985) and function in the viral polymerase complex (Chinchar and Portner, 1981; Deshpande and Portner, 1985). Alterations of these proteins are associated with persistence (Sheshberadaran et al., 1985), and the persistence is characterized by suppressed levels of viral gene transcription and the abnormally high production of read-through transcripts (Cattaneo et al., 1987). In addition, unique ultrastructural paramyxoviral nucleocapsid variants have been associated with persistent viral infections (Rozenblatt et al., 1979). Thus, examination of N and P gene expression in persistent paramyxoviral infection is warranted and requires viral cDNA clones and core protein antibodies for use as probes.

This paper describes a technique for the rapid isolation of high yields of intact CDV-NC, free from contaminating viral non-core or host cellular proteins. Nucleocapsids isolated in this manner are amenable to ultrastructural evaluation, isolation of intact genomic RNA suitable for the production of a CDV cDNA genomic clone bank, and gel purification of high yields of both N and P proteins suitable for the production of monospecific antisera. In addition, this method allows recovery of Df-NC, making possible a biochemical and functional analysis of these structures as well.

Materials and Methods

Virus harvest

Vero cells in the log phase of growth were infected in suspension at a multiplicity of infection of 0.01 with the Onderstepoort strain of CDV (Ond-CDV). Virus-infected cells were seeded into 150 cm² tissue culture flasks containing Dulbecco's minimum essential medium, 10% fetal calf serum, and incubated at 37°C until cytopathic effect was pronounced (approximately 22.5 h post infection). At this point, the media was made 1.0 mM phenylmethylsulfonyl fluoride (PMSF) and 0.23 trypsin inhibitor units (TIU)/ml Aprotinin. The cell monolayer was disrupted by scraping and cell-associated virus and cytoplasmic NC released by two successive freeze-thaw cycles. Cellular debris was removed by centrifugation at 200 × g for 10 min at 4°C and an aliquot removed for virus titration.

Nucleocapsid isolation

All steps were carried out on ice or at 4°C. The clarified medium was made with 2% Triton-X 100 to release viral NC from intact virions and to disperse macromolecular aggregates held together by hydrophobic interactions. A high speed clarification of the media was then performed (10000 × g, 4°C, for 20 min). Remaining material sedimentable at 100000 × g was recovered by centrifugation at 4°C for 1.5 h and the NC-containing pellets resuspended in a final volume of 1.0 ml gradient buffer (25 mM Tris-HCl, 50 mM NaCl, 2 mM EDTA disodium salt, 0.2% w/v sodium dodecyl sarcosinate, pH = 7.4). The resuspended pellets were briefly vortexed and incubated for 12 h on ice. The sample was then loaded onto a step-gradient formed in a 14 × 19 mm Ultraclear^R (Beckman) centrifuge tube. Steps were constituted in gradient buffer and consisted of 5% w/w sucrose (3.5 ml), 25% w/w CsCl (3.0 ml), 30% w/w CsCl (3.0 ml) and 40% w/w CsCl (1.5 ml). Isopycnic banding of nucleocapsid was achieved by centrifugation at 170000 × g at 4°C for 2.0 h. Visualized bands were harvested by needle aspiration through the wall of the tube and refractive indices (RI) of samples measured at 25°C. Sample density was computed from the refractive index using the relationship described by Bruner and Vinograd (1965); density at 25°C = (10.2402) (refractive index at 25°C) - 12.6483 for densities between 1.00 and 1.38 g/ml.

Ultrastructural evaluation of band material

A fraction of the gradient sample was spotted onto 0.05 μm (pore size) dialysis filters (Millipore Corp., Bedford Massachusetts 01730) floated onto 500 ml of distilled deionized water. Following dialysis at 4°C for 1.0 h, the sample was mixed with an equal volume of 3% w/v phosphotungstic acid (pH = 6.9), 1% w/v sucrose and spotted onto 300-mesh formvar coated, carbonized copper grids (Ladd Research Industries, Inc., Burlington, VT 05402). Air-dried specimens were examined using a Phillips 300 transmission electron microscope.

Protein analysis

Gradient samples were dialyzed extensively against 100 mM ammonium bicarbonate (pH = 8.0), containing 0.01% sodium azide, followed by dialysis against 100 mM ammonium bicarbonate (pH = 8.0) at 4°C. Lyophilized samples were resuspended in TNE buffer (0.01 M Tris-HCl, pH = 8.0, 0.01 M NaCl, 0.01 M EDTA disodium salt).

Samples were denatured utilizing an SDS-reducing buffer system and separated electrophoretically through 12% polyacrylamide gels according to the method of Laemmli (1970). Gels were stained for 12 h in a 0.1% Coomassie blue staining solution (0.1% w/v Coomassie blue, 40% methanol, 10% acetic acid) followed by destaining in 40% methanol, 10% acetic acid. A molecular weight assignment was given to each protein band based upon a correlation of migratory distance to a linear regression equation relating mass to migratory distance. The latter was based on the migration of a series of pre-stained molecular weight standards ranging from 12.4 to 95.5 kDa (Diversified Biotech, Newton Centre, MA 02159). Proteins were quantitated by comparing the optical density of each Coomassie stained protein band to that of known dilutions of stained bovine serum albumin (BSA) using a laser densitometer. Densitometer readings were quantitated using a Gelscan™ computer program as previously described (Oglesbee, 1986). Based on these quantitations, a linear regression equation was established relating optical density of a Coomassie stained protein band to the mass of protein BSA present. Silver staining of gels was performed to detect the presence of minor protein bands. Coomassie stained gels were extensively destained in 40% methanol, 10% acetic acid. Silver staining was performed using a commercial system (BioRad Laboratories) based on the method of Merrill et al. (1981).

Production of monospecific hyperimmune serum

Specific NC proteins were separated by SDS-PAGE, stained, and the protein content of band material quantitated as described above. The N and P protein bands were excised and a crushed gel suspension of each created in a small volume of distilled water. Suspensions were emulsified in Freund's complete adjuvant (Life technologies, Inc.) and injected intradermally into adult goats. Subsequent immunizations were performed at 2-week intervals and employed gel suspensions

emulsified in Freund's incomplete adjuvant. Serum was harvested prior to each immunization. The level and specificity of reactivity of the sera for CDV-NC proteins was determined by using a 1:50 dilution of the sera as probe in Western blot analyses of capsid proteins. Briefly, NC proteins separated by 7.5% SDS-PAGE were electroblotted onto nitrocellulose membranes under conditions recommended for use with the Trans-blotTM electrophoretic transfer apparatus (Biorad Laboratories, Richmond, CA 94804). Air-dried nitrocellulose strips were blocked for 12 h at room temperature in Dulbecco's phosphate buffered saline (PBS) (Life Technologies Inc., Grand Island, NY 14072) containing 20% porcine serum. Incubation with the hyperimmune serum diluted in PBS, 1% BSA, 1% Tween-20 (polyoxyethylene sorbitan monolaurate) was performed for 1.5 h at room temperature followed by incubation with a swine anti-goat IgG alkaline phosphatase conjugate (Boehringer Mannheim Biochemicals, Indianapolis, IN 46250) at 37°C for 1 h. Strips were washed in PBS, 1% BSA, 1% Tween-20 and developed using a 5-bromo-4-chloro-3-indolyl-phosphate (BCIP)/nitroblue tetrazolium (NBT) substrate system (Kirkegaard and Perry Laboratories, Gaithersburg, MD 20897). In addition, serum samples were employed in viral neutralization assays according to the procedure of Confer et al. (1975) to test for the presence of cross-reactivity to viral envelope glycoproteins.

Immunocytochemical staining of persistently infected cells

The ability of each antiserum to detect viral proteins within CDV persistently infected cells by indirect immunofluorescence was assessed. RCDV-CCL64 is a CDV persistently infected cell line obtained by cocultivation of the mink lung cell line CCL64 with peripheral blood lymphocytes derived from a CDV viremic feral racoon (Axthelm et al., 1987). Cell monolayers were fixed using methanol-acetic acid (95:5 v/v ratio) fixation for 5 min at -20°C. A 1:10 dilution of specific antiserum was then used as a source of primary antibody in an indirect immunofluorescence assay as described by Krakowka et al. (1973). Fluorescein-conjugated affinity purified rabbit anti-goat IgG was the secondary antibody (Cappel, Cooper Biomedical, Inc., Malvern, PA 19355). Monolayers of Ond-CDV infected VERO cells were similarly prepared to serve as positive controls.

Genomic RNA isolation and cDNA cloning

Nucleocapsid fractions were desalted by diluting with TNE buffer and pelleting at $100\,000 \times g$ for 1.5 h at 4°C. Pellets were resuspended by heating to 37°C in a small volume of 2% SDS in TNE buffer. Genomic RNA was then extracted using 3 successive phenol:chloroform:isoamyl alcohol (25:24:1) extractions, followed by a single chloroform:isoamyl alcohol (24:1) extraction. To examine the integrity of the isolated RNA, the migration of 1 µg of isolated genome was compared to that of a 0.24-9.5 kb RNA ladder (Bethesda Research Laboratories, Gaithersburg, MD 20877) separated by 1.0% agarose gel electrophoresis under formaldehyde-formamide denaturation conditions (Miller, 1987). Sample was visualized following

staining with ethidium bromide and the mass of the RNA band determined by regression analysis as described above. To detect the presence of minor nucleic acid species not detected by ethidium bromide staining, 0.2 pmol of genomic RNA (based on 0.202 pmol/ μ g) was end-labeled with 32 P by the kinase exchange reaction described by Berkner and Folk (1977) using gamma- 32 P-dATP and T4 polynucleotide kinase (New England Biolabs, Beverly, MA 01915). The migration of the radiolabelled product was compared to that of similarly radiolabelled 0.24–9.5 kb RNA ladder subjected to the same electrophoretic conditions as before.

For the production of a genomic RNA cDNA library, a modification of the Gubler and Hoffman (1983) method of cDNA synthesis was employed using a commercially available system (Amersham Corporation, Arlington Heights, IL 60005). Calf thymus DNA oligomers, six base pairs in length, mediated random base priming of the first strand reaction (Rice and Strauss, 1981). Size selected cDNAs were blunt end ligated into the *Sma*I site of pUC 9 and the recombinants used in the transformation of competent *E. coli* DH5-alpha cells (Bethesda Research Laboratories). Transformants bearing recombinant plasmids were identified on the basis of differential complementational activation of the beta-galactosidase gene when grown on selective media (ampicillin, 100 μ g/ml) containing the colorimetric substrate X-gal. Size determinations of linearized plasmids were made based on electrophoretic migration in 0.7% agarose gels. Definitive virus specificity of selected inserts was based on differential reactivity of insert, radiolabelled with 32 P by nick translation, to blots of virus infected and uninfected Vero cells according to a previously described dot-blot hybridization procedure (Oglesbee et al., 1986). To identify N gene specific sequences present in the genomic library, blots of recombinant plasmid were incubated with a previously obtained CDV N gene specific cDNA clone (Oglesbee et al., 1986), radiolabelled by nick translation, using the aforementioned hybridization and post hybridization wash conditions.

Results

Nucleocapsid isolation

NC was isolated from 5.5×10^7 infected cells. A broad and dense band, consisting of a finely globular particulate material, was readily visualized in the upper portion of the 30% cesium step of the density gradients (Fig. 1). The average refractive index of sampled band material was 1.3619 ± 0.0006 ($N = 11$), which corresponds to an average density of 1.2976 ± 0.0033 g/ml. A less well defined homogeneous band of similar thickness was identified higher in the gradients. The average refractive index of samples from this level was 1.3568 ± 0.0006 ($N = 7$), which corresponds to a density of 1.2458 ± 0.0056 g/ml. Increased centrifugation times did not alter band positions, indicating that the banded material had sedimented to a position of equivalent density.

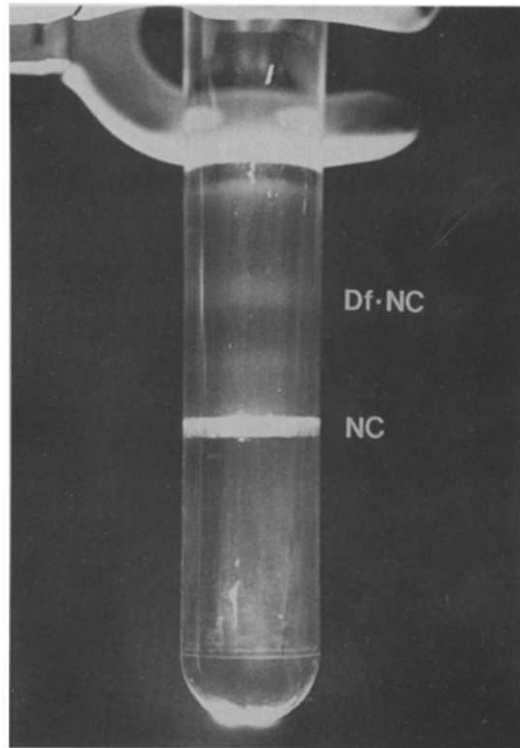


Fig. 1. Cesium step-gradient illustrating isopycnic banding positions of CDV NC. The well-defined NC band and the less dense defective (Df)-NC band are readily visualized in the superficial portion of the 30% w/w CsCl step (density at 25°C = 1.2976 ± 0.0033 g/ml) and the 25% w/w CsCl step (density at 25°C = 1.2460 ± 0.0046 g/ml), respectively.

Ultrastructural examination of band material

Ultrastructural evaluation of the dense band material revealed characteristic paramyxoviral nucleocapsid strands consisting of a double row of well-defined capsid subunits (Fig. 2). The average length of the NC strands was 1.6 ± 0.1 μm and the average outer diameter was 21.2 ± 1.7 nm. Evaluation of material from the lighter band revealed similar structures, except that they were truncated to a uniform length (85.8 ± 7.1 nm). The width of these defective nucleocapsid structures (Df-NC) was 24.5 ± 1.3 nm.

Protein analysis

Electrophoretic analysis of proteins present in NC preparations demonstrated the presence of two major and one minor species (Fig. 3). A linear regression equation relating gel migratory distance to relative mass was established with a correlation coefficient of 0.9995. Using this equation, assignments of 61 and 75 kDa

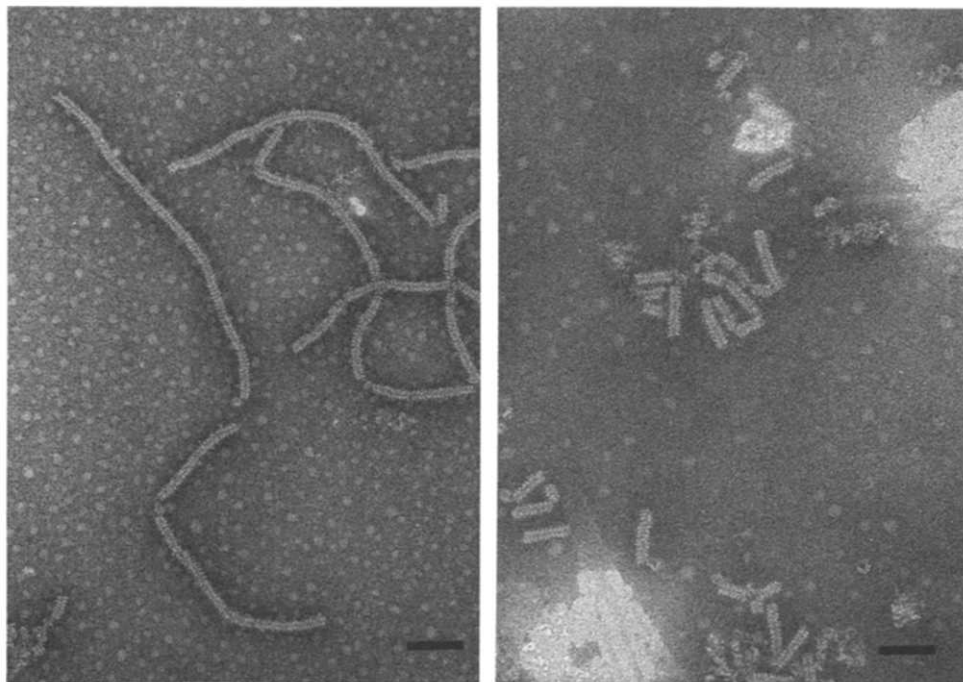


Fig. 2. Transmission electron micrographs of negatively stained nucleocapsid preparations. Left panel: long uninterrupted NC strand, $1.6 \pm 0.1 \mu\text{m}$ in length, $21.2 \pm 1.7 \text{ nm}$ in diameter, with well-defined capsid subunits. Bar = 131 nm. Right panel: truncated Df-NC structures, $85.8 \pm 7.1 \text{ nm}$ in length, with a $24.5 \pm 1.3 \text{ nm}$ diameter. Bar = 111 nm.

were given to the two major protein species, corresponding to published values for CDV N and P proteins, respectively (Rima et al., 1986). The third minor species was a high molecular weight protein, migrating in a position compatible with the expected location of the 160–200 kDa distemper virus L protein (Rima et al., 1986). Based on quantitation of Coomassie staining intensity of viral protein bands compared to that of known quantities of similarly banded and stained BSA, an average of $2.1 \pm 0.2 \mu\text{g}$ of total NC protein was obtained for every 1×10^6 cells infected. This corresponded to an average of $3.63 \times 10^6 \pm 6.7 \times 10^5$ infectious units derived from every 1×10^6 infected cells. If each infectious unit represents a single encapsidated genome, and the morbillivirus RNA genome, with a mass of 4.5×10^6 (Baczko et al., 1983), represents 5% of the total NC mass (Waters and Buswell, 1974), then it may be established that non-infectious nucleocapsid is contributing approximately 99.98% of the NC protein yield. The N protein represents an average of 89.7% of the total yield, with P contributing 8.4% and the putative L protein contributing 1.9%. When protease inhibitors were not used in the isolation procedure, the total yield of intact core protein was reduced by as much as 56% and resulted in disproportionate decreases in P and L relative to N protein (data not shown). In the latter, lower molecular weight bands were evident on ac-

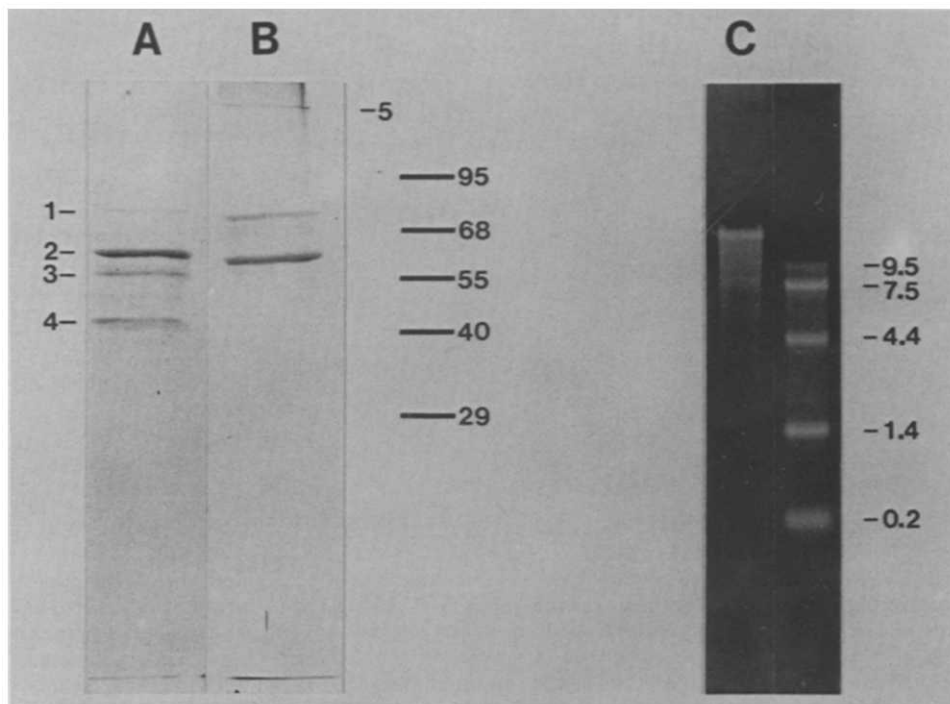


Fig. 3. Silver-stained 12% acrylamide gel of NC proteins, isolated in the absence and presence of protease inhibitors, and an ethidium stained 1.0% formamide/formaldehyde agarose gel of RNA isolated from NC. Lane A: 1.0 μg of total NC protein, isolated in the absence of protease inhibitors. Small amounts of undegraded P protein is evident (band No. 1), while the N protein band (No. 2) as well as N protein degradation product bands (Nos. 3, 4) are readily apparent. Signal from a high molecular weight protein (i.e., L) is not present. Lane B: 1.0 μg of total NC protein, isolated in the presence of protease inhibitors. The P protein is more apparent and a high molecular band (No. 5) is identified in a migratory position compatible with the L protein. Positions of molecular weight standards are indicated to the right of lane B, with the assignments given in kilodaltons. Lane C: 0.8 μg of RNA isolated from NC. A single high molecular weight species is present, in a position compatible with the 14.6 kb morbillivirus genome. Some smearing of lower molecular weight degradation products is evident. Size standards are present in the adjacent lane with the assignments given in kilobases.

rylamide gels of the isolated NC (Fig. 3) and ranged in size from 55 to 44 kDa. This corresponds to published values for the mass of major N protein degradation products (Croce et al., 1980). Silver staining of NC protein gels where NC was isolated in the presence of protease inhibitors did not reveal degradation products or other minor bands even when 1.5 μg of NC protein was examined.

Production of monospecific hyperimmune serum

Three immunizations with N protein were employed, consisting of 650, 685, and 657 μg of protein per injection. Slight specific reactivity of the resultant hyperimmune serum to the N protein on Western blots was demonstrated at the time of

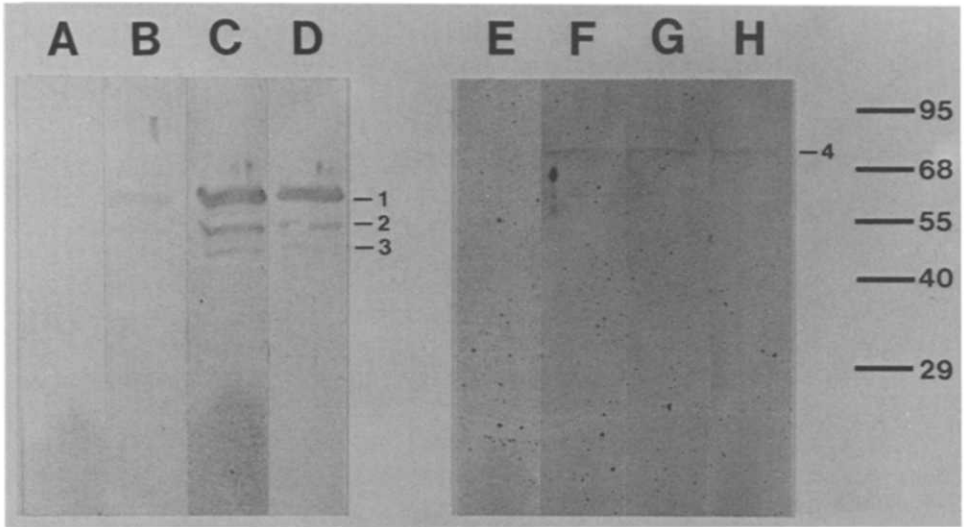


Fig. 4. Western blot analysis of CDV core proteins, separated by 12% polyacrylamide gel electrophoresis, using N protein hyperimmune serum (lanes A–D, 1.2 μg of total core protein per lane) and P protein hyperimmune serum (lanes E–H, 48 ng of total core protein per lane). Positions of molecular weight standards are indicated at the right of each set of blots and the corresponding mass indicated in kilodaltons. Lane A: pre-bleed serum. Lane B: serum collected the 2nd week following the initial immunization with N protein. Slight reactivity to the N protein (band No. 1) is evident. Lanes C, D: serum collected the 4th and 6th week post initial N-protein immunization, respectively. Reactivity to the N protein is increased. In addition, N protein degradation product bands (Nos. 2 and 3) are readily evident. Reactivity to the 0.1 μg of P protein present per lane is lacking. Lane E: pre-bleed serum. Lanes F–H: serum collected 3, 5, and 7 weeks post initial immunization with P protein. The hyperimmune serum demonstrates an early and constant level of reactivity for P protein (band No. 4), where 4 ng is detected. Cross-reactivity to N protein, present in a 10-fold excess of P, is lacking.

the second immunization. Strong specific reactivity was obtained by the third immunization and was maintained for the following four weeks in which serum was collected. When 1.2 μg of total core protein was probed, the aforementioned 55 and 44 kDa bands were detected in addition to the parental 61 kDa N protein band (Fig. 4).

For the production of P protein hyperimmune serum, two immunizations of 24 μg each were used. Strong specific reactivity for the P protein was demonstrated following the second immunization, in which as little as 4 ng of P protein was detected (Fig. 4). When 1.2 μg of total core protein was employed in the Western blot analysis, additional lower molecular weight bands were detected which did not correspond to previously detected N protein or N protein degradation products. They were interpreted to represent P protein degradation products. Viral neutralizing activity was not demonstrated with either N or P hyperimmune serum.

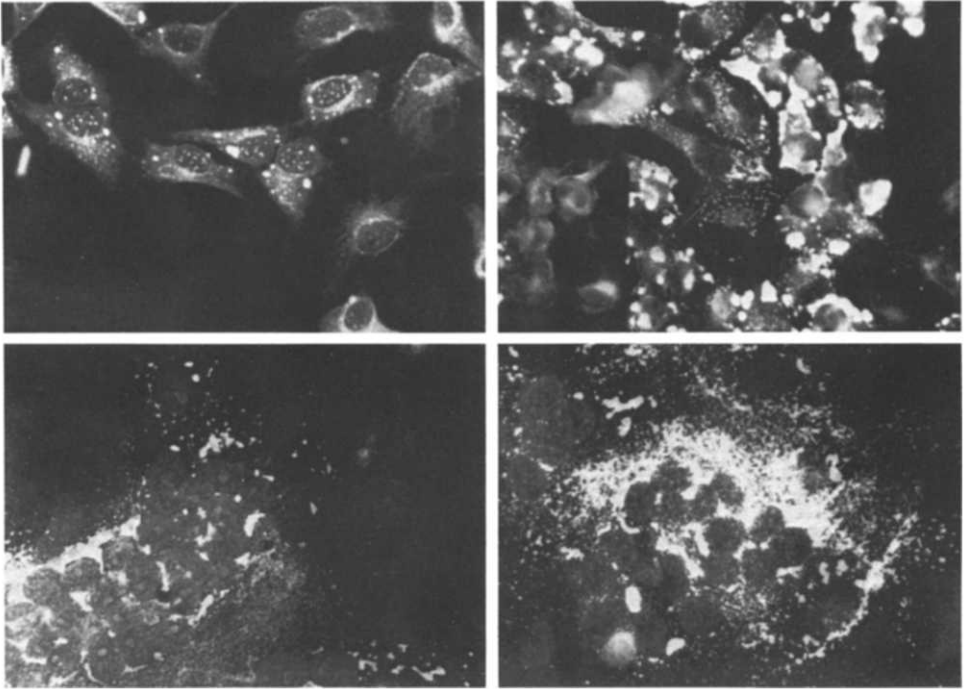


Fig. 5. Indirect immunofluorescence of persistently infected cells (RCDV-CCL64) and lytically infected cells (Ond-CDV infected VERO cells) using N and P hyperimmune sera. Top left panel: N-protein hyperimmune serum yields small globular foci of fluorescence, present at low density, over approximately 30% of persistently infected cells. Top right panel: With P-protein hyperimmune serum, antigen is detected in 80–90% of persistently infected cells, with frequently coalescing globular foci of fluorescence present at a high concentration. Bottom left panel: N-protein hyperimmune serum detects antigen associated with a virus-induced syncytium in lytically infected cells. Fluorescence is of low intensity. Bottom right panel: P-protein hyperimmune serum also detects viral antigen within a syncytium of lytically infected cells.

Immunocytochemical staining of persistently and lytically infected cells

Both N and P antisera were capable of detecting viral antigen in persistently infected cells, though with variable efficacy. When RCDV-CCL64 cells were probed with canine CDV convalescent serum, approximately 90% of the cells were positive for cytoplasmic viral antigen. N protein hyperimmune serum, however, detected viral antigen in only 30% of the cells (Fig. 5). Positive staining consisted of a fine stippling to small globular foci of cytoplasmic fluorescence. The cytoplasmic density of such staining was low. Small foci of fluorescence were also detected within nuclei of infected cells. In contrast, anti-P sera detected viral antigen in 85–90% of the cells. Fluorescence was occasionally characterized by a fine stippling, but more often was in the form of coalescing cytoplasmic globular foci of high density. Staining was restricted to the cytoplasm. Both antisera were capable

of detecting cytoplasmic viral antigen within foci of Ond-CDV infected VERO cells, although the staining intensity with the P antisera was greater. With both, no reactivity to uninfected control cells (CCL64 and VERO) was demonstrated. Preimmune sera were also negative.

Genomic RNA isolation and cDNA cloning

RNase sensitive high molecular weight nucleic acid was isolated from purified nucleocapsid as demonstrated by formaldehyde-formamide denaturing 1.0% agarose gels (Fig. 3). The migratory position of the predominant ethidium-stained band is compatible with a 14.6 morbillivirus genome (Udem and Cook, 1984). Some smearing below the major band is evident, and is interpreted to represent genomic RNA degradation products. Gel electrophoresis of radiolabelled nucleic acid followed by autoradiographic exposure of X-ray film did not reveal the presence of additional minor nucleic acid species.

Purified genomic RNA (1.5 μ g) was used for cDNA cloning. Based on incorporation of alpha-³²P-dCTP, it was determined that 5% of the genomic template was transcribed in the first strand reaction and 100% of the first strand reaction product was converted into cDNA by the second strand reaction. Of the 56.4 ng of double stranded cDNA obtained, 85% (47.9 ng) was larger than 500 base pairs in length with an average size of 1500 bp. This material was size-selected on a 1% agarose gel and the product blunt end ligated into the *Sma*I site of pUC 9. Twenty transformants were selected for further characterization, bearing inserts with an average size of 1.75 kbp. Fifty percent of these were shown to be virus specific, 4 of which demonstrated positive reactivity with an N gene specific radiolabelled cDNA probe in a dot-blot hybridization of recombinant plasmid.

Discussion

The procedure for NC isolation is a modification of a technique described by Udem and Cook (1984). The purpose of the modified isolation scheme is to minimize the number of manipulations required in order to maximize yield of recovered intact nucleocapsid while maintaining purity of the isolated product. In addition to examining the applicability of such a scheme to CDV NC isolation, a single approach to obtaining a product amenable to ultrastructural examination, core protein isolation, and genomic RNA isolation was desired.

Several isolation techniques have been described in which ultrastructure of the resultant NC has been addressed (Waters and Bussell, 1974; Bussell et al., 1974; Robbins and Abbott-Smith, 1985). Such an assessment was not performed by Udem and Cook (1984). In each, significant NC fragmentation or loss was encountered. This may be the result of the method of cell disruption (Waters and Bussell, 1974), mechanical disruption resulting from increased manipulation (Robbins and Abbott-Smith, 1985) or protease degradation of core proteins (Yeh and Iwasaki, 1972; Waters and Bussell, 1974). Fragmentation precludes critical evaluation of nucleocapsid

capsid length, which is required for the identification of defective NCs. In addition, mechanical fragmentation and loss of capsid proteins via protease degradation may result in fragmentation/loss of genomic RNA since only encapsidated genomic RNA is resistant to nuclease degradation (Baczko et al., 1983). Yeh and Iwasaki (1972) employed BSA as a competitive protease inhibitor while Robbins and Abbott-Smith (1985) used a rapid isolation technique to minimize the effects of protease activity. However, the latter still encountered less than desirable NC ultrastructural results and the integrity of the genomic RNA was not evaluated.

Our technique combines rapid isolation with the use of the protease inhibitors PMSF and Aprotinin. NC isolation in media containing high levels of fetal calf serum (10%) probably also assists protease inhibition. The result is the isolation of intact NC structures, attested by a mean length slightly in excess of that reported for other paramyxoviral nucleocapsids (Finch and Gibbs, 1970; Kingsbury, 1972) and the isolation of predominantly intact genomic RNA. Only minimal strand breakage is observed on electron micrographs which is the result of air-drying on the grid surface (unpublished observation).

By decreasing NC fragmentation, the resolution of a well-defined gradient band containing NC structures of uniformly truncated length is achieved. Increased NC fragmentation produces particles of heterogeneous length. Smaller fragments may have a sufficiently low sedimentation coefficient that they do not reach an isopycnic gradient position during centrifugation, thereby obscuring the less dense Df-NC bands. The identity of the Df-NC as a unique NC subset is supported by their lesser isopycnic density and uniform length. Further characterization, including correlation to functional defective interfering particles, remains to be accomplished.

The yield of an isolation procedure is an important consideration for paramyxoviruses due to the difficulty in obtaining virus in large quantity (Baczko et al., 1983). It is for this reason that cytoplasmic nucleocapsid is used, as there is a very low level of nucleocapsid incorporation into complete virions with these viruses. For MV, this has been shown to be less than 5% (Moore et al., 1978). The contribution of infectious virus to the total nucleocapsid yield in our hands is much less. If each infectious unit represents a single encapsidated genome, and since morbillivirus RNA genome, with a mass of 4.5×10^6 (Baczko et al., 1983), represents 5% of the total NC mass (Waters and Bussell, 1974), then it may be calculated that non-infectious NC contributes approximately 99.98% of the NC protein yield. This could be due to a greater contribution by cytoplasmic nucleocapsid in addition to interference with infectivity of intact virions by the protease inhibitors.

Detergents, particularly Sarcosyl, were employed by Udem and Cook (1984) to increase the yield of recovered NC. However, they also suffered a concomitant loss of P and L proteins, which they attributed to dissociation facilitated by the presence of detergent. Utilizing our technique, we recovered substantial amounts of these proteins in the absence of contaminating host cellular material. The constituency of the NC obtained (89.7% N, 8.4% P, 1.9% L) is in agreement with NC composition data derived from well characterized systems such as Sendai virus (88.7% NP, 10.2% P and 1.0% L protein) (Lamb et al., 1976). This suggests that

it is not detergents that are effecting loss of core proteins, but rather lack of protection from proteases or increased exposure to the high salt conditions of cesium gradients with more involved purification schemes. The enhanced loss of P and L proteins in the absence of protease inhibitors and the effect on total protein yield has been demonstrated. Thus, our modified procedure is applicable to examination of NC composition as well as a preparative technique.

The use of protease inhibitors is also important if gel purification is to be adequate for the isolation of core proteins of sufficient purity to produce monospecific antisera. If P-origin antigenic fragments were present in high quantity, P-derived antigen would likely be present in N protein bands in sufficient quantity to induce anti-P antibodies. This would seem a likely possibility based on the apparent propensity for P to undergo degradation. In addition to demonstrating the specificity of the N protein hyperimmune serum by Western blot analysis, in which high levels of P antigen were not detected, immunocytochemical data illustrated the specificity *in situ*. Reactivity of N hyperimmune serum to intranuclear antigen in RCDV-CCL64 cells, with lack of such reactivity by the P hyperimmune serum, is compatible with the differential cellular distribution of these antigens in paramyxoviral infected cells (Norrby et al., 1982).

Using repeated phenol:chloroform:isoamyl alcohol extractions, followed by a chloroform:isoamyl alcohol extraction, we calculated a 14.2% recovery of genomic RNA from NC starting material. This determination was based on the total core protein isolated from every 1×10^6 infected cells, the fact that the RNA genome represents 5% of the total NC mass, and the amount of 260/280 nm absorbable material isolated from NC samples. Attempts to increase this recovery by including prior pronase digestion (Baczko et al., 1983) failed to increase the yield and increased the degradation of RNA (unpublished observation). Comparing the RNA yield (based on total number of cells infected) of Baczko et al. (1983) to that of Udem and Cook (1984), in which such digestion was omitted, it would appear that pronase digestion does not increase yields significantly. In either event, using our extraction scheme, ample RNA may be recovered for gel analysis and cDNA cloning.

The occurrence of non-viral cDNAs in the genomic bank was unexpected, since we did not demonstrate contaminating nucleic acids on ethidium-stained gels or gels of radiolabelled extracts. Baczko et al. (1983), however, describe the recovery of trace amounts of high molecular weight DNAs and small RNAs (likely ribosomal in origin) which have a tendency to co-purify with MV nucleocapsids. This group suggests that non-specific interactions between the contaminating nucleic acids and the viral ribonucleoprotein complexes occur, and that attempts to remove these products by increased stringency of NC purification only results in further reductions of the genomic RNA yield. The reason for the high representation of non-viral cDNAs in our bank is likely due to the use of random base oligomers of eukaryotic origin as primer for the first strand cDNA reaction. These primers would be expected to preferentially prime eukaryotic sequences, thereby overrepresenting host sequences in the resultant cDNA product. This preferential priming might also explain the low efficiency of the first strand reaction. To avoid the

high representation of host sequences and to increase the first strand reaction yield, random base priming with viral cDNA oligomers may be used. The latter approach is being employed presently in this laboratory.

The ability to generate high yields of pure nucleocapsid, amenable to ultrastructural evaluation, quantitative and qualitative protein analysis/recovery, and nucleic acid recovery in a single procedure is important in terms of maximizing the utilization of scarce starting material. Our approach assures that sufficient substrate of the appropriate purity and integrity may be obtained for the generation of probes necessary for the characterization of transcriptional and translational activity in this paramyxoviral system. It also allows for more carefully controlled analyses of specific nucleocapsid preparations, in that ultrastructural and biochemical analyses may utilize the same starting material. The potential for such a methodology to examine further biochemical and functional properties of Df-NC structures should prove rewarding.

Acknowledgements

This work was supported by the State of Ohio Canine Research Fund and grant RO1-14821, N.I.H., P.H.S. Stipend support for the author was provided by the DuPont Corporation and an N.I.H., P.H.S., National Research Service Award F32-107811.

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