Purification and biochemical characterization of chicken anaemia agent

Daniel Todd,1* Julie L. Creelan,1 Dermot P. Mackie,1 Frazer Rixon2 and M. Stewart McNulty1

1Virology Section, Veterinary Research Laboratories, Stormont, Belfast BT4 3SD and 2Institute of Virology, Church Street, Glasgow G11 5JR, U.K.

Chicken anaemia agent (CAA) was purified using differential centrifugation and successive cycles of equilibrium density gradient centrifugation using sucrose and CsCl. The purification method was dependent on the use of an antigen-detecting ELISA based on a CAA-specific monoclonal antibody. Virus particles banded at a density of 1.33 to 1.34 g/ml in CsCl and measured 23.5 ± 0.8 nm in diameter. Purified preparations contained one major polypeptide (M, 50 000) and a single-stranded, circular DNA (2.3 kb). CAA shares some of the biochemical characteristics possessed by porcine circovirus and the virus associated with psittacine beak and feather disease.

Introduction

Chicken anaemia agent (CAA), which causes anaemia, aplasia of the bone marrow and atrophy of the lymphoid organs in susceptible chicks was first isolated in Japan (Yuasa et al., 1979). Serological evidence indicates that CAA infections are common in many parts of the world (Yuasa et al., 1983; McNulty et al., 1989).

The agent can be serially propagated in a transformed chicken lymphoblastoid (MDCC-MSB1) cell line and its infectivity resists heating at 60 °C for 30 min, treatment with chloroform and passes through a 25 nm filter (Yuasa et al., 1987). In CsCl density gradient separations the infectivity was associated with spherical virus particles measuring 19.1 ± 0.2 nm in diameter which banded at a density of 1.35 to 1.36 g/ml (Goryo et al., 1989). Although some of the physicochemical properties of the agent resemble those of parvoviruses, CAA has not yet been fully characterized or classified.

In this paper we describe the purification and biochemical characterization of the virus.

Methods

Cells and virus growth. The Marek’s disease virus-transformed chicken lymphoblastoid (MDCC-MSB1) cell line and the Cuxhaven-1 isolate of CAA were obtained from Dr V. von Bulow, Institut fur Gefuegetrankheiten, Freie Universitat, Berlin, F.R.G. For purification purposes 22 ml suspension cultures (3 x 105 MDCC-MSBI cells/ml) contained in 80 cm2 plastic flasks were infected with 1 ml virus inocula (105 to 106 TCIDs0/ml). Virus growth was checked after 48 h by the indirect immunofluorescence (IIF) technique described by McNulty et al. (1988) and cultures were usually harvested after 72 h. Infectivity titres, expressed as TCID50/ml, were determined by the method of Goryo et al. (1987). Alternatively, infectivity titres of virus samples were determined by growing 10-fold dilutions of virus samples in cluster plate cultures and, following two subcultures of the infected cultures, cells from each culture were checked for CAA infection using IIF. This method in which values were expressed as infectious units/ml was more rapid (5 or 6 days) but 10 to 100-fold less sensitive than the method described by Goryo et al. (1987) which involved up to eight subcultures of infected cells (19 or 20 days).

Enzyme-linked immunosorbent assay. Virus antigen was detected in density gradient fractions using a sandwich ELISA. This test utilized a CAA-specific monoclonal antibody, designated 2A9, which was produced and characterized in this laboratory (M. S. McNulty, D. P. Mackie, D. A. Pollock, J. McNair, D. Todd, K. A. Mawhinney, F. McNeilly & T. J. Connor, unpublished results). Tests were performed using ELISA microtitre plates (Immulon, Dynatech) and comprised four successive addition and incubation steps prior to reaction with enzyme substrate. Each step involved 100 µl volumes/well and was followed by thorough washing cycles using 0.01 M-phosphate, 0.15 M-NaCl, pH 7.2 (PBS) containing 0.05% Tween-20. The four addition and incubation steps were as follows.

(i) Plates were coated overnight at 4°C with the immunoglobulin fraction (10 µg/ml) of 2A9 ascites diluted in 0.05 M-bicarbonate buffer pH 9.5. The immunoglobulin fraction was prepared by precipitation with 50% ammonium sulphate. (ii) Virus samples diluted (1/100) in PT buffer (PBS containing 0.05% Tween-80) were added and incubated for 2 h at 37°C. (iii) Biotinylated CAA-specific (2A9) monoclonal antibody (0.2 µg/ml PT) was added and incubated for 30 min at 37°C. The conjugate was prepared by the method of Hofmann et al. (1982) using the immunoglobulin fraction of ascites fluid and aminohexanoyl biotin succinate (Zymed). (iv) Streptavidin–peroxidase (Amersham) which had been diluted (1/300) in PT buffer was added and incubated for 30 min at room temperature. The enzyme substrate o-phenylenediamine (OPD; 0.4 mg/ml) in 0.05 M-citric acid, 0.1 M-Na2HPO4 (pH 5.0) containing 0.01% H2O2 was incubated for 10 min at 37°C. The reaction was terminated by the addition of 2.5 M-H2SO4 (25 µl) and the absorbance at 492 nm was read using a Titertek Multiskan.

Virus purification. Infected cell lysates (400 ml volumes) which had been stored at −70°C prior to virus purification were thoroughly
sonicated and treated with 0.5% SDS for 30 min at 37°C. Cellular debris was removed by centrifugation at 10000 g for 30 min at 15°C and the supernatant centrifuged at 80000 g for 3 h at 15°C. The crude virus pellet obtained was dispersed with a brief period of sonication in 3 ml TE (pH 8.7) buffer (0.01 M-EDTA, 0.01 M-Tris-HCl), clarified at 2000 g for 5 min and layered onto a 9 ml continuous gradient of 30 to 60% (w/w) sucrose in TE (pH 8.7). Gradients were centrifuged at 80000 g for 17 h at 15°C using a 6 x 14 ml swing-out MSE rotor. Fractions (0.5 ml) were collected from the bottom of the tube through a carefully pierced pin-hole. Aliquots from the fractions were tested immediately for virus antigen by ELISA and subsequently for virus infectivity. Peak fractions (1 to 2 ml) were pooled and semi-purified virus was collected for nucleic acid extraction by centrifugation at 80000 g for 3 h at 15°C. Alternatively, the purification was continued by layering the pooled fractions, after dilution with an equal volume of TE (pH 8.7) onto a discontinuous CsCl gradient containing 1.30 g/ml (4.5 ml) and 1.35 g/ml (4.5 ml) CsCl in TE (pH 8.7). Following centrifugation at 80000 g for 17 h at 15°C, gradients were eluted from the bottom and 0.5 ml fractions collected. Aliquots from each were tested by ELISA and for infectivity. Peak fractions (1 to 1.5 ml) were pooled, dialysed against 11 TE (pH 8.7) and concentrated 10-fold by freeze-drying. On two occasions CsCl fractions containing peak amounts of virus antigen were subjected to a second CsCl density gradient centrifugation step. The densities of the CsCl fractions were determined by refractometry.

Polyacrylamide gel electrophoresis. Samples containing CAA virus were analysed by electrophoresis in gels containing 12.5% polyacrylamide as described by Todd et al. (1987). M, protein standards (Bio-Rad) were included in each analysis. Gels were stained with Coomassie blue R or with silver using the Bio-Rad silver stain kit. In order to stain the CAA polypeptide it was necessary to subject the gel to a second reaction cycle with the silver reagents as recommended by Bio-Rad.

Nucleic acid extraction. Virus samples diluted in TE (pH 8.0) were treated with SDS (1%) and Proteinase K (1 mg/ml) for 2 h at 37°C and extracted successively with phenol, phenol: chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1) followed by three washes with diethyl ether.

Agarose gel electrophoresis. Nucleic acid samples were electrophoresed in neutral 1% agarose gels (11 x 14 x 1 cm) prepared in 0.01 M-EDTA, 0.04 M-Tris, 0.02 M-sodium acetate-acetic acid pH 8.2 at 20 V for 18 h as described previously (Todd et al., 1988). Alternatively, samples, which had been denatured by treatment in 0.3 M-NaOH, 0.02 M-EDTA at 65°C for 30 min, were electrophoresed in gels of similar size prepared in 0.03 M-NaOH, 0.02 M-EDTA at 50 V for 18 h. Following electrophoresis the alkaline gels were washed in H2O for 30 min, neutralized in 0.5 M-Tris–HCl pH 6.8 for 30 min and rinsed in H2O for an additional 2 to 4 h prior to staining with ethidium bromide (1 mg/ml). The HindIII restriction fragments of λ phage DNA were included as M, markers.

Denaturation treatment. Samples of virus DNA (0.1 μg/20 μl TE, pH 8.0) which had been extracted from sucrose density gradient-purified preparations were heated at 100°C for 10 min and incubated at 37°C for 15 min. Following electrophoresis in neutral 1% agarose gels the DNA was Southern-blotted (Southern, 1975) onto Hybond-N (Amersham) using the manufacturer's protocol. The blots were hybridized with 32P-labelled virus DNA probe which was prepared by the oligo-priming method of Feinberg & Vogelstein (1984) using virus DNA fractionated by electrophoresis in low melting point agarose (Gibco-BRL).

Nuclease treatments. Samples of nucleic acid (approximately 0.1 μg) in TE (pH 8.0) which had been extracted from sucrose density gradient-purified virus were separately treated with deoxyribonuclease I (Sigma; 20 units/ml) for 1 h at 37°C in the presence of 0.0025 M-MgCl2, ribonuclease A (Sigma; 1 μg/ml) for 1 h at 37°C in the presence of 0.1 M-NaCl, and 31 nuclease (Amersham; 10 units/ml) for 30 min at 37°C in 0.001 M-Zn2SO4, 0.1 M-sodium acetate-acetic acid pH 4.5. Nuclease acid (0.5 to 1 μg) comprising DNA and RNA extracted from MDCC-MSBI cells and the HindIII fragments of λ phage DNA (0.5 μg) were used in control digests with each of the nuclease. The effects of nuclease treatments were assessed by analysis of the digests using agarose gel electrophoresis followed by Southern blot hybridization using a 32P-labelled virus DNA probe, which had been prepared as described above.

Electron microscopy. Preparations of purified CAA were mounted on carbon-coated grids and negatively stained with 4% ammonium molybdate.

The DNA extracted from virus purified by sucrose density gradient centrifugation was examined by electron microscopy using the method of Kleinschmidt (1968) as described by Rixon et al. (1984). The size of the virus DNA was estimated by incorporating single-stranded φX174 DNA as an internal standard.

Results

Virus purification

Harvests of MDCC-MSBI cells (400 ml volumes) infected with the Cuxhaven-1 isolate of CAA usually contained 104 to 105 infectious units/ml as determined by the modified, IIF-based method. Following SDS treatment and differential centrifugation, suspensions (2 to 3 ml) of the crude virus pellets contained 105 to 107 infectious units/ml. Equilibrium density centrifugation of the virus in 30 to 60% sucrose gradients resulted in the detection by ELISA of one major peak of virus antigen about half-way down the gradient and a minor peak near the top of the gradient. Subsequent infectivity determinations showed that the major peak of virus antigen corresponded to one of virus infectivity (Fig. 1). Omission of the SDS treatment prior to differential centrifugation resulted in a reduction of approximately

![Fig. 1. Equilibrium density centrifugation of CAA in 30 to 60% (w/w) sucrose gradient. Fractions collected from the bottom were tested by ELISA (O, A492). The infectivities of selected fractions are included.](image-url)
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10-fold in the amount of virus antigen detected by ELISA in sucrose gradients.

When peak sucrose gradient fractions (Fig. 1; fractions 10 and 11) were subjected to equilibrium density centrifugation in gradients of CsCl a major peak of ELISA-detectable virus antigen and infectious virus was obtained at a density of 1.33 to 1.34 g/ml (Fig. 2). In two experiments, the CsCl fractions containing peak amounts of virus antigen were re-centrifuged on a second density gradient. Although a single band was recovered at a density of 1.33 to 1.34 g/ml in CsCl, ELISA testing showed that the amount of virus antigen detected was significantly reduced (>80% loss). A second cycle of CsCl density gradient centrifugation was not therefore included in the purification scheme.

Peak CsCl fractions from the first gradient (Fig. 2; fractions 7 and 8) were pooled, dialysed and concentrated. Negative contrast electron microscopy revealed the presence of numerous spherical virus-like particles which measured 23.5 ± 0.8 nm (n = 50) in diameter. Ten well defined peripheral protrusions could be resolved in some of the particles (Fig. 3).

Protein composition

One major polypeptide (M, 50000) was detected when purified virus collected from CsCl gradients was analysed by PAGE and stained with Coomassie blue (Fig. 4). The 50K polypeptide did not stain with silver after one reaction cycle with the silver reagents. Staining of this polypeptide band was observed when a second silver staining cycle was completed. When purified CAA preparations containing approximately 0.5 µg of the 50K polypeptide were analysed, no other protein was detected by silver staining.

Fig. 2. Equilibrium density centrifugation of CAA in CsCl gradient. The densities (○) of CsCl fractions were determined by refractometry. Fractions were tested by ELISA (□, A492). The infectivities of selected fractions are included.

Fig. 3. Negative contrast electron microscopy of purified CAA. Particles with 10 well-defined peripheral protrusions were sometimes observed (arrow). Bar marker represents 50 nm.

Fig. 4. Protein composition of purified CAA. Samples were analysed by electrophoresis on SDS-12.5% polyacrylamide gels. Lane 1, M, protein standards; lane 2, CAA polypeptide.

Fig. 5. Analysis of CAA DNA by electrophoresis in neutral (lanes 1 and 2; sizes in base pairs) and alkaline (lanes 3 and 4; sizes in bases) agarose gels. Lanes 1 and 3, HindIII fragments of λ DNA; lanes 2 and 4, CAA DNA.

Nucleic acid composition

A single ethidium bromide-stained band was observed when the nucleic acid extracted from virus obtained from sucrose density gradients or CsCl density gradients was analysed by electrophoresis in neutral agarose gels (Fig. 5).
Fig. 6. Electron microscopy of CAA DNA (black arrows). Single-stranded φX174 DNA (open arrows; 5386 bases) was used as an internal standard. Bar marker represents 250 nm.

5, lane 2). The mobility of this band relative to the HindIII fragments of λ phage DNA indicated a genome size of about 0.8 kbp. Resistance to ribonuclease A and sensitivity to deoxyribonuclease I indicated that the nucleic acid species was DNA. Digestion of this molecule by S1 nuclease under conditions in which the fragments of λ DNA remained intact suggested that the virus DNA was single-stranded (data not shown).

It was therefore necessary, if the genome size of CAA virus DNA was to be validly estimated, to compare the mobilities of virus DNA with those of the Mr DNA standards under conditions in which all molecules were single-stranded. When this was done by electrophoresing alkali-denatured samples in alkaline agarose gels, the mobility of the CAA virus genome relative to the denatured, single-stranded λ DNA HindIII fragments corresponded to a size of about 2.3 kb (Fig. 5, lane 4).

Electron microscopic examination using the Kleinschmidt (1968) technique showed that the single-stranded virus DNA was circular. Fifty-nine CAA virus DNA molecules and 31 φX174 single-stranded DNA molecules were measured from a single electron micrograph. The length of the CAA virus DNA relative to that of the known φX174 standard (5386 bases) indicated that the avian virus possessed a genome of approximately 2170 ± 43 bases (Fig. 6).

To determine whether the virus DNA molecules were covalently linked circles or were circular due to the presence of cohesive ends, samples were heat-denatured and, after incubation at 37 °C for 15 min, were analysed by electrophoresis in neutral agarose gels. Southern blot hybridization using a 32P-labelled virus DNA probe revealed that the denatured DNA migrated with the same mobility as the undenatured sample (Fig. 7). This finding suggested that the CAA virus DNA was probably covalently linked since molecules with cohesive ends would be expected to form oligomers upon renaturing and therefore migrate with reduced mobility.

Discussion

In this paper the biochemical composition of purified CAA is described for the first time. We have found that CAA virus particles measure 23.5 ± 0.8 nm in diameter and band at a density of 1.33 to 1.34 g/ml in CsCl. The virus contains one major polypeptide (Mr 50000) and a circular, DNA genome, the single-stranded nature of which was indicated by its sensitivity to S1 nuclease, its electrophoretic behaviour in neutral and alkaline gels and its appearance using electron microscopy. The genome size values obtained by electrophoresis in denaturing gels (2.3 kb) and by electron microscopy (2.17 kb) were in close agreement.

This composition is very different from those of paroviruses, to which CAA has been likened on the basis of size and physical characteristics. Paroviruses contain a linear, single-stranded DNA (approximately
SDS. This detergent solubilized much of the cellular infected cells and the IIF-based assay requiring two in the absence of a convenient method for monitoring the presence of virus. The infectivity assay reported by another problem encountered during purification was the close correlation between ELISA absorbance and infectivity (Figs. 1 and 2) support the view that virus reductions in the amounts of virus recovered. Purifying CAA in amounts sufficient for biochemical analysis was made difficult due to the moderate titre (10^5 to 10^6 TCID₅₀/ml) to which the virus grew in MDCC-MSB1 cell line. To overcome this problem we have developed and used a sandwich ELISA for detecting virus antigen. The relationship of these three viruses obviously requires further investigation. As a group they do not appear to belong to any known animal virus family and represent animal viruses with the smallest known genomes. It is also interesting that the capsid polypeptides of CAA and pig circovirus both account for between 50% and 60% of their genomic coding potentials. Purifying CAA in amounts sufficient for biochemical analysis was made difficult due to the moderate titre (10^5 to 10^6 TCID₅₀/ml) to which the virus grew in MDCC-MSB1 cell line and to its apparent close association with cellular material. We have found that the amounts of virus recovered after differential centrifugation were increased by treatment of the cell/virus lysate with 0.53% SDS. This detergent solubilized much of the cellular material and appeared to facilitate the release of virus. Another problem encountered during purification was the absence of a convenient method for monitoring the presence of virus. The infectivity assay reported by Goryo et al. (1987) requiring up to eight subcultures of infected cells and the IIF-based assay requiring two subcultures are both time-consuming and laborious. To overcome this problem we have developed and used a sandwich ELISA for detecting virus antigen. The neutralizing activity of the CAA-specific monoclonal antibody used in the test (M. S. McNulty, D. P. Mackie, D. A. Pollock, J. McNair, D. Todd, K. A. Mawhinney, F. McNeilly & T. J. Connor, unpublished results) and the close correlation between ELISA absorbance and infectivity (Figs. 1 and 2) support the view that virus particles are being detected by ELISA. Density gradient fractions containing virus could be identified by ELISA within 3 h instead of the 5 or 6 days required by the modified, IIF-based, infectivity assay. Such delays particularly at the CsCl stage could well have resulted in reductions in the amounts of virus recovered. Large numbers of particles were detected by negative contrast electron microscopy in purified preparations. These measured 23.5 ± 0.8 nm in diameter and had an obvious surface structure (Fig. 3). No similar surface structure was described by Goryo et al. (1987) who estimated the particle diameter as 19.1 ± 0.2 nm. The density in CsCl (1.35 to 1.36 g/ml) reported by Goryo et al. (1987) was also different from that (1.33 to 1.34 g/ml) determined in the present study. Such differences in size and density may be due to variation in the purification methods adopted or to inter-laboratory technical variation.

References

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