Two Particle Types of Avian Infectious Bronchitis Virus

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SUMMARY

Two distinct types of avian infectious bronchitis virus (IBV) particles were isolated on sucrose density gradients. The higher density particles banded at 1.18 g/ml, had typical coronavirus morphology and contained all the structural polypeptides and a complete genome. The less dense particles of density 1.13 g/ml appeared to have typical coronavirus morphology, although they were much more flattened than the more dense particles. Furthermore, these particles lacked the ribonucleoprotein polypeptide and the genome, although all the other polypeptides were present in the same amounts as in the denser particles.

INTRODUCTION

The coronavirus, avian infectious bronchitis virus (IBV) is an enveloped, RNA-containing virus that buds from endoplasmic reticular membranes (McIntosh, 1974). IBV particles are extremely heterogeneous in sucrose density gradients and they generally sediment in a broad band of density varying from 1.14 to 1.22 g/ml (Tannock, 1973; Bingham, 1975; Collins et al. 1976; Alexander & Collins, 1977; Macnaughton & Madge, 1977a). Within this band, individual virus peaks containing particles of similar morphology and molecular structure can sometimes be distinguished (Bingham, 1975; Collins et al. 1976; Alexander & Collins, 1977; Macnaughton & Madge, 1977a). These particles have been shown to have typical coronavirus morphology (Tannock, 1973; Bingham, 1975; Collins et al. 1976; Macnaughton & Madge 1977a), consisting of roughly spherical particles with characteristic widely spaced club-shaped surface projections (McIntosh, 1974). They have been shown to have six or seven major polypeptides (Macnaughton & Madge, 1977a; Macnaughton et al. 1977), most of which are glycosylated (Alexander & Collins, 1977; Macnaughton & Madge, 1977a). We consider that the 74,000 mol. wt. polypeptide which is not always present and then is only loosely attached to the virus particle (Macnaughton et al. 1977) is a contaminant. A non-glycosylated polypeptide of mol. wt. 51,000 is the protein component of the ribonucleoprotein (Macnaughton et al. 1977). The genome of these particles is a large unsegmented single-stranded RNA molecule with a mol. wt. in the range 9.0 x 10^6 to 5.6 x 10^6 (Watkins et al. 1975; Lomniczi & Kennedy, 1977; Macnaughton & Madge, 1977b; Schochetman et al. 1977; Macnaughton, 1978).

An additional peak of virus has also been detected (Macnaughton & Madge, 1977a), at a density of 1.13 g/ml, and in this paper the morphological, molecular and biochemical characteristics of particles from this peak have been analysed for the first time. This low density virus consists of empty, presumably non-infectious, particles which lack ribonucleoprotein (RNP). Other coronaviruses also band at about 1.18 g/ml in sucrose density gradients, although with less variation in density than IBV (McIntosh, 1974). However, no lower density incomplete particles have so far been reported for these other coronaviruses.
METHODS

Virus strains. IBV strains Beaudette (IBV 42) and Connecticut (IBV 46) were used and both had many previous passages in embryonated chicken eggs.

Virus growth. IBV was grown in embryonated chicken eggs or primary chick kidney cell cultures and 0.2 ml containing $10^4$ to $10^5$ TCD$_{50}$ of virus was inoculated by the allantoic route into 10-day-old embryonated chicken eggs which were then incubated at 37°C for 24 h. The eggs were chilled at 4°C overnight, the allantoic fluid harvested and immediately clarified by centrifugation at 2000 g for 30 min at 4°C.

IBV was also grown in primary cultures of kidneys from 3 to 7-week-old specific-pathogen-free chickens (Bingham, 1975). Monolayer cultures of these were generally infected at an input multiplicity of 0.1 infectious particles/cell and, if appropriate, 20 µCi/ml of $^{14}$C-leucine (sp. act. 0.2 Ci/mmol) or 20 µCi/ml of $^3$H-uridine (sp. act. 24 Ci/mmol), both from the Radiochemical Centre, Amersham, Bucks., were added just after infection. The cells were harvested after 25 h, subjected to three freeze-thaw cycles and then clarified at 2000 g for 30 min at 4°C (Macnaughton & Madge, 1977b).

Virus purification. Virus grown in eggs or chick kidney cells was purified by pelleting at 75,000 g followed by two cycles of isopycnic centrifugation in sucrose gradients (Macnaughton & Madge, 1977a; Macnaughton, 1978).

Electron microscopy. Separate aliquots from virus fractions were either negatively stained with 2% (w/v) potassium phosphotungstate, pH 6.5, or shadowed with carbon/platinum at an angle of 12° and then examined in a Philips EM 300 electron microscope. Latex particles were used for the internal calibration of the shadowing angle, which showed some variation.

Virus polypeptide analysis. Purified virus preparations were treated with 5% sodium dodecyl sulphate–2% 2-mercaptoethanol at 100°C for 1.5 min. A trace amount of bromophenol blue was added to the reduced polypeptides which were then electrophoresed through 7.5% polyacrylamide gels (Macnaughton & Madge, 1977a). The polypeptides were visualized by staining with 0.1% Coomassie brilliant blue and the gels were scanned at 620 nm using a Joyce-Loebl Chromoscan (Macnaughton & Madge, 1977a).

Virus RNA analysis. Purified virus particles from pooled sucrose gradient fractions were pelleted at 75,000 g for 1 h, then extracted using proteinase K. The virus was suspended in 15 mM-KCl, 10 mM-tris, pH 7.5, containing 1.5% sodium dodecyl sulphate and proteinase K (40 µg/mg virus, Merck) for 10 min at 0°C, then incubated for 30 min at 25°C. The solution was adjusted to 0.4 M in NaCl and 1 mM in EDTA, then 3 vol. of ethanol were added and stored at 4°C for 16 h. The RNA was recovered by centrifuging at 1500 g for 15 min. Unlabelled marker rRNA was extracted from chick kidney cells in a similar way. The RNAs were electrophoresed through 2:2% polyacrylamide gels as described previously (Macnaughton & Madge, 1978). Frozen gels were sliced into 1 mm discs, which were dissolved in hydrogen peroxide; scintillation fluid was then added and radioactivity was determined in a Packard Tri-Carb scintillation counter.

Infectivity assay. Infectivity was estimated by inoculating 0.2 ml amounts of a 10-fold dilution series of virus suspension into rolled tube cultures of chick kidney cells containing 1 ml of maintenance medium; two tubes were used for each dilution. The tubes were incubated at 37°C and c.p.e. in the cells were observed at 48 h after inoculation.
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1.1.2 ~

8

X

2L

I

5 10 15 20

Fraction number

Fig. 1. Centrifugation of partially purified IBV Beaudette particles grown in chick kidney cells and labelled with ^3H-uridine (○—○) and ^14C-leucine (●—●) on 25 to 55 % (w/v) sucrose density gradients for 16 h at 90,000 g. □—□, Density.

Table 1. IBV Beaudette* particle counts in peak fractions from sucrose density gradients

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Major (× 10^6)</th>
<th>Low density (× 10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>150</td>
<td>1.5</td>
</tr>
<tr>
<td>2</td>
<td>120</td>
<td>10.0</td>
</tr>
<tr>
<td>3</td>
<td>105</td>
<td>2.7</td>
</tr>
<tr>
<td>4</td>
<td>7.1</td>
<td>2.5</td>
</tr>
<tr>
<td>5</td>
<td>1.7</td>
<td>3.4</td>
</tr>
</tbody>
</table>

* Grown in chick kidney cells.

RESULTS

Fractionation of virus particles on sucrose density gradients

IBV particles grown in eggs or chick kidney cells were analysed on sucrose density gradients. Fig. 1 shows a typical experiment in which a broad band of virus material was located at 1.16 to 1.20 g/ml, with a peak at about 1.18 g/ml. Virus particles in this band incorporated both ^14C-leucine and ^3H-uridine and corresponded to previously described major particles (Macnaughton & Madge, 1977a). A second band of virus of about 1.13 g/ml, in the range of 1.11 to 1.15 g/ml, was usually observed which incorporated more ^14C-leucine compared with ^3H-uridine than major particles. We have called these particles low density particles (Macnaughton & Madge, 1977a). The results presented in Fig. 1 are from a sucrose gradient in which approximately equal numbers of major and low density particles were observed. However, the results from five typical experiments in which virus particles were prepared from chick kidney cells infected with a low multiplicity of 0.1 infectious particles/cell (Table 1) show that the numbers of major virus particles and the ratio of major to low density particles can vary considerably. Similar results were obtained from cells infected at other m.o.i. No obvious pattern was detected in a number of experiments to suggest an explanation for this variation.
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Fig. 2. Egg-grown IBV Connecticut particles from sucrose density gradients, negatively stained with 2% potassium phosphotungstate, pH 6.5. (a) Major particles; (b) low density particles.

Fig. 3. Egg-grown IBV Connecticut particles from sucrose density gradients, shadowed with carbon/platinum at an angle of 12°. (a) Major particles; (b) low density particles.

Table 2. Dimensions of shadowed* egg-grown IBV Connecticut particles from sucrose density gradients†

<table>
<thead>
<tr>
<th>Particle</th>
<th>Diam. of particle (nm)</th>
<th>Length of shadow (nm)</th>
<th>Height of particle (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Major</td>
<td>123 ± 15 (102–186)</td>
<td>64 ± 17 (25–107)</td>
<td>12.3 ± 3.4 (4.8–20.6)</td>
</tr>
<tr>
<td>Low density</td>
<td>126 ± 18 (107–195)</td>
<td>42 ± 15 (16–84)</td>
<td>8.2 ± 2.9 (3.0–16.1)</td>
</tr>
</tbody>
</table>

* Shadowing was at an angle of 12° using carbon/platinum. Measurements were performed on 57 complete and 41 incomplete particles.
† Results are expressed as mean value ± standard deviation with range in parentheses.

Morphology of the virus particles

Fig. 2 shows electron micrographs of negatively stained preparations of major (Fig. 2a) and low density (Fig. 2b) particles obtained from peak sucrose density gradient fractions. There were no morphological differences between the two virus populations. In each case, typical coronas of surface projections were observed around approximately spherical virus particles of mean diam. 155 nm.

The major and low density virus particles were then examined by electron microscopy after shadowing with carbon/platinum at an angle of 12° (Fig. 3). The diameters of both
Fig. 4. Histograms of the heights of (a) major and (b) low density egg-grown IBV Connecticut particles determined by shadowing with carbon/platinum (results from Table 2).

Fig. 5. Densitometer tracings of egg-grown IBV Connecticut polypeptides on 7.5% polyacrylamide gels after staining with Coomassie brilliant blue. Polypeptides were from (a) major and (b) low density particles. Numbers on peaks indicate polypeptide mol. wt. × 10⁻⁶.

virus types were similar although the mean values were approx. 30 nm smaller than that of particles negatively stained with potassium phosphotungstate, pH 6.5. The results from several experiments (Table 2) show that the mean lengths of the shadows of major particles were 1.5 times greater than those of low density particles. A comparison of the height of the particles with diameter and also the shape of the shadow showed that particles of both types were flattened and not spherical as can easily be assumed from negative staining. The low density particles were somewhat more flattened than major particles, possibly indicating the
absence of some internal components. Fig. 4 shows histograms of the heights of major (Fig. 4a) and low density (Fig. 4b) virus particles. The difference in mean heights of 12.3 nm for major particles and 8.2 nm for low density particles is statistically significant ($P < 0.001$), although there was some overlap between the two virus particle populations.

**Virus polypeptides**

Fig. 5 shows typical densitometer tracings of virus polypeptides from major and low density particles. Six structural polypeptides with mol. wt. of 130 000, 105 000, 97 000, 82 000, 51 000 and 33 000 were found (Macnaughton et al. 1977) in both major and low density particles. In all experiments the RNP polypeptide of mol. wt. 51 000 (Macnaughton et al. 1977) was considerably reduced in amount in the low density particles (Fig. 5b), although the other polypeptides were found in approximately the same amounts in particles of both types.

**Virus genome RNA**

Fig. 6 shows that very little $^3$H-uridine was incorporated into low density particles compared with major particles. As major virus particles have been shown to contain a genome consisting of only one large single-stranded RNA molecule (Watkins et al. 1975; Lomniczi & Kennedy, 1977; Macnaughton & Madge, 1977b; Schochetman et al. 1977), this result implies that the low density particles lack this genome or contain only a segment of it. $^3$H-uridine-labelled RNA from both virus populations was analysed on 2.2% polyacrylamide gels to characterize the RNA of these particles. Fig 6(a) shows the profile of RNA extracted from major particles. A single peak of activity was obtained corresponding to an RNA species of mol. wt. about $5.8 \times 10^6$. On the other hand, essentially no RNA species were resolved on gels prepared from low density particles (Fig. 6b).

**Infectivity assays**

A number of titration methods are available for assay of infectious IBV particles (Bingham, 1975; Watkins et al. 1975; Collins et al. 1976; Lomniczi, 1977; Schochetman et al. 1977). However, all these methods produced variable and relatively low infectious titres compared with the number of particles in the sample. For example, assaying infectious virus by tube
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Titration, which in our study produced the highest infectivity titres, revealed that generally only 1 in $10^4$ to $10^6$ major IBV particles, detectable by electron microscopy, were infectious. Furthermore, low density IBV particles showed low and extremely variable infectivity using this method of assay with an infectivity to particle ratio of between 1 to $10^6$ and 1 to $10^7$. The significance of these results is discussed below.

Discussion

In this paper, we have shown the presence of low density IBV particles, which can be separated from major particles by centrifugation on sucrose density gradients. Both virus particle populations form heterogeneous peaks on density gradients with the low density particles banding at about 1.13 g/ml and the major particles at about 1.18 g/ml. Low density particles were identical to the major particles after negative staining, although they were much more flattened after shadowing with carbon/platinum. Furthermore, shadowing experiments indicated that there was a certain amount of cross contamination of the low density and major virus populations. Additional sucrose gradient purification steps did not produce much greater separation, but did lead to considerably lower virus yields.

Analysis of the polypeptide and RNA content of low density particles revealed that they lacked most of the ribonucleoprotein polypeptide and most, if not all, of the genome found in major particles. It seems likely that the small amounts of these components that were found in low density particle preparations were due to contaminating denser major particles. This conclusion is confirmed by certain atypical virus preparations where a complete separation of the virus population was obtained and no RNP components were obtained in the preparations of low density particles. Thus, the low density particles are structurally the same as major particles, except that they lack RNP, and can be considered as incomplete particles.

The study of the infectivity of IBV particles has been hindered in the past by the lack of a suitable method for detecting high levels of infectivity (Bingham, 1975; Watkins et al. 1975; Collins et al. 1976; Lomniczi, 1977; Schochetman et al. 1977). In our study only about 1 in $10^4$ to $10^5$ major or complete particles, detectable by electron microscopy, was infectious using tube titrations. Such low infectivity of IBV particles may be due to a lack of infectivity in a high proportion of particles or, more likely, due to the insensitivity of the virus titration methods used. Thus, the apparently high particle to infectivity ratio makes it difficult to compare the infectivity of incomplete and complete particles. The low infectivity observed in incomplete virus preparations can easily be attributed to slight contamination by complete particles. Furthermore, it would be extremely unlikely that IBV particles, lacking RNP, were infectious since these particles contain no genomic material to initiate replication.

This is the first report showing the presence of incomplete coronavirus particles and studies are in progress to identify similar particles from other coronaviruses. Other studies are attempting to show whether incomplete IBV particles are degraded forms of complete particles or whether they are produced during virus multiplication and have some role in infection. Certainly, the proportion of incomplete and complete particles varied considerably between experiments and at present no obvious pattern in the production of incomplete particles has been detected.

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REFERENCES


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