A Regular Subunit Pattern Seen on Non-infectious Newcastle Disease Virus Particles

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SUMMARY

A pseudo-crystalline array of subunits has been observed on particles of the La Sota, in contrast to the Ulster, strain of Newcastle disease virus (NDV) grown in MDBK tissue culture without trypsin. This regular arrangement of subunits was associated with the semi-permissive nature of the tissue culture system, as it disappeared when trypsin, which allows infectious virus to be made, was added. The phenomenon described was considered to be related to the crystalline array of matrix protein which has been described inside the envelope of Sendai virus and NDV by others.

INTRODUCTION

Although lentogenic Newcastle disease virus (NDV) can be propagated with ease in embryonated eggs, it can be grown in tissue culture only with difficulty and the virus harvested is biologically inactive. However, incorporation of trypsin into the medium or post-treatment of virus with trypsin yields infectious virus (Nagai et al., 1976). We have undertaken a morphological study of egg-grown compared to tissue culture-grown virus either with or without the presence of trypsin. Two lentogenic strains of NDV (Ulster-2C and La Sota) were used for the study.

In order to obtain a suitable base-line, the morphology of egg-grown virus was studied and it was found that NDV particles can be divided into two populations: those with long, 15 nm, glycoprotein projections, and those with a short, 9 nm, fringe. The same particle morphology was seen on tissue culture-grown virus except when the La Sota strain was grown on Madin & Darby bovine kidney (MDBK) cells without trypsin. Under these conditions, a subpopulation of particles showed regular arrays of subunits on the lipoprotein membrane. How these features can be affected by growth conditions and relate to theories of virus structure will be addressed.

METHODS

Viruses. Seed stocks of NDV (Ulster-2C) and NDV (La Sota) were donated by Dr D. J. Alexander of the Central Veterinary Laboratory, Weybridge, Surrey, U.K. Egg-grown virus was produced by inoculating 10-day-old eggs with virus and incubating for 3 days at 35 °C. Allantoic fluid was then harvested and yielded about 10^8.3 i.u./ml as measured by a microtitre method (Russell et al., 1983) with a haemagglutination (HA) titre of 10^3.

Tissue culture virus stocks were grown in MDBK cells which were infected with a 1 in 3 dilution of allantoic fluid for 1 h, washed five times and then overlaid with maintenance medium (RPMI 1640). After 24 h at 37 °C, the medium was removed, clarified, and the virus stocks were kept on ice until examination the next day.

In some experiments 10% tryptose phosphate broth (TPB, Gibco) and various levels of acetylated bovine trypsin (Type V-S, Sigma) were incorporated into the medium. Tissue culture harvests had a titre of 8 to 64 HA units. The infectivity was 10^{6.7 ± 0.4} i.u./ml for virus grown in medium containing no trypsin or 0.1 µg trypsin/ml and this was unaltered by TPB. The infectivity was 10^{6.8 ± 0.6} i.u./ml when virus was grown or post-treated with 0.3 to 3.0 µg trypsin/ml of medium.

Electron microscopy. Because of current legislation, NDV-containing specimens had to be inactivated at the Royal Veterinary College before examination by electron microscopy. This was carried out by adding a 1 in 4000 dilution of β-propiolactone at 4 °C overnight. The specimens were then heated to 37 °C for 30 min to inactivate the
$\beta$-propiolactone and transferred onto ice for examination by negative staining using the standard method of Almeida (1980).

To prepare egg-grown virus for negative staining, 500 µl of allantoic virus was diluted with phosphate-buffered saline (PBS) to 2 ml and centrifuged for 1 h at 15000 g. For tissue culture-grown virus, 1 ml of supernatant was diluted to 2 ml using PBS and centrifuged as before. In both cases the supernatant was decanted and the pellet resuspended in approximately 50 µl distilled water. This suspension was then used for negative staining employing 4% phosphotungstic acid at pH 6.

**RESULTS**

*Electron microscopy of egg-grown virus*

Particles seen showed typical parainfluenza morphology, with many having a disrupted outer membrane which allowed the internal helical component to be seen. In every specimen there were particles with long surface glycoprotein projections of approximately 15 nm (Fig. 1) and others with a short projection of 9 nm (Fig. 2). The particles with a short fringe were the more usual. After prolonged searching, occasional particles which carried both lengths of projection were found (Fig. 3). On no occasion did these surface projections exhibit any regular pattern and it was impossible to distinguish the two strains of NDV.

*Electron microscopy of tissue culture-grown virus*

Harvests of both the Ulster and La Sota strains of NDV from MDBK cells with added trypsin showed morphology identical to that of the egg-grown virus. Particles with both the long and short glycoprotein projections were seen and the ratio of long to short fringed particles could not be shifted by post-treating or growing virus with trypsin at 0.03, 0.3, 0.9 or 3 µg/ml.

The same standard paramyxovirus morphology was also seen when the Ulster strain was grown in the absence of trypsin. However, when the La Sota strain was grown without trypsin a distinctive pseudo-crystalline array was seen on some 10 to 20% of particles (Fig. 4). It was then found that if TPB was added to the maintenance medium of cultures without trypsin, the number of well-defined NDV particles in each specimen of NDV increased and, as far as the La Sota strain was concerned, the number of particles exhibiting the pseudo-crystalline array increased. However, the regular array was never seen on the Ulster strain even with TPB in the medium. Many of the subunit-bearing La Sota particles appeared to be empty, flattened, membranes, but it was not difficult to find others with typical internal ribonucleoprotein (RNP). However, since the empty forms show the substructure arrangement best, these have been used for illustration.

The regular surface array was seen mainly on particles having a distinctive surrounding fringe 7 to 8 nm long, but could occasionally be seen on particles with the long glycoprotein fringe (Fig. 5). Almost all of the subunits showed a 'one surrounded by six' arrangement although occasional 'one surrounded by five' arrangements were also seen. The individual subunits were 6 nm in diameter and could be seen to have a further substructure. Centre-to-centre measurement of subunits in the arrays gave a value of 8 to 9 nm. This regular subunit array appeared to be related to the surface of the particles, as the arrangement could be seen to extend beyond the membrane component when the subunits were in profile at the edge of the virus (Fig. 6). Many particles gave the impression that the regularly arranged subunits were unstable in nature, because only limited areas of them remained (Fig. 7). Addition of trypsin to the maintenance medium at a level sufficient to produce infectious virus led to the disappearance of all particles displaying the regular surface array. However, incorporation of trypsin at 0.1 µg/ml, which is insufficient to produce infectious virus, appeared to increase the proportion of particles showing regular subunits.

Infectivity can also be increased by post-harvest treatment of virus with 0.3 µg/ml trypsin for 2 h. This treatment appeared to destroy the population of particles bearing regular subunits. However, close examination revealed occasional particles where remnants of the subunit array could still be seen (Fig. 8). In these instances, the subunits could just be resolved but they appeared to be degraded and relaxed, so that the pattern was obscured.
NDV envelope structure

Fig. 1. Part of control egg-grown NDV (Ulster) particle showing the long form of the surface glycoprotein. Projections are approx. 15 nm long and hence similar in size to the 17 nm diam. of the internal helix. Bar marker represents 25 nm. This micrograph had been chosen deliberately so that the dimensions of the projections and helix could be compared.

Fig. 2. Particles of egg-grown NDV (Ulster) with the type of fringe more usually associated with the paramyxovirus group. Projections are 8 to 9 nm long and are not individually resolved. Bar marker represents 25 nm.
Fig. 3. A single egg-grown NDV (Ulster) particle that shows both the long and short surface projections. The fringe at the left of the virus is 9 nm while that at the right is 15 nm long. Bar marker represents 25 nm.

Fig. 4. When NDV (La Sota) is grown in tissue culture without trypsin, 10 to 20% of the resultant particles carry a regular surface arrangement. Individual subunits are 6 nm diam. and the centre-to-centre spacing is 8 to 9 nm. This particle also shows a superimposition or moiré pattern at the top. Bar marker represents 25 nm.
Fig. 5. Although unusual, an occasional long-fringed particle of tissue culture NDV (La Sota) displayed the regular array of subunits. However, as here, the pattern is not clearly seen and is visible only on small areas. Bar marker represents 25 nm.

Fig. 6. Closer examination shows that the subunit arrangement of tissue culture NDV (La Sota) is 'one surrounded by six' although the occasional 'one surrounded by five' can also be found. This micrograph also shows subunits extending to the edge of the particle where they can be seen in profile. Bar marker represents 25 nm.
Fig. 7. Many particles of tissue culture NDV (La Sota) were only partially covered by the regular subunit arrangement. The particle here demonstrates this effect and also that peripheral projections occur only at areas where there are surface subunits. Bar marker represents 25 nm.

Fig. 8. Post-harvest treatment of tissue culture NDV (La Sota) with trypsin increases infectivity but also destroys the regular subunit arrangement. However, an occasional particle shows remnants of this arrangement although the subunits appear degraded and relaxed both on the particle surface and around the periphery. Bar marker represents 25 nm.
DISCUSSION

Although present legislation has limited this study to two strains of NDV, Ulster and La Sota, comparison of egg- and tissue culture-grown virus has revealed some interesting morphological features. First, in all preparations, virus could be found with either long or short surface glycoprotein projections. Long projections measured approximately 15 nm, while the others had a length of 9 nm. In most instances the fringe remained constant in dimension, although on the occasional particle, transition from one fringe length to another could be seen (Fig. 3). Such particles suggest that, rather than two separate populations we are looking at one particle type whose appearance can alter. Our interest in the appearance of the NDV particles was to establish a morphological base-line against which any variation could be compared. However, since the work undertaken meant relating infectivity of virus to treatment with trypsin and also the comparison of non-infectious tissue culture-grown virus with fully infectious egg-grown virus, it was feasible to relate these various virus groups to morphological appearance. An obvious interpretation of the two projection lengths was that we were visualizing the cleavage of the surface glycoproteins, from an inactive precursor form to the active form (Garten et al., 1980). However, whilst trypsin treatment enhanced infectivity 100-fold and increased the quantity of cleaved haemagglutinin–neuraminidase molecules by about 10-fold (as detected by $[^{35}S]$methionine labelling; results not shown), this activation was not accompanied by an obvious shift in the proportion of particles with long or short spikes. Similarly, it might be expected that egg-grown virus should contain mainly cleaved, short-fringed particles because it is infectious and because this is the morphology normally associated with the family Paramyxoviridae (Matthews, 1982). This was not the case, and the best micrographs of the long-fringed form came from egg-grown preparations. One possible explanation is that the long fringe, which bears a close resemblance to the surface projections of respiratory syncytial virus (Zakstelskaya et al., 1967), may be involved in fusion. However, the main reason for studying the appearance of this virus was to establish normal variation in its appearance, before interpreting the particular appearance that was encountered when the La Sota strain was grown in MDBK cells.

NDV particles with a regular surface subunit array were found only when the La Sota strain was grown in MDBK cells without trypsin. In the presence of maintenance medium alone, around 10% of all particles showed the regular surface arrays. Addition of TPB to the medium increased this amount to 15 to 20% and seemed to improve the quality of the specimens. This may be related to the fact that TPB is known to help maintain cells in culture without serum (Ginsberg et al., 1955). It is interesting that the yield of virus from MDBK cells without trypsin was the same for both the La Sota and Ulster strains of NDV, and that although both of these strains are lentogenic, only La Sota yielded the particles of interest. It would obviously be important to test other strains of the virus but at the moment this can only be carried out in specially licensed laboratories.

Examination of these particles with the regular surface pattern showed that the subunits building up the pattern were superficial to the lipoprotein membrane on which they were arranged. This could be seen from the fact that the regular arrays of subunits extend across the particle and can then be seen in profile at the edge of the membrane. This appearance could only occur if the subunits were superficial to the underlying lipoprotein. The superficiality of the subunits was also confirmed by the many particles that were incompletely covered by them. In these instances, projections at the periphery of the particles occurred only where subunits occurred on the main body of the particle. Finally, and once again supporting the idea that the subunits are external to the membrane, treatment with trypsin destroyed the regular arrays except for a few particles where the much altered remains of the subunits could still be seen.

The subunits seen in profile at the edge of the particles showed a morphology that did not resemble either the long or the short fringe described for control virus. The regular arrangement of the subunits on the edge of the La Sota virus gave a distinctive palisaded effect with greater spacing between the individual components than was seen for the glycoprotein fringe.

Using the freeze-fracture technique, Bächli (1980) has shown that newly formed Sendai virus particles have a crystalline arrangement of subunits on the inner lipid leaflet of the virus envelope. An equivalent array occurs on the cytoplasmic surface of the plasma membrane of the
cells near the RNP strands (Buechi & Bächi, 1982). The pseudo-crystalline array described by Bächi has a periodicity of 7.5 nm, which accords closely with the 8 to 9 nm centre-to-centre spacing of the 6 nm-wide subunits we describe here. Bächi makes the point that the ordered arrangement does not extend to the viral surface and suggests that it is probably composed of matrix protein which may function as a scaffolding structure between nucleocapsid and envelope spikes (Bächi, 1980; Buechi & Bächi, 1982). Bächi observed the crystalline arrangement in both allantoic-grown and tissue culture-grown Sendai virus, and mentioned that the same arrangement occurred in NDV and respiratory syncytial virus. He also pointed out that the regular arrays of the subunits were associated with the early stages of virus maturation and that aged virus lost the pattern. He suggested that this was associated with increases in membrane permeability and fluidity as described for Sendai virus by Shimizu et al. (1976).

We suggest that the particular combination of the La Sota strain of NDV grown without trypsin on MDBK cells is a semi-permissive system, allowing the same intermediate stage of matrix protein formation on the inner lipid leaflet to be visualized. If Bächi is correct in his suggestion that the matrix protein is used to form a bridge locating the RNP inside the particle with the glycoprotein projections on the outer surface of the virus, then we are visualizing subunits of matrix protein either before or soon after glycoprotein insertion. Weight is added to this supposition by the occasional particle of the type illustrated in Fig. 5 where it is just possible to see a regular arrangement of subunits even though the particle possesses a glycoprotein fringe. In these instances, it would appear that although the outer of the two phospholipid bilayers is present it is incomplete and still allows the pattern of matrix subunits to be seen on the inner lipid leaflet.

In their classic paper of 1956, Crick & Watson predicted that viruses would be constructed from small identical subunits packed together in a regular manner. This prediction was fulfilled by the subunits of the RNP of viruses with helical symmetry and by the capsid subunits of viruses with cubic symmetry. It is also known that some enveloped negative-stranded RNA viruses, e.g. influenza C and rabies, carry a hexagonal arrangement of envelope projections on their lipoprotein membrane (Herrler et al., 1981; Hummeler et al., 1967). Yet another category, represented by influenza A, would appear to have an occasionally recognizable regular surface pattern associated with the projections located on the lipoprotein membrane (Almeida & Waterson, 1967).

The present study suggests that construction based on geometrically arranged subunits may also extend to viruses of the paramyxovirus group. The arrangement that we describe adheres to the rules of 'one surrounded by six' and 'one surrounded by five' which are essential for producing a three-dimensional structure. However, this arrangement is confined to the inner of the two phospholipid bilayers and it appears that the regular construction is no longer necessary after the RNP and glycoproteins have been located. In fact, it may well be that in order for the virus to become able to fuse cells, the regular arrangement must be lost, although it is an essential part of viral morphogenesis.

In summary the present study shows that control NDV particles can possess either a long or a short glycoprotein fringe. It was not possible to establish the significance of these two fringe lengths. Of greater interest, use of the particular combination of the La Sota strain of NDV with MDBK cells without trypsin gave rise to particles with a distinctive geometric surface arrangement. The pattern described corresponds well with that found by Bächi (1980) for the matrix protein on the inner lipid leaflet. It is therefore suggested that the semi-permissive system supplied by MDBK cells allows examination of a step during virus synthesis in the normal growth cycle that is not revealed by the negative staining process.

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REFERENCES


**NDV envelope structure**


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