Isolation of a New Avian Paramyxovirus from Budgerigar 
(*Melopsittacus undulatus*)

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SUMMARY

In 1974 an epizootic occurred among budgerigar flocks in Kunitachi, Tokyo, and a causative agent which possessed haemagglutinating, neuraminidase, and haemolytic activities was isolated from the lung of a dead budgerigar. This agent was 100 to 300 nm in diameter and pleomorphic. The width of the ribonucleoprotein was estimated to be about 20 nm. These results indicated that the virus, designated Kunitachi virus, was a member of the paramyxovirus group. The virus contained in the amniotic fluid from infected embryonated hen’s eggs, however, at times displayed no haemagglutinating activity with different erythrocytes and complete haemagglutination could only be detected in purified preparations. The Kunitachi viruses including three strains recently isolated from the same host were found to be serologically distinct from the known paramyxovirus strains and appeared to constitute a new subtype of avian paramyxovirus.

INTRODUCTION

A number of paramyxovirus strains have been isolated from domestic and wild birds (Bankowski, Corstvet & Clark, 1960; Dinter, Hermodsson & Hermodsson, 1964; Wagner & Enders-Ruckle, 1966; McFerran et al. 1973; G. Lang, unpublished results) and, at present, paramyxovirus strains of avian origin are classified into four serological types: Newcastle disease, Yucaipa, Turkey/Wisconsin/68 and Bangor virus isolated from finch. However, the incidence and distribution of these paramyxovirus strains, other than NDV, in domestic fowls and wild birds are still not fully understood. In the course of epidemiological surveys for influenza virus, a haemagglutinating agent was isolated from the lung of a dead budgerigar in 1974 in Kunitachi, Tokyo, Japan. In the present paper biological, structural, and serological properties of this virus are described and its classification is discussed.

METHODS

*Virus isolation and cell culture.* For virus isolation, lung tissues were collected from dead budgerigars and isolation was performed by intra-amniotic inoculation of 9-day-old fertile hen’s eggs. Chick embryo fibroblast cultures were prepared from 7-day-old chick embryo by the method previously described (Kondo, 1965; Kondo, Takashima & Suzuki, 1974).

*Viruses.* The following strains of influenza and parainfluenza virus were used: A/PR/8/34-
(HoN1), A/NWS/33(HoN1), A/FM/1/47(H1N1), A/RI/53/57(H2N2), A/Aichi/2/68(H3N2),
C/JJ/50, parainfluenza viruses 67-10 strain of type 1, Greer strain of type 2, HA-1 strain of
type 3, 66-348 strain of type 4A, 68-334 strain of type 4B, Paramyxo/Yucaipa/chicken/
California/60(PMY), Turkey/Wisconsin/68(Ty/Wis), Bangor/finch/N. Ireland/73(Bangor),
Ishii strain of Newcastle disease virus (NDV), and MN strain of haemagglutinating
virus of Japan (HJV). In addition, Toyoshima strain of measles, the Enders strain of
mumps, the M-33 strain of rubella, and the VR-3 strain of herpes type 1 were used.

**Growth and purification of virus.** All viruses except Kunitachi virus, herpes, measles,
and mumps viruses, were grown in the allantoic cavity of 10-day-old embryonated hen’s eggs.
Kunitachi virus was grown in the amniotic cavity of 8-day-old embryonated hen’s eggs.
Measles and mumps viruses were kindly supplied by Dr Y. Akao, Central Virus Diagnostic
Laboratory of this Institute and rubella virus was kindly supplied by Dr M. Otawara,
Department of Measles Virus of this Institute. For virus purification, the centrifuged
concentrate was banded in a linear 10 to 50 % (w/w) sucrose gradient at 24000 rev/min for
3 h at 5 °C in the Spinco SW 25.1 rotor.

**Antisera.** Hyperimmune sera against the myxo- and paramyxovirus strains listed above,
except Kunitachi virus, were prepared by intravenous (ear-vein) and intradermal injection
of purified virus into rabbits. Antiserum against Kunitachi virus was prepared in rabbits
by subcutaneous and intramuscular injections with Freund’s incomplete adjuvant. Before
use, all sera were treated with RDE as described previously (Burnet & Stone, 1947).

**Haemagglutination and haemagglutination-inhibition (HI) tests.** Haemagglutination and
haemagglutination-inhibition assays were done in microplastic trays as described previously
(Lennette & Schmidt, 1969) using 0.5 % erythrocytes from several species of animals.

**Plaque assays.** Infectivity assays for p.f.u. were carried out in primary culture of chick
embryo cells as described previously (Kondo, 1965; Kondo et al. 1974). Infectivity titres,
expressed as log p.f.u./ml were determined after inoculation of the virus into three dish
cultures for each dilution.

**Stability test of virus.** For ether sensitivity, virus suspensions were mixed with an equal
volume of ethyl ether and the mixture was held for 4 h at 4 °C with vigorous shaking.
Thermostability was tested according to McFerran et al. (1974). Sensitivity to acid was
tested according to Suganuma (1967).

**Identification of the type of nucleic acid.** The type of nucleic acid in Kunitachi virus was
determined indirectly by the use of 5-iodo-2'-deoxyuridine (IdUrd) as described previously
(Dinter et al. 1964).

**Test for haemolysis.** Aliquots (0-8 ml) of serial dilutions of virus were mixed with 0-2 ml of
10 % chicken erythrocytes and the mixture was held for 30 min at 4 °C. This mixture was
subsequently incubated with shaking at 37 °C for 60 min, after adding 0-8 ml of a 1 %
EDTA solution. The haemoglobin content of the supernatant fluids from centrifugation at
2000 rev/min for 10 min was measured as E540 in a Coleman spectrophotometer.

**Neuraminidase assay.** Neuraminidase activity assays were made using the method
recommended by WHO (1973).

**Electron microscopy.** The structure of the virus was examined in a Hitachi H-500 type by
staining with 2 % phosphotungstic acid as described previously (Nerome, Kumagi & Aoyagi,
1972).

**Experimental infection.** Groups of 23 immature budgerigars were infected intranasally
and intratracheally with approx. 10^6 p.f.u. of freshly propagated Kunitachi virus. All the
exposed or uninfected budgerigars were held for 4 weeks and the dead budgerigar was
subjected to laboratory investigation.
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Table 1. Effect of IdUrd on the multiplication of influenza, herpes, and Kunitachi viruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>Virus yield (p.f.u./ml)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No IdUrd</td>
</tr>
<tr>
<td>Kunitachi virus</td>
<td>$2.5 \times 10^4$</td>
</tr>
<tr>
<td>Influenza virus (NWS)</td>
<td>$8.0 \times 10^5$</td>
</tr>
<tr>
<td>Herpes virus</td>
<td>$9.0 \times 10^6$</td>
</tr>
</tbody>
</table>

* The media, with and without IdUrd ($10^{-4.5}$ M), were added to the chick embryo cells infected with each virus. The amount of infectious virus in the tissue culture fluids was titrated in primary cultures of chick embryo cells after incubation at $34^\circ C$ for 72 h.

Table 2. Effect of ether, acid, and heating on the infectivity of influenza and Kunitachi viruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>Infectivity (p.f.u./ml)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ether</td>
</tr>
<tr>
<td>Kunitachi virus</td>
<td>$&lt; 10$†</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^6$</td>
</tr>
<tr>
<td>Influenza virus (NWS)</td>
<td>$&lt; 1.25$</td>
</tr>
<tr>
<td></td>
<td>$3.7 \times 10^5$</td>
</tr>
</tbody>
</table>

* After treatment, residual virus infectivity was titrated in primary cultures of chick embryo cells.
† After treatment Before treatment

RESULTS

Identification of nucleic acid

The effect of IdUrd on the replication of Kunitachi virus, the NWS strain of influenza virus and herpes virus was determined by assaying the production of infectious virus (p.f.u.) in chick embryo monolayers exposed to IdUrd during the virus multiplication period. Table 1 gives the results of experiments using a concentration $10^{-4.5}$ M-IdUrd. There was no significant difference in virus replication in chick embryo cells with and without IdUrd. These results showed that Kunitachi virus contains RNA rather than DNA.

Stability of virus

As shown in Table 2 Kunitachi virus, like influenza virus, was inactivated by ether treatment, whereas the yield of Kunitachi virus was not affected by acid treatment and was relatively susceptible to heating at $56^\circ C$ for 30 min. Inactivation of Kunitachi virus by ether treatment indicates that this virus contains lipid.

Biological properties

The Kunitachi virus failed to grow in the allantoic cavity of fertile hen's eggs and reproduction was only possible in the amniotic cavity. However, when propagation in primary cultures of chick embryo cells was attempted, Kunitachi virus grew and reached a titre of $2.5 \times 10^6$ p.f.u./ml.

Kunitachi and influenza viruses were tested for their ability to agglutinate the erythrocytes of a number of animal species at both $4^\circ C$ and $25^\circ C$. Haemagglutination tests with Kunitachi virus were performed on infected amniotic fluids and purified preparations on
Fig. 1. Neuraminidase activity of viruses isolated from budgerigar and influenza viruses. Neuraminidase activity assays were performed as described by the WHO Expert Committee (1973). Serial two-fold diluted virus samples were incubated with an equal volume of fetuin solution for 18 h at 37 °C, and liberated sialic acid was measured at 549 nm. ○—○, Paramyxobudgerigar/Kunitachi/1/71; □—□, Paramyxobudgerigar/Ueno/1/75; ■—■, Paramyxobudgerigar/Ueno/4/76; △—△, Paramyxobudgerigar/Ueno/10/76; ●—●, A/RI/5+/57(H2N2); ▲—▲, C/JH/50.

the basis of neuraminidase activity since native amniotic fluids, in spite of occasional high neuraminidase activity, caused little or no haemagglutination. The erythrocytes from chicken, goose, duck, guinea pig, and human O blood were agglutinated at both 4 °C and 25 °C by purified Kunitachi virus. The haemagglutinating activity of the Kunitachi virus in the infected amniotic fluids was extremely unstable but stable haemagglutinating activity was obtained by purifying this virus through a sucrose density gradient.

The reaction of Kunitachi virus and other avian paramyxovirus strains with fetuin was investigated. Since haemagglutination by the Kunitachi virus was inhibited by fetuin itself, it appears that the receptor against this virus is a glycoprotein containing sialic acid as in the case of ortho- and paramyxoviruses.

We examined the neuraminidase activity of Kunitachi virus using fetuin as a substrate. As shown in Fig. 1, Kunitachi virus, like A/RI/5+, caused the release of sialic acid from fetuin, although influenza C virus failed to liberate sialic acid even at a high concentration. In addition to Kunitachi virus, as seen in Fig. 1, three other strains (Ueno-175, Ueno-4-76, and Ueno-10-76) from the budgerigar showed remarkable neuraminidase activity.

We next determined whether or not the isolate from budgerigar contains haemolytic activity. As seen in Fig. 2, Kunitachi virus and NDV showed a significant degree of haemolysis which depended on the virus concentration, whereas influenza virus A/NWS, which was used as negative control, did not cause haemolysis of chicken erythrocytes. The
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haemolytic activity was also determined in the three strains from budgerigar. Under the conditions of assay, there was a significant difference between the haemolytic activity of the four strains from budgerigar as compared with NDV on the basis of haemagglutinating activity.

Size and structure

The morphology of the virus is shown in Fig. 3. The diameter of the rounded forms was quite variable, ranging from 100 nm to 300 nm. They were quite pleomorphic, about 900 to 1500 nm in length. The spikes seem to be released from the envelope, since in some pictures spikeless particles were discernible (Fig. 3a and b). The loss of the spikes may take place during the concentration and purification of the virions. The internal components also seem to be spontaneously released from the virions. In some pictures, released coiled ribonucleoprotein which was previously demonstrated for other paramyxoviruses (Horne & Waterson, 1960; Dinter et al. 1964; Webster et al. 1976), was discernible in the field (Fig. 3c). The diameter of the helical structure measured about 20 nm and the central hole measured about 5 nm. On the basis of morphology, Kunitachi virus thus resembled paramyxoviruses not only in the form of its intact particles, but also in the structure of the ribonucleoprotein.

Antigenic analysis

Cross haemagglutination-inhibition (HI) tests were performed with hyperimmune sera to the Kunitachi virus and various strains of ortho- and paramyxoviruses. No reaction was observed with antisera to reference strains of orthomyxovirus and the isolate. All sera against measles, mumps, rubella, and parainfluenza viruses type 1 to 4 of mammalian origin did not react in cross HI tests with the isolate (data not shown). Paramyxoviruses of avian origin have
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Table 3. Cross haemagglutination-inhibition tests with Kunitachi virus and other avian paramyxovirus strains

<table>
<thead>
<tr>
<th>Virus strains</th>
<th>Kunitachi</th>
<th>NDV</th>
<th>Yucaipa</th>
<th>PI/Ty/Wis/68</th>
<th>Bangor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kunitachi†</td>
<td>4096</td>
<td>&lt;16</td>
<td>&lt;16</td>
<td>&lt;16</td>
<td>&lt;16</td>
</tr>
<tr>
<td>Ueno-1-75†</td>
<td>4096</td>
<td>16</td>
<td>&lt;16</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td>Ueno-4-76†</td>
<td>4096</td>
<td>&lt;16</td>
<td>&lt;16</td>
<td>&lt;16</td>
<td>&lt;16</td>
</tr>
<tr>
<td>Ueno-10-76†</td>
<td>2048</td>
<td>&lt;16</td>
<td>&lt;16</td>
<td>&lt;16</td>
<td>&lt;16</td>
</tr>
<tr>
<td>NDV</td>
<td>16</td>
<td>4094</td>
<td>64</td>
<td>&lt;16</td>
<td>512</td>
</tr>
<tr>
<td>Yucaipa</td>
<td>&lt;16</td>
<td>512</td>
<td>512</td>
<td>&lt;16</td>
<td>8192</td>
</tr>
<tr>
<td>PI/Ty/Wis/68</td>
<td>&lt;16</td>
<td>128</td>
<td>&lt;16</td>
<td>256</td>
<td>&lt;16</td>
</tr>
<tr>
<td>Bangor</td>
<td>&lt;16</td>
<td>512</td>
<td>64</td>
<td>&lt;16</td>
<td>4096</td>
</tr>
</tbody>
</table>

* HI titres represent reciprocals of serum dilution. All antisera were treated with RDE.
† Paramyxovirus strains isolated from budgerigar: Kunitachi, Paramyxo/budgerigar/Kunitachi/1/74; Ueno-1-75, Paramyxo/budgerigar/Ueno1/75; Ueno-4-76, Paramyxo/budgerigar/Ueno/4/76; Ueno-10-76, Paramyxo/budgerigar/Ueno/10/76.

been divided into four subtypes on the basis of HI and neutralization tests; NDV, Yucaipa PI/Ty/Wis/68, and Bangor. These four paramyxoviruses were not antigenically related to the Kunitachi virus (Table 3).

Recently, Webster et al. (1976) have isolated seven paramyxoviruses from wild ducks, which were found to be unrelated to the Kunitachi virus. In addition to the Kunitachi virus, three haemagglutinating agents were isolated from budgerigars in 1975 and 1976 but all three isolates (Ueno-1-75, Ueno-4-76, Ueno-10-76) were closely related to Kunitachi virus.

Pathogenicity

After infection of 23 budgerigars with Kunitachi virus, 18 showed signs of depression, dyspnoea, diarrhoea and rare torticollis before death. All the budgerigars that developed signs of disease eventually died within 2 weeks. At death the budgerigar had viraemia and subsequently the causative virus was again isolated from brain, lung, spleen, kidney, liver, and blood.

DISCUSSION

The myxovirus groups are classified into ortho (true)- and paramyxoviruses based on morphological, biological, physicochemical and serological properties (Andrewes, 1962; Finch & Gibbs, 1970; Mountcastle, Compans & Choppin, 1971; Andrewes & Pereira, 1972). The results described above suggest that the isolate described (Kunitachi virus) is a member of the paramyxovirus group according to biological and morphological characteristics, and stability to physicochemical treatment.

However, it differs from the avian paramyxovirus strains described so far on the basis of growth and haemagglutinating properties. There is no reproduction in the allantoic cavity.

Fig. 3. Electron micrographs of Kunitachi virus stained with phosphotungstic acid; (a) typical rounded and filamentous forms were seen, projections present in the outer coat of the particles seem to be arranged in a regular fashion; (b) a number of stripped virions were also present; (c) spontaneously released ribonucleoproteins which measure about 20 nm in diam.
of fertile hen’s eggs and reproduction is possible only in the amniotic cavity, whereas all avian paramyxovirus strains grow well in the allantoic cavity. Stable haemagglutinating activity appears only in purified virus suspensions, suggesting that crude samples contain some inhibiting components.

Several paramyxoviruses have been isolated from domestic and wild birds (Bankowski et al. 1960; Dinter et al. 1964; Wagner & Enders-Ruckle, 1966; McFerran et al. 1973; Webster et al. 1976) but Kunitachi virus has no serological relationship to known paramyxovirus strains of mammalian or avian origin including the recently isolated paramyxoviruses from ducks (Webster et al. 1976). We have already isolated three paramyxoviruses from budgerigars, which were found to be serologically related to Kunitachi virus (Table 3). Moreover, the paramyxovirus (TI strain) which is related to Kunitachi virus has been isolated from the same host in Tokyo (N. Yoshida et al., personal communication, 1975). It is assumed that paramyxoviruses such as Kunitachi virus are widespread among budgerigars in the Kanto area of Japan.

From the serological analysis and biological properties, it seems reasonable to assume that Kunitachi virus represents a new subtype of paramyxoviruses infecting budgerigars in nature. However, the final classification of Kunitachi virus must wait until its physico-chemical properties become clear.

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