Studies on a Paramyxovirus Isolated from Japanese Sparrow-hawks (*Accipiter virgatus gularis*)

IV. Hemagglutinating Activity of Two Clones of the Virus

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Abstract. The Takavirus, a variant of Newcastle disease virus (NDV), was found to agglutinate chicken erythrocytes unstably at room temperature. Two clones were obtained from the plaques formed in the chick embryo fibroblasts (CEF): one showed an constant hemagglutinating (HA) reaction only at 4°C (L⁻R⁺); while the other showed no significant reversion in an HA reaction both at 4°C and room temperature (L⁺R⁺). There was no difference in the structural polypeptides and serological identity among two clones of Takavirus and two strains of NDV. The hemolytic activity of the Takaviruses (L⁻R⁺, L⁺R⁺ and parent) was similar to that of NDV-Sato strain, but much lower than that of NDV-BI strain. The elution of L⁺R⁺ from the chicken erythrocytes occurred more rapidly at room temperature. The amount of the eluted virus was greater than that obtained from L⁺R⁺ or NDVs. The reaction did not occur when fresh viruses were added to the erythrocytes which had eluted the viruses.

Many strains of Newcastle disease virus (NDV) have been isolated; they show a broad spectrum of virulence for chickens and chicken embryos [21]. However, they do not have any significant antigenic difference, and they are not classified into distinct antigenic groups.

The Takavirus, a variant of NDV, isolated from Japanese sparrow-hawks (*Accipiter virgatus gularis*), was found to be serologically related to NDV, but was different from NDV in some biological aspects [2, 3]. The hemagglutinating (HA) reaction of Takavirus was especially temperature-dependent. A constant HA reaction was observed when tested at 4°C but not at room temperature (25°C) or at 37°C [2]. On the other hand, though the HA activity with NDV is best read at 4°C [1], all strains of NDV can agglutinate chicken erythrocytes at room temperature. Recent studies suggest that the negative conversion of the Takavirus in HA reaction at room temperature may be due to the high neuraminidase activity (NA) of the virus under a condition of pH 7.2 [17].

Influenza and paramyxoviruses contain neuraminidase on their envelopes [15]. The enzyme destroys the viral receptors on the erythrocytes, making the cells that eluted the viruses incapable of absorbing the virus any more.

In the present study, we found two clones in the stock of Takavirus: one showed an constant HA reaction only at 4°C, while the other showed no significant reversion in HA reaction at both 4°C and room temperature (25°C). The purpose of the present study is to describe the properties of two clones of the Takavirus.
Materials and Methods

Cell culture: Primary chick embryo fibroblast (CEF) cultures were prepared from 10-day-old specific pathogen-free (SPF) embryos as described by Hitchner et al. [9]. Cells were grown in 50 mm glass plates in Eagle's minimal essential medium (MEM) containing 10% of calf serum (CS) and 10% of tryptose phosphate broth (TPB, Difco) for growth medium, and 5% of CS and 10% of TPB for maintenance medium.

Embryo inoculation: Ten-day-old chick embryos were used for inoculation by allantoic cavity (AC) route. Death of embryos was recorded daily for three days.

Viruses: Takavirus, Sato (velogenic) and Hitchner B1 (gentleotic) strains of NDV, Yucaipa/chicken/California/60 (Yucaipa) and Turkey/Wisconsin/68 (Ty/Wis) were used in the present experiment. Ty/Wis was kindly supplied by Dr. K. Nerome (National Institute of Health, Tokyo). The source of other viruses was described in the previous paper [2]. Except for the viruses used for plaque formation which were propagated in CEF, all the viruses were propagated in the AC of embryonated eggs and stored at -80°C. Takavirus and NDV-Sato strain were harvested in 48 hr, other viruses were harvested in 60 hr. Viruses with three to five passages in embryonated eggs constituted the original stock.

Hemagglutination and hemagglutination-inhibition (HI) tests: HA and HI tests were carried out by the micromethod developed by Sever [19]. HA titers at 25°C were expressed as reciprocals of minimum and maximum dilutions of virus preparation that showed HA reaction against 0.5% chicken erythrocytes, which were suspended in phosphate buffered saline (PBS, pH 7.2), and the titers at 4°C were expressed as reciprocals of the maximum dilution of virus preparation that showed HA reaction. HI titers were measured by adding 4 HA units of virus to two-fold dilutions of antiserum. After 1 hr at room temperature, 0.5% chicken erythrocytes were added and the observations were carried out after 1 hr of incubation at 4°C.

Plaque formation and isolation of clones: The confluent monolayers of the primary culture were used for the plaque formation. After removal of the growth medium the monolayer was infected with 0.2 ml of virus preparation suitably diluted with MEM. At the end of a 30 min absorption period at 37°C, the excess virus fluid was removed and the cells overlaid with an agar overlay medium (A.O.M.) containing 0.75% special agar noble (Difco) and 0.01% DEAE-DEAE-Dextran in the maintenance medium. Twenty minutes were allowed for the agar to harden, and the plates were then returned to the CO2 incubator in an inverted position. Sixty-eight hours after infection, a second A.O.M. was added consisting of the same formula as the first A.O.M. except for the neutral red was added (0.01%). Five hours after addition of the second overlay, well isolated plaques were observed in some plates. Cloning of virus was made by picking the plaques with a 2-mm-bore Pasteur pipette and the cloned virus was propagated in the second culture of CEF. They were then tested for HA reaction. In order to purify the clone, the picking, seeding, and growing of plaques was repeated three consecutive times.

Serial passages in embryonated eggs: The virus preparation of the third passage in CEF of each clone (L-R+ and L-R-) was further passed in embryonated eggs. The virus preparations were separately harvested from each embryonated eggs. Then they were separately tested for HA activity. The progenies having an HA activity of either L-R- or L-R+ were used for next passage. The passage was repeated four times.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE): The procedures for the preparation of gels and electrophoresis have been described [22]; 7.5% acrylamide gels, approximately 11 cm long, were used. Each sample was dissolved with 1% SDS and 1% 2-mercaptoethanol. At the end of the run, gels were removed from the tubes, fixed and stained with 0.25% Coomassie brilliant blue R-250 in 50% methanol and 10% acetic acid and destained by the leaching method with 7.5% acetic acid. Alternatively, glycoproteins were stained with Schiff's reagent as described [6]. Molecular weight determinations were based on electrophoretic mobilities using NP (m.w. 56,000) and M (m.w. 41,000) [12] as internal standards.

Purification of virus: For preparation of purified virus samples, infected allantoic fluid was given a low speed spin and the virus was concentrated by centrifugation at 100,000×g for 1 hr. The pelleted virus was resuspended in 0.01 M sodium phosphate buffer (pH 7.2) and purified by centrifugation through sucrose density gradients (20-50%) dissolved in the buffer at 70,000×g for 1 hr. The fractions having HA activity were pooled and used for SDS-PAGE.

Elution of the viruses from chicken erythrocytes: Fifty µl of virus preparation was mixed three times with 50 µl of 2% chicken erythrocytes, which were suspended in PBS (pH 7.2), at intervals of 20 min. After the mixtures were kept for an additional 20 min at 4°C, they were warmed to 25°C, and then
PARAMYXOVIRUS FROM JAPANESE SPARROW-HAWKS

Table 1. Hemagglutinating reaction of 3 avian paramyxoviruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>HA reaction at</th>
<th>4°C for 60 min</th>
<th>25°C for various lengths of time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>45</td>
</tr>
<tr>
<td>Taka</td>
<td>128&lt;sup&gt;3)&lt;/sup&gt;</td>
<td>16-128&lt;sup&gt;4&lt;/sup&gt;</td>
<td>32-128</td>
</tr>
<tr>
<td>NDV-Sato&lt;sup&gt;1)&lt;/sup&gt;</td>
<td>256</td>
<td>4-256</td>
<td>16-256</td>
</tr>
<tr>
<td>NDV-B1&lt;sup&gt;2)&lt;/sup&gt;</td>
<td>1,024</td>
<td>4-1,024</td>
<td>4-1,024</td>
</tr>
</tbody>
</table>

1) NDV-Sato strain (velogenic).
2) NDV-Hitchner B1 strain (lentogenic).
3) HA titer.
4) HA titers at 25°C are expressed by range as reciprocals of minimum and maximum dilutions of virus preparations that showed HA reaction against chicken erythrocytes.

Portions of the mixtures were removed at an interval of 15 min for HA test. The suspensions were well mixed each time. The erythrocytes in the samples were quickly sedimented at low centrifugal speed, and the supernatant fluids were examined for HA activity. The amount of eluted viruses was expressed as a percentage of the titer of the starting materials.

Hemolysis: Chicken erythrocytes were washed three times in PBS (pH 7.2). Assays were performed by incubation of 1 ml of virus suspension and 1 ml of 2% chicken erythrocyte suspension for 3 hr at 37°C in a water bath. The tubes were centrifuged at 1,500 rpm for 5 min; the supernatant fluids were diluted and measured for hemolysis according to their absorbance at 540 nm.

Agar gel precipitation (AGP) test: The AGP test was performed as previously described [10]. The viral antigens for AGP test were prepared from the purified viruses (L-R-, L-R, NDV-Sato, NDV-B1, Yucaipa and Ty/Wis) which were adjusted to 5 mg protein per ml in PBS-containing 1 M KCl and solubilized with Triton X-100 (final concentration at 2%) at 37°C for 30 min.

Antisera: Antisera against viruses used in both HI and AGP tests were prepared in 6-month-old chickens. Details for preparation have already been described in the previous paper [2].

Results

Hemagglutination tests

Three avian paramyxoviruses were tested for HA activity at 4°C and room temperature (25°C). In the case of 4°C, the results were observed after 60 min, because the reaction was always constant for over 90 min.

At room temperature, the HA reactions were read at intervals of 15 min. The results read at room temperature were inconsistent, depending on the virus strains and the reading time (Table 1). The positive HA reaction of the Takavirus converted into a negative reaction after 75 min. On the other hand, no significant reversion in the titers of the two strains of NDV could be noted during the observation periods. In particular the titer of NDV-B1 strain was unchanged for 90 min.

Cloning of the virus with plaque formation and serial passages in embryonated eggs

The plaques of the Takavirus formed in CEF were heterogeneous in size. Several clones were isolated from the plaques. These clones could be divided into two groups: one showed an constant HA reaction only at 4°C, similar to the parent Takavirus, while the other showed no significant reversion in HA reaction when observed even at 25°C. The former was designated L-R- and the latter was designated L-R+.

Attempts were made to purify two clones by plaque cloning technique in CEF (Table 2) and also by serial passages in embryonated eggs (Table 3).

As shown in Table 2, the ratio of L-R+ or L-R- in each passage of the two clones
Table 2. The ratio of L'R⁺ or L'R⁻ in each passage of two clones

<table>
<thead>
<tr>
<th>No. of passage</th>
<th>L'R⁺ clone</th>
<th>L'R⁻ clone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of L'R⁺ clone / No. tested (%)</td>
<td>No. of L'R⁻ clone / No. tested (%)</td>
</tr>
<tr>
<td>1</td>
<td>11/24 (45.8)</td>
<td>12/16 (75.0)</td>
</tr>
<tr>
<td>2</td>
<td>12/27 (44.4)</td>
<td>15/16 (93.8)</td>
</tr>
<tr>
<td>3</td>
<td>9/16 (56.3)</td>
<td>22/24 (91.7)</td>
</tr>
</tbody>
</table>

1) Number of clones of which the HA reaction was L'R⁺ (progeny of L'R⁺ clone).
2) Number of clones of which the HA reaction was L'R⁻ (progeny of L'R⁻ clone).

Table 3. Passages of two clones in embryonated eggs

<table>
<thead>
<tr>
<th>No. of passage</th>
<th>L'R⁺ progenies 1)</th>
<th>L'R⁻ progenies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of L'R⁺/ No. of embryos tested (%)</td>
<td>No. of L'R⁻/ No. of embryos tested (%)</td>
</tr>
<tr>
<td>1</td>
<td>6/6 (66.7)</td>
<td>6/10 (60.0)</td>
</tr>
<tr>
<td>2</td>
<td>7/10 (70.0)</td>
<td>5/6 (83.3)</td>
</tr>
<tr>
<td>3</td>
<td>9/13 (69.2)</td>
<td>11/11 (100.0)</td>
</tr>
<tr>
<td>4</td>
<td>11/16 (68.8)</td>
<td>15/33 (45.5)</td>
</tr>
</tbody>
</table>

1) Progenies were tested for HA activity in each embryonated eggs.

was relatively unchanged. The materials of the third passage of each clone in CEF were further passed in embryonated eggs for four times. Table 3 shows the results of the HA reaction of virus preparations harvested separately from each embryonated eggs. Although the two clones having the HA properties of either L'R⁺ or L'R⁻ were passed separately, the heterogeneity of the two clones remained unchanged. These results indicate that we were unable to obtain as a purified virus being characteristic of either L'R⁺ or L'R⁻. In some progenies of L'R⁻ propagated in different embryonated eggs, however, the negative conversion in HA reaction occurred in 30 or 45 min, faster than that of the parent virus (Table 4). Therefore, two progenies composing mainly of either L'R⁻ or L'R⁺ were used in the following experiments.

Serological tests

No apparent serological difference between Takaviruses (L'R⁺, L'R⁻ and parent) and NDVs was observed in both HI and AGP tests. Furthermore, there was no serological difference between two clones of Takavirus (L'R⁻ and L'R⁺) (data is not shown).

SDS-polyacrylamide gel electrophoresis

When the polypeptides of the two progenies of Takavirus and the two strains of NDV were compared by SDS-PAGE, six major polypeptides were detected in all viruses. Two of the polypeptides were glycoproteins, which appear to be HN and F glycoproteins and molecular weights estimate to be 74,000 and 56,000, respectively. Other proteins had a molecular weight of 130,000, 51,000, 46,000 and 41,000. However, the molecular weight of the viral proteins among four viruses were identical (data is not shown).

Hemolysis

Hemolytic activity of Takaviruses (L'R⁺, L'R⁻ and parent) and NDVs were com-
Table 4. Comparison of HA reaction of two kinds of clones propagated in different embryonated eggs

<table>
<thead>
<tr>
<th>Virus</th>
<th>HA reaction&lt;sup&gt;1) at 4°C for 60 min&lt;/sup&gt;</th>
<th>25°C for various lengths of time (min)</th>
</tr>
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<tr>
<td></td>
<td>30</td>
<td>45</td>
</tr>
<tr>
<td>L&lt;sup&gt;+&lt;/sup&gt;R&lt;sup&gt;+&lt;/sup&gt;</td>
<td>128&lt;sup&gt;2) &lt;/sup&gt;</td>
<td>4-128&lt;sup&gt;3) &lt;/sup&gt;</td>
</tr>
<tr>
<td>L&lt;sup&gt;-&lt;/sup&gt;R&lt;sup&gt;-&lt;/sup&gt;</td>
<td>128</td>
<td>4-128</td>
</tr>
<tr>
<td>Parent</td>
<td>128</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>128</td>
<td>16-128</td>
</tr>
</tbody>
</table>

<sup>1) HA titers showed in this table are two examples of each progeny.</sup>
<sup>2) HA titer.</sup>
<sup>3) HA titers at 25°C are expressed by range as reciprocals of minimum and maximum dilutions of virus preparations that showed HA reaction against chicken erythrocytes.</sup>
<sup>4) Negative.</sup>

Fig. 1. Hemolytic activity of the parainfluenzoviruses. Viruses (HA titer 1:128) were suitably diluted and mixed with 2% chicken erythrocyte suspension. After incubation at 37°C for 3 hours, the mixtures were centrifuged; the supernatants were measured for hemolysis. Hemolytic activities were expressed as optical density (OD) reading at 540 nm.

- - - Takavirus L<sup>+</sup>R<sup>+</sup>, △ — △ Takavirus parent, ■ — ■ Takavirus L<sup>-</sup>R<sup>-</sup>, ○ — ○ NDV-Sato, △ — △ NDV-B1, □ — □ Yucaipa, ▽ — ▽ Ty/Wis.

Fig. 2. The elution of the viruses from chicken erythrocytes.

The amount of the eluted viruses was measured by HA reaction. For this experiment, the absorption was carried out for 60 minutes at 4°C, and the removed at 25°C.

- - - Takavirus L<sup>-</sup>R<sup>-</sup>, △ — △ Takavirus parent, ■ — ■ Takavirus L<sup>+</sup>R<sup>+</sup>, ○ — ○ NDV-Sato, △ — △ NDV-B1.

Elution of the viruses from chicken erythrocytes

The elution pattern of the viruses from chicken erythrocytes was examined at room temperature (25°C). The elution of L<sup>-</sup>R<sup>-</sup> from the chicken erythrocytes occurred more rapidly and the amount of the eluted virus was greater than that obtained from L<sup>+</sup>R<sup>+</sup> or NDV (Fig. 2). The HA reaction did not occur when fresh viruses were added to the
erythrocytes which had eluted the viruses.

Discussion

Takavirus can agglutinate chicken erythrocytes unstably at room temperature (25°C). This virus appears to be a variant of NDV, but the positive HA reaction of the virus converted into the negative reaction at room temperature (25°C) [2]. This phenomenon may be due to the high NA of the virus [17]. The present studies indicate that Takavirus could be divided into two groups by plaque cloning technique; one of which showed an constant HA reaction only at 4°C (L' R-); and the other showed no significant reversion in an HA reaction both at 4°C and 25°C (L' R'). However, we could not isolate them as a single population in the CEF. The results suggest that these characteristics may be due to not only genetic factors but also non-genetic interactions such as phenotypic mixing [7, 8].

Although two clones were passed four times more in embryonated eggs to obtain the virus having HA characteristics of either L' R- or L' R', the heterogeneity of HA activity remained unchanged. The reason for the heterogeneity is not known, however, it was suggested that the heterogeneity was not caused by the factor of host cells but by the specific characteristics of Takavirus or NDV in general. Therefore, to prepare the experimental stocks for each progeny virus, L' R- or L' R' clone was propagated in embryonated eggs, and the HA activity of allantoic fluid from individual eggs were separately examined. The progenies that indicated either L' R- or L' R' were pooled and used for the characterization of Takavirus in the present experiment.

The clones of Takavirus were serologically identical to NDVs. The results of the present study agree with those reported by Arias-Ibarrondo et al. [2, 3], and the two clones of Takavirus were also demonstrated to be antigenically identical.

NDV contains at least six polypeptides [5, 11, 12]. Two of the polypeptides are glycoprotein with molecular weights of 74,000 and 56,000 [12]. The surface antigens, hemagglutinin and neuraminidase of paramyxoviruses have been identified as glycoprotein [4] and both activities of NDV reside on the same glycoprotein molecule [16, 18]. Nagai et al. [13, 14] reported the precursor proteins of HN and F proteins which existed in low-pathogenic strains of NDV propagated only in cultured cells. Neither of them had biological activity. If the Takavirus had such precursor protein when propagated even in embryonated eggs, the negative conversion of HA reaction might occur. In the present experiment, we could not find the precursor proteins of the Takaviruses (L' R' and L' R-) propagated in embryonated eggs. Furthermore, no difference was found in the molecular weights of viral proteins among Takaviruses (L' R' and L' R-) and the two strains of NDV which were propagated in embryonated eggs. Therefore, above mentioned possibility is denied. The thermolabile HA reaction of Takavirus appears not to be attributed to the precursor protein.

Hemagglutination of chicken erythrocytes is known to occur by absorption of virus particles onto erythrocytes. The interaction of virus and erythrocytes at 37°C occurred in two phases: first, a combination of the virus and cells and second, some alteration or modification of the cells accompanied by an elution of the viruses. Erythrocytes which had absorbed and then fully eluted the viruses would no longer agglutinate upon addition of fresh virus suspensions, and thus such cells considered to be incapable of reabsorbing a detectable amount
of fresh viruses. In the latter stage of the interaction, the modification of cells may have been caused by an enzyme carried by the viruses. The enzyme is identified as neuraminidase, and exists on the surface glycoprotein of influenza and paramyxoviruses [15]. Spalatin et al. [20] showed that wide variations existed in elution of NDV strains and even clones of some strains from chicken erythrocytes. However, the elution of L⁺R⁻ appears to be much rapid than that of NDV strains described. The significant difference in the elution time among the paramyxoviruses was found in the present experiment. The rate of the elution of Takavirus-parent from chicken erythrocytes was faster than that of L⁺R⁺ or NDVs but slower than that of L⁺R⁻ (Fig. 2, Table 4). Similarly, when the ratio of the neuraminidase to hemagglutinin titers compared, the ratio for Takavirus-parent was much higher than that for L⁺R⁺ but much lower than L⁺R⁻ [17]. These results indicate that Takavirus-parent consists of the mixture of L⁺R⁻ and L⁺R⁺, and that the negative conversion in HA reaction of L⁺R⁻ at room temperature may be due to the elution of the virus from erythrocytes and occur under the influence of the NA of the virus [17].

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References


要約

タカ（ツミ）から分離された paramyxovirus に関する研究。2 種のクローンの赤血球凝集能：関崎 勁・伊沢久夫・小沼 操・見上 雄（北海道大学獣医学部家畜伝染病学講座）——ニューカッスル病ウイルス（NDV）の変異株であるタカウイルスは、室温での赤血球凝集（HA）反応が不安定である。ニワトリ胚腺雛を供試細胞に形成させたプラックにより、本ウイルスをクローン化することによって、4℃でのみ一定した HA 反応を起こすウイルス（L⁺R⁻）と、4℃および室温の両温度で HA 反応を起こすウイルス（L⁺R⁺）が得られた。これらウイルスは、構成蛋白においても、血清学的性状においても互いに類似しており、2 株の NDV との間にも差異が認めなかった。一方、タカウイルス（L⁺R⁺, L⁺R⁺ および親株）の溶血能は、NDV 佐藤株と同程度であったが、NDV-B 1 株に比べ低かった。室温におけるニワトリ赤血球からのウイルス遊出は、L⁺R⁺ や 2 株の NDV に比べて L⁺R⁻ が最も早く、またその遊出量も L⁺R⁺ が最も多かった。ウイルスを遊出した後の赤血球に、新たにウイルスを加えても、HA 反応は起こらなかった。