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

I. Isolation and Characterization of the Virus

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Abstract. A virus isolated from the brains of young Japanese sparrow-hawks suffering from an acute infection with a slight nervous symptom was shown to have the morphological, physical and chemical characteristics of a paramyxovirus. The virus was serologically related to Newcastle disease virus and Bangor virus but differed from them in some biological aspects mainly in hemagglutination (HA) activity. The HA reaction of the virus was temperature-dependent. The constant HA reaction was observed when tested at 4°C but not at room temperature or 37°C.

Paramyxoviruses other than Newcastle disease virus (NDV) have been isolated from several avian species including chicken [4], turkeys [Tumova and Lang, unpublished results], finch [16], parrots [6], budgerigars [18, Nerome, unpublished results], pigeon [Kida and Yanagawa, unpublished results] and ducks [26]. Also, NDV has been iso-

lated from many avian species [10, 11, 20]. Among avian paramyxoviruses, there are at least 3 or possibly 4 serotypes with various degrees of cross reaction when examined by the hemagglutination inhibition (HI) and the serum neutralization tests [1, 16, 18]. These include NDV, Yucaipa, Bangor and a parainfluenza isolated from turkey. Al-

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オオタカ (ツミ) から分離された paramyxovirus に関する研究 I. ウイルスの分離・同定: アリラス-イバロンド・ヘスース・見上 彪・山本 博・古田有一・石岡佐平 (北海道大学獣医学部家畜伝染病学教室), 岡田幸助 (北海道大学獣医学部比較病理学教室), 佐藤儀平 (帯広畜産大学獣医公衆衛生学教室)

though most of the paramyxoviruses have both hemagglutinin and neuraminidase, they are not classified into distinct antigenic groups such as the influenza viruses.

The present communication describes the investigation of an infectious agent isolated from young Japanese sparrow-hawks suffering from an acute infection with a slight nervous symptom. The agent, designated the Taka virus, was serologically related to NDV and Bangor virus, but differed from them in some biological aspects mainly in hemagglutination (HA) activity. Discussion was made on the possibility that the virus isolated was a new variant of NDV.

Materials and Methods

Eggs: The fertile eggs for embryo inoculation and chick embryo fibroblast (CEF) cultures, and chicks for the source of the kidney cultures were obtained from a specific pathogen free (SPF) flock maintained in our laboratory.

Cell culture: The methods for the preparation of CEF culture and for the titration of the virus by plaque assay were those described [12]. Briefly, CEF were prepared from 9–10 day old embryos and grown in 60 mm plastic plates using Eagle's minimal essential medium (MEM) containing 10% calf serum for growth medium and 5% calf serum for maintenance medium. Chick kidney (CK) cultures were prepared as described [19].

Embryo inoculation: Nine to 10 day old embryos were used for inoculation by chorioallantoic cavity (CAC) route. Titrations were made in the usual manner using 0.1 ml as infectious dose and four eggs per dilution. Death of embryos was recorded daily up to 7 days after inoculation and titers were calculated by the method described by Reed and Muench [22].

Viruses: A virus designated the Taka virus, was isolated from the brains of Japanese sparrow-hawk (*Accipiter virgatus gularis*). Virus with three to five passages in chicken embryos was used throughout the experiments. The details will be described in the following section. Yucaipa/chicken/California/60 (Yucaipa) was kindly supplied by Dr. T. Horiuchi (National Institute of Animal Health, Seki), Bangor/Finch/N. Ireland/73 (Bangor) was kindly supplied by Dr. K. Nerome (National Institute of Health, Tokyo), and Sato (velogenic), Miyadera (mesogenic) and Hitchner B1 (lentogenic) strains of

NDV were kindly supplied by Dr. C. Morita (Research Center for Veterinary Science of the Kitasato Institute, Kashiwa). All six viruses were propagated in the allantois of 10-day-old embryos; Taka virus, Sato and Miyadera strains of NDV, and Bangor were harvested 2–3 days after inoculation, Yucaipa and NDV Hitchner B1 were harvested 5–6 days after inoculation. For serological examination the propagated viruses in eggs were used, and for physical and chemical characterization studies, the Taka virus propagated in CEF was used.

Serological tests: Both the HA and the HI tests were carried as described [12]. The tests were done by using a microtiter system. Unless specified HA and HI tests using chick erythrocytes were carried out at 4°C.

The antigenic relationship between two viruses were calculated, using the formula $r = \sqrt{r_1 \times r_2}$ described by Archetti and Horsfall [2], in which r_1 was the ratio of dividing the heterologous titer obtained with virus 2 by the homologous titer obtained with virus 1, and r_2 was the ratio of dividing the heterologous titer with virus 1 by the homologous titer with virus 2. If r equals 1, there is no antigenic difference and an r value of 4 indicates an antigenic cross relationship of 25 percent. The larger r value indicates the less antigenic relationship and an r value of 4 or greater is considered as a significant antigenic difference between strains [16].

Antisera: Anti-NDV serum used in the present experiments was the convalescent serum from chickens infected with NDV during the outbreak in the field. Antisera against Taka virus, Bangor and Yucaipa were separately prepared in 6-month-old chickens by three intramuscular inoculations of each antigen at intervals of 1 or 2 weeks. Two ml of antigen mixed with an equal amount of Freund's complete adjuvant (Yatoron Co. Ltd., Tokyo) were used for the first inoculation, and 1.5 ml of antigens without adjuvant were used for the second and third inoculations. Anti-sera were harvested 8–10 days after the last inoculation. The antigens used for immunization were originated from the infectious allantoic fluid, which was inactivated with formalin in a final concentration of 0.04% for 1 week at 4°C. The inactivated viruses were given a low speed spin and the clarified supernatants were recentrifuged at 27,000 rpm for 90 minutes. The resuspended pellets in the appropriate buffer were further purified by centrifugation using a SW 65 rotor through sucrose gradients (10–50%) at 45,000 rpm for 90 minutes. The fractions having HA activity were pooled and used as the antigens. HA titer of Taka antigen was 1:5000 and those of

Yucaipa and Bangor were 1:20,000. All preimmune sera were found to be no inhibition with any paramyxoviruses used. Before using in HI tests, all sera were treated with double amount of receptor destroying enzyme (Takeda Chemical Industries, Ltd.) than that recommended by the manufacturer, incubated overnight at 37°C, and then heated at 56°C for 30 minutes.

Electron microscope: The infected CEF was fixed in glutaraldehyde and osmium tetroxide and embedded in Epon 812 as described [19]. Ultra-thin sections were stained with uranyl acetate and lead citrate. For negative staining, the infectious allantoic fluid clarified at 5,500 rpm for 20 minutes was pelleted at 27,000 rpm for 90 minutes, in the Hitachi RP 30 rotor and stained with 2% silicotungstate (pH 7.2). Both materials were examined with HU-12A type electron microscope (Hitachi Co. Ltd.).

Temperature sensitivity: Suspensions of tenfold dilutions of the virus were divided into test tubes and held at 56°C for different periods of time up to 1 hour. The infectivity for each dilution was examined in CEF.

Chloroform sensitivity: Chloroform sensitivity of the virus was determined as suggested [15]. After treatment the virus was titrated in CEF for infectivity.

pH sensitivity: A serial dilution (10^1 – 10^8) of the virus was made in McIlvaine buffer of pH 7 and pH 3. Following storage at 4°C for 24 hours, each dilution was examined for infectivity in CEF.

Filtration experiment: Suspensions of the virus (1/100 dilution in MEM) were filtrated through Millipore filters of 300, 220 or 100 nm by the method described previously [17]. Each filtrate was examined for infectivity in CEF.

Nucleic acid type: Determination of nucleic acid type was achieved by testing 5-iododeoxyuridine (IUDR, 100 µg/ml) for inhibition of multiplication of the virus in CEF cultures. As control for a known DNA virus, herpes virus of turkey (HVT) was used.

Results

History and isolation of the Taka virus

On June 28, 1973, our laboratory was requested to investigate a disease on a commercial falcon breeder operation in Shikoku. This organization had been having problems in young Japanese sparrow-hawks (*Accipiter virgatus gularis*) aging from 10 to 20 days. The disease was first recognized

with a slight nervous symptom with unstable legs and tremor. Within a few days, the birds showed signs of anorexia and vomiting. Death preceded by prostration and respiratory difficulty occurred in 5–7 days. No diarrhea was observed. The spread of the disease in young birds was very rapid and the mortality was usually up to 90–100%. The epizootic form of the disease in young birds occurred for three consecutive years whenever they were introduced into the flock. However, the adult birds kept in this flock never showed any symptom of the disease and remained healthy up to today. At necropsy, no significant changes were observed. A similar disease was also observed in Japanese hawk-eagles (*Spizaetus nipalensis orientalis*) reared in the same house with the diseased birds. Pools of the brains from four dead young birds were ground in sterile sand and tryptose phosphate broth. A portion of the suspensions was left untreated and the second portion treated with 1,000 units of penicillin and 0.5 mg of streptomycin per ml, and the suspensions were allowed to stand at room temperature for 2 hours before inoculating into chicken embryonating eggs. The untreated portions were tested for the presence of bacterial pathogens, mycoplasma and chlamydia, and found to be negative. The treated suspensions were inoculated into chicken embryonating eggs via yolk sac or chorioallantoic cavity. Embryos died between 24 and 48 hours after inoculation. The most constant lesions were subcutaneous and intracerebral hemorrhage. No pocks were found on the chorioallantoic membrane. The allantoic fluids harvested from dead embryos were pooled and stored at –20°C and served as an original virus.

Embryo inoculation

The original virus was passed three times

in embryos via chorioallantoic route and the titration was made. The most embryos died between 24 to 48 hours after inoculation. However, some embryos died at 4th day when inoculated with higher dilutions of virus. The titer was found to be $10^{8.37}$ ELD₅₀/ml, and the mean death time of minimum lethal dose in hours was 46.

Tissue culture inoculation

The virus was cytopathic for CK or CEF cultures, the cytopathic effects (CPE) being characterized by the appearance of elongated swollen cells which detached later from monolayers. The CPE appeared within 2 days and the monolayers were completely destroyed by 4th to 5th days. The plaques formed in CEF were heterogeneous in size, varied from 0.5 to 3 mm in diameter. Upon staining coverslip cultures with May-Grünwald Giemsa, the cytological changes were characterized by the presence of polykaryotic multinucleated cells. Titer as measured by plaque formation was 1×10^8 PFU/ml.

Electron microscopy

Infected CEF cultures were examined by the thin sectioning technique of electron microscopy and the concentrated culture fluid by centrifugation were examined by negative staining. Sections of the cells showed the presence of typical pleomorphic paramyxovirus particles in the extracellular spaces (Fig. 1). The particles with spikes had an average diameter of 125 nm with a range of 96–160 nm. Many filamentous forms having an average length of 730 nm with a range of 300 nm–2,100 nm were observed. The negatively stained preparation showed that the diameter of the nucleocapsid was 17–19 nm and the length of the envelope projections were approximately 8 nm.

Table 1. The effect of different treatments on the infectivity of the Taka virus

Treatment		Titer (PFU/0.1 ml)
56°C	5 min	3×10^4
	15 min	6×10^2
	30 min	0
	60 min	0
pH	3.0 for 24 hr	5×10^4
	7.0 for 24 hr	5×10^6
Chloroform		0
Control (TC propagate)		1×10^7

Physical and chemical nature of the Taka virus

Results of temperature, pH and chloroform sensitivity of the virus were shown in Table 1. The virus found to be partially thermolabile and acidlabile. The treatment of the virus at 56°C for 5 minutes or in McIlvaine buffer of pH 3 resulted in decrease of titer by more than 2 log. Furthermore, the virus was sensitive to chloroform treatment.

Attempts to determine the average size of the virus by filtration experiments showed that the virus consistently passed filter membrane of 300 nm but not 100 nm pore diameter. When the filter membrane of 220 nm was used in three separate experiments, the results were inconsistent, in which the virus passed through in one attempt but did not in other experiments.

The addition of IUDR to the medium of CEF cultures at the time of infection did not prevent the development of CPE. The titers measured by CPE in both infected cultures with or without the presence of IUDR were similar. On the other hand, the multiplication of HVT used as control was completely inhibited by using the same medium. These results indicate that the nucleic acid type of the Taka virus is RNA.

Serology

Hemagglutination: Four avian para-

Table 2. HA titers of four avian paramyxoviruses tested at different reaction temperature

Reaction time (minutes)	Virus	HA titer		
		4°C	Room temp.	37°C
30	Taka	80	<10	<10
	NDV B1	1280	1280	1280
	PMY	160	160	160
	Bangor	320	320	320
45	Taka	80	<10	<10
	NDV B1	1280	1280	1280
	PMY	160	80	160
	Bangor	320	160	160
60	Taka	80	<10	<10
	NDV B1	1280	1280	1280
	PMY	160	80	160
	Bangor	160	160	160
90	Taka	80	<10	<10
	NDV B1	1280	1290	*
	PMY	160	90	160
	Bangor	160	80	160

Remarks.

*: Hemolyzed.

myxoviruses were tested for HA activity at 4°C, 22°C and 37°C, and the reactions were read at different times (Table 2). The HA reaction of the Taka virus was found to be temperature-dependent. The constant HA reaction was observed when tested at 4°C but not at room temperature (22°C) or 37°C. When the 10 times concentrated Taka virus was tested for HA at room temperature, some HA reaction was observed after 20 minutes. The titer was always 8 to 16 times lower than that obtained at 4°C, however, the reaction became negative within 30 to 45 minutes. On the other hand, no significant difference in titer of other three paramyxoviruses could be detected when the HA tests were carried at 4°C, 22°C or 37°C.

Thermostability of hemagglutinin: The thermostability of the hemagglutinin of the paramyxoviruses was examined after treatment at 56°C for different periods (Table 3). The hemagglutinin of the Taka virus

Table 3. Thermostability of the hemagglutinin of avian paramyxoviruses after treatments at 56°C for various times

Time in minutes	HA titer					
	Taka	B1	Miyadera	Sato	Yucaipa	Bangor
0	128	256	256	256	128	256
5	128	<2	128	128	32	256
10	64	<2	64	128	<2	256
15	16	<2	16	64	<2	32
20	16	<2	16	64	<2	16
30	<2		<2	8		8
45	<2		<2	2		4
60	<2			<2		4
90	<2			<2		<2
120	<2					<2

Table 4. Cross hemagglutination-inhibition tests between four strains of avian paramyxoviruses

Virus	Antiserum titer			
	Taka	NDV	Yucaipa	Bangor
Taka	1024	129	8	256
NDV	256	256	<8	128
Yucaipa	32	8	512	32
Bangor	128	128	64	512

and NDV-Miyadera was inactivated after treatment for 30 minutes. However, the inactivation of the hemagglutinin of NDV-B1 and Yucaipa took 10 minutes, and that of Bangor and NDV-Sato took 90 minutes and 60 minutes, respectively.

Cross HI test between Taka virus and other paramyxoviruses: The results of cross HI test between Taka virus and other paramyxoviruses are shown in Table 4. Various degrees of cross reaction among avian paramyxoviruses were observed. The Taka virus cross-reacted with both NDV and Bangor but did not significantly with Yucaipa. The fourfold or more difference in HI titer was found between NDV and Yucaipa, and Yucaipa and Bangor. If the formula of Archetti and Horsfall [2] is applied for analysis of these cross-HI results, Taka

virus against NDV and Bangor gave r values of 2.8 and 4, respectively. Bangor against NDV gave an r value of 2.8. These analysis indicate that antigenic cross relationship of Taka virus against NDV is 74.7%, and against Bangor is 25%, and that of Bangor against NDV is 74.7%.

The Taka antiserum did not react significantly with antigens prepared from Turkey/Wisconsin, hemagglutinating virus of Japan and Kunitachi virus which is a paramyxovirus isolated from budgerigars (Nerome, unpublished results).

Discussion

The observations presented are concerned with isolation of a virus from a flock of young Japanese sparrow-hawks with a slight nervous symptom and unusually high mortality. The virus, referred to as the Taka virus, was morphologically indistinguishable from paramyxoviruses.

According to its physical and chemical properties, the Taka virus belongs to the paramyxoviruses; of these it most closely resembles NDV. Like NDV, the multiplication of the Taka virus was not inhibited by IUDR indicating the virus contains RNA. The Taka virus is sensitive to chloroform and partially resistant to acid. Furthermore, the virus was readily propagated in CEF and formed clear plaques of different size, ranging from 0.5 to 3 mm in diameter. Mortality in infected embryo was rather rapid and the death usually occurred within 48 hours. Thermostability of the hemagglutinin of the virus is similar to mesogenic strain of NDV (Miyadera) and the virus lose the hemagglutinating activity on treatment at 56°C for 30 minutes. However, hemagglutinins of some velogenic strains of NDV [10], and Bangor virus [6, 16] were stable on treatment at 56°C for 1 hour or more.

The serological relationship among avian paramyxoviruses are of interest. Analysis of our cross-HI results by the method of Archetti and Horsfall [2] indicates little or no antigenic relationship between the Taka virus and Yucaipa, but some relationship to Bangor (25%) and a much closer relationship to NDV (74.7%). Our results of cross-HI test confirm those of others [1, 6, 16] in which hemagglutinin of Bangor and Yucaipa show some relationship to each other but have significant antigenic difference. However, if the results of cross-HI test between NDV and Bangor are subjected to the analysis, an r value of 2.8 is obtained. These results are very different from those reported by others [6, 16], who obtained about 64 times greater r value than our r value. The reason for the discrepancy is not known.

Although the Taka virus is antigenically related to NDV, the virus has an unusual HA activity and can agglutinate chicken erythrocyte at 4°C but fail to do it at room temperature or 37°C. Furthermore, following transferring the plates showing HA positive reaction at 4°C to room temperature, the reaction became negative within 30–45 minutes. With the exception of one paramyxovirus isolated from a budgerigar [18], all avian paramyxoviruses are able to agglutinate chicken erythrocytes. It is reported that while the HA activity with NDV is best read at 4°C [2], all strains of NDV can agglutinate chicken erythrocyte at room temperature. The failure of the Taka virus to agglutinate chicken erythrocytes at room temperature, may be due to a very rapid elution of the virus from erythrocytes. If the neuraminidase activity is associated with the release of virus from cells [14, 25], it seems that the neuraminidase of Taka virus has a strong activity at room temperature to break the receptors

on the erythrocytes, resulting in negative HA reaction. Another difference between Taka virus and NDV was that the optimal pH of the neuraminidase of Taka virus found to be 4.2 [3], whereas that of NDV was 5.0–5.2 [1, 5].

Concerning the pathogenicity in chickens, Taka virus is similar to the velogenic strain of NDV [13]. However, while Taka virus killed almost all chickens inoculated via intravenous at different dilutions, and in a less degree via intratrachea, the transmission of this virus to chickens by direct or indirect contact exposure was not as we could expect for a velogenic strain of NDV. Most of the controls remained without showing signs of the disease, even though they developed HI antibodies against the Taka virus [3].

Individuals in a natural population of NDV are not all identical [9]. Mutants among NDV which differ in virulence [23], heat resistance [8], agglutination of horse erythrocytes [7] and neuropathogenicity [21] have been reported. Since many strains of NDV with different biological and antigenic properties were isolated from many avian species [10, 24], the Taka virus could be a variant strain of NDV derived from one selective process that could take place in wild birds in nature. However, it is difficult at the present time to determine the origin of the Taka virus, further serological and pathological characterization will be required to obtain more conclusive evidences.

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Explanation of Figures

Fig. 1. Electron micrographs of thin sections of CEF cultures infected with Taka virus, showing typical paramyxovirus particles in the extracellular spaces. In all virus particles, nucleo-

capsids aligned just beneath viral envelope. $\times 34,000$. Inset: Viral particle is budding from the surface of an infected cell. $\times 70,000$.