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

II. Serology and Pathogenesis in Chickens

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Abstract. A virus isolated from the brains of Japanese sparrow-hawks and designated Taka virus was serologically studied by serum neutralization and neuraminidase inhibition tests. It was found to be antigenically related to Newcastle disease virus (NDV) with regard to hemagglutinin and neuraminidase, and to a lower degree to Bangor virus. The optimal pH of its neuraminidase activity was pH 4.2, which was different from that of the enzyme of NDV or Bangor virus. Taka virus was pathogenic for chickens, like a velogenic strain of NDV. In winter, however, its transmission to chickens was very low by direct or indirect exposure by contact. The environmental temperature might play a predominant role in the pathogenicity and transmission of Taka virus. These results indicate that Taka virus may be a new variant strain of NDV.

During their investigation of an etiological agent of an acute disease occurring in young Japanese sparrow-hawks, the authors isolated a virus and designated it Taka virus. This virus has morphological, physical and chemical characteristics of a paramyxovirus [3]. It was found to be serologically related to Newcastle disease virus (NDV) and Bangor virus when examined by the hemagglutination inhibition (HI) test, but was different from them in some biological aspects, particularly 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.

The present communication describes a further serological comparison of the virus

by serum neutralization and neuraminidase inhibition tests with other avian paramyxoviruses and the pathogenesis in chickens.

Materials and Methods

Cell culture: Primary chick embryo fibroblast (CEF) cultures were prepared from 10-day-old specific pathogen free (SPF) embryos on 60 mm plastic plates with Eagle's minimal essential medium, as described by Hitchner et al. [8].

Viruses: Taka virus, the Sato (velogenic) and Miyadera (mesogenic) strains of NDV, Yucaipa/Chicken/California/60 (Yucaipa) and Bangor/Finch/N. Ireland/73 (Bangor) were used in the present experiment. The source of these viruses was described in the previous paper [3].

All the viruses were propagated in the allantois of 10-day-old embryos obtained from an SPF flock maintained at the authors' laboratory. For the virus titration and cross serum neutralization test, eggs obtained from a local hatchery were used.

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Antisera: Antiserum against NDV used was the convalescent serum from chickens infected with NDV in the field. Antisera against Taka virus, Bangor, and Yucaipa were prepared in 6-month-old chickens. The detail for preparation was described in the previous paper [3].

Hemagglutination and hemagglutination inhibition test: Both HA and HI test (beta procedure) with 4HA units were carried out at 4°C, as described by Hitchner et al. [8]. The microtiter system was used for them.

Serum neutralization test: The serum neutralization (SN) test was carried out by using both 9-day-old fertile eggs and CEF cultures, as described by Hitchner et al. [8]. When embryos were tested, 100 ELD₅₀ (NDV, Taka, Bangor) or 100 EID₅₀ (Yucaipa) of virus was added to various serum dilutions. The plaque reduction test was conducted with 100 pfu/ml of virus. The SN titer in CEF culture was expressed by the reciprocal of the serum dilution which induced a 50% plaque-reducing end point calculated by using the formula described by Mitchel [14]. The SN titer in embryo was examined by 50% reduction of infectivity. Neutralization and neuraminidase inhibition values were used for the estimation of the degree of relationship expressed as percent by using the following formula of Archetti and Horsfall [2]:

$$\% \text{ relationship} = 100$$

$$\times \sqrt{\frac{\text{Heterologous titer 1}}{\text{Homologous titer 1}}} \times \frac{\text{Heterologous titer 2}}{\text{Homologous titer 2}}$$

Neuraminidase assay and neuraminidase inhibition tests: The neuraminidase activity and the neuraminidase inhibition tests were performed with fetuin as substrate in the same manner as reported by Webster and Campbell [19], except that 0.03 M citrate buffer was used at the optimal pH of a given enzyme. One unit of neuraminidase activity was defined as the dilution of virus that gave an optical density (OD) of 0.500 at 549 nm under standard conditions.

The titer in the neuraminidase inhibition test was expressed by the reciprocal of the serum dilution which caused 50% inhibition of one unit of neuraminidase activity [19].

For both tests, the allantoic fluid was collected from embryos inoculated with each of Taka virus, the Sato and Miyadera strains of NDV, and Bangor virus, and centrifuged at 5,500 rpm for 20 minutes. The resulting supernatant was again centrifuged at 19,000 rpm for 60 minutes. The resulting pellet was resuspended in such amount of phosphate-buffered saline, pH 7.2, as to make the resultant suspension one-tenth volume of starting material. The suspen-

sion was stored at -20°C.

Protein estimation: Protein was determined by the method of Lowry et al. [12].

Chicken inoculation: Three separate experiments were conducted with SPF and conventional chickens in summer and winter. The SPF chickens were derived from a flock maintained in the authors' laboratory. Seventeen conventional hens were obtained from a local breeder and these chickens had been immunized with NDV vaccine.

The chickens were inoculated with 0.2 ml of various dilutions of Taka virus by different routes. The virus used for the experiments was the 4th passage of an original isolate propagated in the allantoic cavity of embryos. Its titer was 10^{8.3} ELD₅₀/ml. In each experiment a group of chickens was placed in each cage of inoculated chickens to serve as a direct contact control. Another group of chickens was kept in the same room with the inoculated groups to use as an indirect contact control.

Virus recovery: For virus recovery, samples were collected from the trachea and cloaca of each chicken with cotton swabs on long applicator sticks every 48 hours after inoculation. The swabs were broken into pieces in screw cap tubes containing 1 ml of Bacto tryptose broth with penicillin (1,000 units/ml) and streptomycin (100 mg/ml).

At postmortem examination, the trachea, lungs, spleen and cloaca were removed aseptically from chickens. A 10% (w/v) suspension of tissues was made in Bacto tryptose broth with penicillin and streptomycin. Tissues and swabs were kept at -20°C until tested. All the samples were assayed for the presence of virus in CEF cultures.

Results

Neuraminidase

Fig. 1 shows the effect of pH on the neuraminidase activity of Taka virus, the NDV Miyadera strain, and Bangor virus. The optimal pH of Taka virus was 4.2. Bangor and the NDV Miyadera strain had an optimum pH of 4.0 and 5.0, respectively.

Thermostability of neuraminidase

Fig. 2 shows the thermostability of the neuraminidase activity of the four viruses. The temperatures which inactivated 50% of the virus-bound enzymes after 5 minutes' exposure were 57°C for Taka virus, 56°C for the NDV Sato strain, 54°C for the NDV Miyadera strain, and 53°C for Bangor

Fig. 1. Effect of pH on neuraminidase activity

Remarks.

The result is expressed as percent enzyme activity at an optimum pH for viruses. Citrate-phosphate buffer was used over the whole range. ○—○ Taka virus, ▲—▲ NDV Miyadera strain and ■—■ Bangor virus.

virus. All the viruses gave similar inactivation curves, except Bangor virus which presented a curve slightly different from that of any other virus.

Serological relationship between virus neuraminidases

Table 1 shows the results of the cross neuraminidase inhibition test among Taka virus, the NDV Miyadera strain, and Bangor virus. All the three viruses had immunologically similar neuraminidases. Taka virus against the NDV Miyadera strain, and Bangor virus gave a cross relationship of 89% and 52%, respectively. The cross relationship of the NDV Miyadera strain against Bangor virus was 90%.

Serum neutralization test

Table 2 shows the results of the cross

Fig. 2. Heat inactivation of neuraminidase activity

Remarks.

Each virus sample was diluted to give one unit of neuraminidase activity and adjusted to a protein concentration of 1 mg/ml with bovine serum albumin. It was incubated at the temperature indicated for 5 minutes, and placed immediately into an ice-bath prior to assaying. The result is expressed as percent of the control value obtained by preincubation of the virus sample at 36°C for 5 minutes. ○—○ Taka virus, ●---● NDV Sato strain, ▲—▲ NDV Miyadera strain and ■—■ Bangor virus.

SN tests carried out in CEF cultures with the four viruses. There was a considerable similarity in the antigenic pattern among the viruses used. When the formula of Archetti and Horsfall [2] was applied, the degree of cross neutralization between Taka virus and the NDV Sato strain was 77.7% and that between Taka virus and the NDV Miyadera strain 95%. The percent relationship of Taka against Bangor virus was 55.6% and that of Bangor virus against the NDV Sato and Miyadera strains 75.8% and 74.9%, respectively.

The cross SN test with the four viruses

Table 1. Neuraminidase inhibition test among three strains of avian paramyxoviruses

Virus	Antiserum titer*		
	Taka	NDV	Bangor
Taka	196	98	155
NDV, Miyadera strain	159	100	159
Bangor	36	93	179

Remarks.

*: Reciprocal of a serum dilution causing 50% inhibition of one neuraminidase unit.
The average of duplicate tests.

Table 2. Plaque-reduction neutralization tests among avian paramyxoviruses

Virus	Antiserum titer*		
	Taka	NDV	Bangor
Taka	724	195	363
NDV, Sato strain	647	288	426
NDV, Miyadera strain	794	234	338
Bangor	295	186	478

Remarks.

*: Reciprocal of a serum dilution causing a 50% plaque-reducing end-point.
The average of duplicate test.

Table 3. Comparison of infectivity of Taka virus administered at various doses by different routes at diverse environmental temperatures

Experiment No.	Chicken	Season	Route of inoculation	Virus dose (dilution)	Age (months)	No. of chickens			HI titer	
						Used	With symptoms	Died	Preinoculation	14 days after inoculation
1	SPF	Winter	IV	10 ⁻⁴	6	4	4 (2.5) *	4 (3.5) **	8	NT
			„	10 ⁻⁵	6	4	4 (3.5)	4 (5.2)	8	NT
			„	10 ⁻⁶	6	4	4 (3.8)	3 (5.7)	8	512
			„	10 ⁻⁷	6	2	2 (4.0)	1 (5.0)	8	512
			DC	—	6	3	2 (6.0)	1 (8.0)	8	16
			IT	10 ⁻⁵	6	2	2 (4.0)	1 (6.0)	8	256
			„	10 ⁻⁶	6	2	2 (4.5)	0 —	8	16-128
			DC	—	6	2	0 —	0 —	8	32-256
			IC	—	6	5	0 —	0 —	8	8-8
2	SPF	Summer	IT	10 ⁻⁴	1.5	2	2 (3.0)	2 (4.5)	8	NT
			„	10 ⁻⁵	1.5	2	2 (4.5)	2 (7.0)	8	NT
			„	10 ⁻⁶	1.5	2	2 (5.0)	2 (8.0)	8	NT
			DC	—	1.5	3	3 (7.0)	3 (8.3)	8	NT
			Cloaca	10 ⁻⁴	6	2	2 (3.0)	2 (5.5)	8	NT
			„	10 ⁻⁵	6	2	2 (4.0)	2 (7.5)	8	NT
			DC	—	6	1	1 (9.0)	1 (8.0)	8	NT
			IC	—	6	1	1 (9.0)	1 (8.0)	8	NT
3	Conventional	Winter	IT	10 ⁻⁵	18	2	2 (5.0)	0 —	8-32	256-512
			„	10 ⁻⁶	18	3	3 (6.5)	0 —	8-16	128-256
			„	10 ⁻⁷	18	3	0 —	0 —	8-32	16-256
			DC	—	18	4	0 —	0 —	8-16	16-64
			IC	—	18	3	0 —	0 —	8-16	16-512

Remarks.

IT: Intratracheal. DC: Direct contact control. IC: Indirect contact control. IV: Intravenous. NT: Not tested.

*: Average day on which chickens started to show symptoms.

**: Average day on which chickens died.

was also carried out in embryos. It gave results similar to those obtained from the same test in CEF. In addition, the difference between Taka and Yucaipa viruses was examined. No relationship, however,

was found between the two viruses.

Pathogenesis in chickens

To observe the pathogenicity of Taka virus for chickens, SPF and conventional chickens were injected with tenfold dilu-

tions of infected allantoic fluid by the intratracheal (IT) and intravenous (IV) routes in winter, and by the IT and cloacal routes in summer. The results obtained are summarized in Table 3. When inoculated by the IT route in winter, chickens exhibited listlessness and an increase in respiratory rate. When inoculated by the IV route in winter and by the IT and cloacal routes in summer, chickens showed an increase in respiratory rate and greenish diarrhea. In addition, some of them were affected with prostration before death. Very severe lesions occurred in the gastrointestinal tract and hemorrhage in the proventriculus, small intestine, cecal tonsils, and cloaca. In the respiratory system only mild tracheitis was observed.

In experiment No. 1 (Table 3), fatality was almost 100% in the group of IV inoculation of chickens. Of the chickens inoculated by the IT route in the same experiment, however, only one died of infection 6 days after inoculation. Furthermore, one of the three direct contact control chickens for the group of IV inoculation died 9 days after inoculation. Both direct and indirect contact controls for the group of IT inoculation showed no signs of disease, but developed HI antibodies against Taka virus. The titer increased two to eight times when examined 9 weeks after contact exposure.

In experiment No. 2 conducted in summer (Table 3), all the chickens inoculated by the IT and cloacal routes and those exposed to direct contact with the inoculated chickens died between 4 and 10 days after inoculation. When the virus recovery was carried out in this experiment, Taka virus was isolated from the trachea, cloaca, lungs and spleen of all the dead chickens. When tracheal swabs were used, the virus was isolated from the IT and cloacal groups on the second and fourth day after inocu-

lation, respectively. When cloacal swabs were used, Taka virus was isolated from both groups on the fourth day after inoculation. No difference was found in virus recovery between the inoculated and contact control chickens.

No death occurred to the conventional chickens of the group of IT inoculation in experiment No. 3 (Table 3). When inoculated with 10^{-5} or 10^{-6} dilution, chickens showed mild respiratory symptoms, which disappeared by 8 to 10 days after inoculation. Since these chickens had been vaccinated against NDV, they showed HI titers against NDV and Taka virus in serum before inoculation. After inoculation these titers increased in all the chickens, except two in which they remained unchanged.

Discussion

The results obtained from the present study lend support to those reported in the previous paper [3], in which Taka virus was found to be related antigenically to NDV and Bangor virus when examined by the HI test. The relationship between the surface antigens, hemagglutinin, and neuraminidase of Taka virus to those of NDV or Bangor virus was examined by the SN and neuraminidase inhibition tests. Since the antiserum against NDV used in the present experiment was convalescent serum from chickens involved in a field outbreak, further studies might be required to compare antigenic relations among the three strains of avian paramyxoviruses by using hyperimmune serum to NDV. Nevertheless, the results obtained indicate that antigenically, Taka virus is more closely related to NDV than to Bangor virus. The antigenic cross relationship between NDV and Bangor virus is of interest. The results of the present study agree with those reported

by Nakayama et al. [16], but not with those by others [1, 6, 13, 17] who found that the hemagglutinin and neuraminidase of Bangor virus were significantly different in antigenicity from those of NDV.

The reason for this discrepancy is not yet known. It was possible, however, that the sample of Bangor virus used in the present experiment might have been contaminated with NDV during passage in many laboratories in the world, and/or changed its immunological character(s) as an original virus by an unknown mechanism during passage in embryos.

The original Bangor virus did not regularly kill embryos at high dilutions, but induced infection in embryos without causing death [13]. The Bangor virus used in the present study, however, killed embryos regularly within 72 hours even when it was diluted. During its passage in embryos, a virulent clone of the virus might have been selected and possessed immunological properties different from that of the original virus, although it might have retained the biological properties of the original Bangor virus.

Taka virus was found to be related antigenically to NDV, but there were differences in some properties between the two viruses. The most striking difference was that the HA reaction of Taka virus was temperature-dependent [3]. Moreover, the optimal pH of neuraminidase was 4.2 for Taka virus and 5.0–5.2 for NDV [1, 5].

Of the avian paramyxoviruses, only the velogenic strains of NDV can readily kill chickens in natural or experimental infection [6], and the others induce disease and occasional death in wild birds [15]. The Taka virus isolated from the brains of Japanese sparrow-hawks was found to be pathogenic for both chickens and wild birds. This virus mainly causes nervous symptoms

in Japanese sparrow-hawks and Japanese hawk-eagles [3], and symptoms similar to those of the Asiatic form of NDV in chickens [9].

It has been suggested that the virulence of an NDV strain for chickens may be affected by age of chickens, routes and dose of administration, and environmental conditions, such as temperature [4, 7, 10, 18]. The influence of temperature on the virulence of Taka virus was clearly demonstrated in the present experiment, in which fatality was higher in chickens inoculated with Taka virus by the IT route in summer than in those in winter. Moreover, NDV was transmitted more readily to susceptible chickens by direct or indirect contact in warm than in cold environmental conditions [18]. In the present experiment, Taka virus was transmitted in cold weather with much more difficulty than was NDV at a moderately low environmental temperature [18].

The possible origin of Taka virus was briefly discussed in the previous paper [3]. So far as the authors know, it has not been found that genetic recombination exists in the paramyxovirus group as in the influenza viruses [11]. Therefore, Taka virus should be considered as a new variant of NDV derived from a selective process that could take place in wild birds in nature.

There are differences in biological properties between Taka virus and NDV. Such differences are of interest for further classification of the avian paramyxoviruses.

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