PATHOGENICITY FOR BABY CHICKS OF A NEWLY ISOLATED PICORNAVIRUS "AVIAN NEPHRITIS VIRUS"

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Introduction

We isolated a picornavirus, immunologically distinct from the virus of avian encephalomyelitis, from the rectal contents of apparently normal 1-week-old broiler chicks in chicken kidney cell (CKC) cultures, in 1976. The virus had the following properties: ribonucleic acid in the viral core; virus growth in the cytoplasm; a particle about 30 nm in diameter; resistance to organic solvents, trypsin, and acid; relative heat-lability; and partial stabilization to molar magnesium chloride. These studies were carried out to elucidate the pathogenicity of the virus for baby chicks by experimental inoculation.

Materials and methods

Virus The isolate, G-4260 strain, at the 3rd passage level in a CKC culture was cloned by the plaque-picking method into a CKC monolayer culture. It was grown once more in a CKC culture. Then it was transmitted serially onto the chorioallantoic membrane of 11-day-old embryonated chicken eggs derived from specific-pathogen-free layer chickens of the PDL-1 strain, which had been produced at the authors' laboratory. Infected chorioallantoic membranes of the 4th and 5th passage levels were prepared into 50% emulsions in the tissue culture medium, respectively. The emulsions were centrifuged at 4,000 rpm for 5 minutes. The resulting supernatants of the 4th and 5th passage levels were freeze-dried separately and stored at −70°C until used.

Chicks Specific-pathogen-free chicks of the PDL-1 strain and commercial broiler chicks without maternal antibodies against the virus were used at one day of age.

CKC culture The procedure used for CKC culture was as described previously[6].

Virus titration Virus titration was performed by the plaque method described in the previous paper[6]. Agar overlay medium contained 0.2 mg of diethylaminoethyl-dextran (Pharmacia products) per milliliter.

Detection of fluorescent antigens The direct method was used for detection of fluorescent antigens in organs. The organs were harvested from infected chicks, frozen in a tube containing n-hexane and kept at −70°C, and cut into sections 4 to 6 μ thick with the Lipshow cryostat. Sections on the coverslip were dried in the air, fixed in acetone, and stained with fluorescent antibody against the G-4260 strain.

Detection of antibodies Antibodies against the G-4260 strain were detected by the indirect fluorescent antibody method. Fluorescent antigens were examined with a Tiyoda FM200 fluorescent microscope.

Pathological examination The chicks were autopsied at regular intervals. For the histopathological examination, the main organs, such as the kidneys, liver, spleen, pancreas, laryngotrachea, lungs, proventriculus, duodenum, free portion of the small intestine, cecum, rectum, bursa of Fabricius, thymus, thigh muscle, bone marrow, and brain, were fixed in a 10% formalin solution and Bouin's fixative, and thin sections made after paraffin-embedding were stained with hematoxylin and eosin. In some cases, the spinal cord was included in the histological examination.

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Electron-microscopic examination A piece of the kidney from the chicks 3 and 5 days after inoculation was fixed in glutaraldehyde and osmium tetroxide. Ultrathin sections, cut from Epon 812-embedded materials, were stained with both uranyl acetate and lead citrate. Stained sections were observed with an electron microscope, model HS-9, Hitachi Company.

Experimental design In experiment 1, 92 one-day-old broiler chicks were divided into 2 groups: 58 chicks for inoculation and 34 chicks as uninoculated controls. Body weight was recorded in 20 chicks of each group before virus inoculation (day-old) and at 8 days of age. Each of the 58 day-old chicks was inoculated intraperitoneally with $10^{4.3}$ plaque-forming units (PFUs) of the virus, which had been passed 4 times on the chorioallantoic membrane. The chicks were reared in isolators. In the inoculated group, 2 chicks were killed 1, 2, 3, 4, 5, 8, 10, 14, 21, and 28 days after inoculation for recovery of the virus in organs and for assay of the antibodies. For gross pathological examination, 2 other chicks were dissected 1, 2, 3, 4, 5, 7, 14, 21, and 28 days after inoculation. In the control group, 2 chicks were killed at 2, 4, 6, 8, 15, 22, and 29 days of age for gross pathological examination.

In experiment 2, 90 one-day-old chicks of the PDL-1 strain were divided into 2 groups: 58 chicks for inoculation and 32 chicks as uninoculated controls. Each of the 58 day-old chicks was inoculated intraperitoneally with $10^{6.0}$ PFUs of the virus, which had been transmitted 5 times on the chorioallantoic membrane. Twenty-two chicks of each group were divided equally into two subgroups and weighed before and 7 days after inoculation. Analysis of variance of body weight was done by the method of Snedecor. In the inoculated group, 2 chicks were killed 1, 3, 5, 7, 9, and 14 days after inoculation for detection of fluorescent antigens in organs. Three other chicks were examined for gross and histopathological lesions and antibodies 1, 3, 5, 7, 9, 14, 21, and 28 days after inoculation In the control group, 2 chicks were killed 3, 5, 7, 14, and 21 days after inoculation for gross and histopathological examination.

Results

Clinical signs In either experiments, chicks inoculated with the virus did not manifest any clinical sign until they were sacrificed.

Gross lesions In the inoculated groups, mild yellowish-tan discoloration was recognized in the kidneys of chicks killed 7 to 21 days after inoculation (Tables 1 and 2). No gross lesions were observed in any other organ of the chicks of the inoculated groups or the control groups.

Body weight In the 2 experiments, inoculated chicks grew more slowly than uninoculated controls (Fig. 1). The groups differed significantly (1% level) in mean body weight 7 days after inoculation.

Distribution of virus and development of antibodies In experiment 1, the virus was recovered from the various organs examined, except for the brain and trachea. The kidneys, jejunum, rectum, and bursa of Fabricius had respective virus titers of $10^{6.2}$, $10^{5.7}$, $10^{5.0}$, and $10^{5.0}$ PFUs per ml, higher than in any other organ. The virus was demonstrated in the kidneys, jejunum, and rectum for 10 days after inoculation. The virus titer in the kidney was highest 4 days after inoculation. The results are shown in Table 1.

Antibodies, as measured by the indirect fluorescent antibody method, appeared at 8 days and were demonstrated even 28 days after inoculation, as shown in Tables 1 and 2.

Fluorescent antigens in organs In experiment 2, lumpy and granular specific fluorescent antigens were seen in the epithelia of the renal tubules 1 to 7 days after inoculation. They were recognized also in the spleen, jejunum, rectum, and bursa of Fabricius, but were smaller in quantity and lower in intensity than in the kidneys. No antigen was detected in the brain, liver, or pancreas. The results are shown in Table 3.

Histopathological changes of the affected kidneys Changes were recognized in the chicks from 3 to 28 days after inoculation, when the experiment was terminated. Initial lesions started focally in the glomerular area of the cortex 3 days after inoculation. The lesions consisted of interstitial lymphocytic and granulocytic infiltration with swelling of the capillary endothelium and

Table 1 Distribution of the virus in organs of broiler chicks inoculated intraperitoneally with the G-4260 strain

Days after inoculation	1		2		3		4		5		8		10		14		21		28	
Chick No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Brain	–[A]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Trachea	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Thymus	–	–	–	–	–	2.4[B]	–	–	–	1.9	–	–	–	–	–	–	–	–	–	–
Lungs	–	–	–	–	–	1.7	–	–	2.9	–	–	–	–	–	–	–	–	–	–	–
Liver	–	–	2.1	2.7	–	4.1	1.4	–	1.4	–	1.4	–	–	–	–	–	–	–	–	–
Spleen	–	1.4	–	3.4	2.9	3.4	3.7	3.5	3.9	2.4	–	–	–	–	–	–	–	–	–	–
Pancreas	–	–	–	–	–	2.9	–	2.7	1.7	–	–	–	–	–	–	–	–	–	–	–
Kidneys	–	–	3.0	3.7	4.9	4.9	4.5	6.2	5.7	4.1	4.0	4.4	–	3.1	–	–	–	–	–	–
Jejunun	–	–	–	4.1	5.4	5.7	5.3	4.8	4.6	5.5	4.0	3.9	–	4.5	–	–	–	–	–	–
Rectum	–	–	3.3	3.6	4.9	4.7	3.4	4.5	5.0	4.7	4.5	4.5	–	3.9	–	–	–	–	–	–
Bursa	–	2.4	–	3.1	4.2	3.9	3.4	4.5	5.0	3.0	3.2	2.4	–	–	–	–	–	–	–	–
Antibody[C]	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	4	4	8	4	32	64	32	32	16	8
Gross lesions in kidneys[D]	N	N	N	N	N	N	N	N	N	N	P	P	P	P	P	P	P	N	N	N

[A]Negative in 10% suspension of the organ.

[B]Log-PFU per ml of 10% suspension of the organ.

[C]The figure indicates the reciprocal of the serum dilution positive for fluorescent antibody.

[D]N and P for lesions: Negative and positive for lesions, respectively.

Table 2 Appearance of gross lesions, viral antigens, and antibodies in chicks inoculated intraperitoneally with the G-4260 strain

	Days after inoculation							
	1	3	5	7	9	14	21	28
Discoloration of kidneys	0/3[A]	0/3	0/3	2/3	1/3	3/3	1/3	0/3
Fluorescent antigens in kidneys	2/2	2/2	2/2	2/2	2/2	0/2	NT	NT
Antibodies in serum	0/3	0/3	0/3	0/3	1/3	3/3	3/3	3/3

[A]Number of positive chicks/number of chicks examined.
NT, not tested.

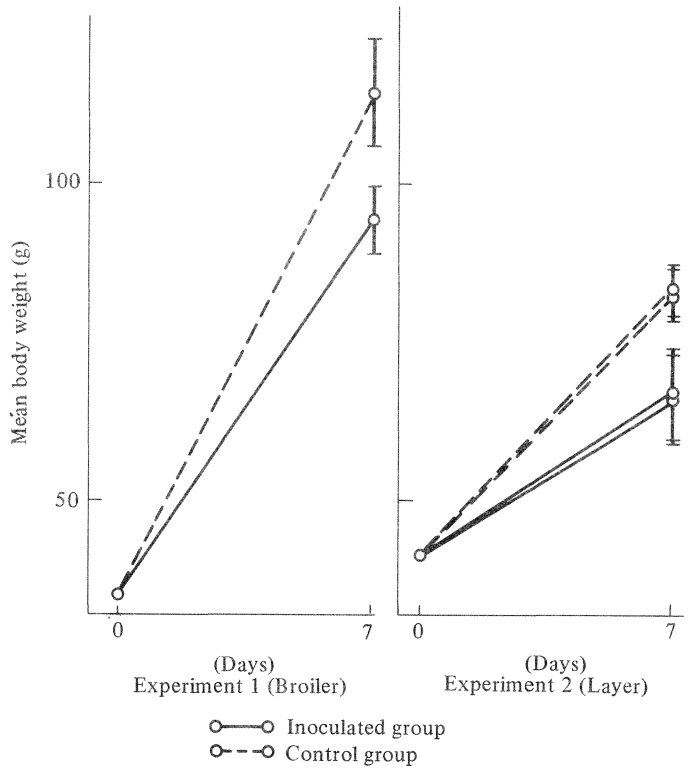

Fig. 1 Effect of the G-4260 strain on increase in body weight

Table 3 Distribution of fluorescent antigens in organs of chicks inoculated intraperitoneally with the G-4260 strain

Days after inoculation	1		3		5		7		9		14	
Chick No.	21	22	23	24	25	26	27	28	29	30	31	32
Brain	–[A]	–	–	–	–	–	–	–	–	–	–	–
Liver	–	–	–	–	–	–	–	–	–	–	–	–
Spleen	–	+[B]	–	–	–	–	–	–	–	–	–	–
Pancreas	–	–	–	–	–	–	–	–	–	–	–	–
Kidneys	+	+	+	+	+	+	+	–	–	–	–	–
Jejunum	–	–	+	–	+	+	+	+	–	–	–	–
Rectum	–	–	+	–	–	–	–	–	–	–	–	–
BF[C]	–	–	+	+	–	–	–	–	–	–	–	–

[A]Negative for antigen.
[B]Positive for antigen.
[C]Bursa of Fabricius.

a slight monocytic infiltration in the blood capillaries. Epithelial cells of the proximal convoluted tubules showed degeneration accompanied by frequent infiltration of granulocytes. The degenerating epithelial cells contained acidophilic granules of various size in their cytoplasm (hereinafter called granular degeneration). Some of their nuclei became swollen, and others fragmented. The focal lesions became more pronounced, extending from the glomerular area to the interlobular venular area in the cortex 5 and 7 days after inoculation. The lesions contained mild proliferation of fibroblasts in the interstitium and some renal corpuscles displayed swelling of epithelium of both glomerulus and Bowman's capsule, although most corpuscles were intact. At the middle stage of the experiment, 9 and 14 days after inoculation, only a few proximal convoluted tubules manifested granular degeneration of the epithelium at the periphery of the lesions, although there was wide interstitial lymphocytic infiltration and fibroblastic reaction. Most renal tubules in the lesions stained pale and looked like the distal convoluted tubules, and some of the tubules were distended with casts. The dense lymphocytic infiltration, composed of medium-sized lymphocytic cells frequently forming demarcated lymphoid follicles, occurred at the center of the lesions. Lymphoid follicles were also observed adjacent to the intralobular venules. Two types of focal inflammatory lesions were recognized at the late stage of the experiment, 21 and 28 days after inoculation. One was characterized by a mild fibrosis in the interstitium, reminiscent of the focal sclerosis in mammalian kidneys. In the lesions, the glomeruli were frequently aggregated. Another type of focal lesion was characterized by follicular lymphoid hyperplasia accompanied by interstitial lymphocytic infiltration (Table 4).

No histopathological changes were observed in the kidneys of the control chicks.

Histological changes in other organs No significant changes were recognized in organs other than the kidneys.

Ultrastructural changes in kidneys Two kinds of discrete lesions were seen in the cytoplasm of the degenerative tubular epithelium. One was evenly high in electron density and did not contain any structures. The other was a phagosomal lesion, demarcated by double membranes, enclosing many tubular and myelinic structures as well as virus particles. Isolated crystal arrays of virus particles occurred also frequently in the cytoplasm. The particles were about 30 nm in size in the array. Degenerative changes of the mitochondria and endoplasmic reticulum were prominent. Microvillous brush border was lost on the surface of affected epithelial cells.

Table 4 Histological changes in kidneys of chicks inoculated intraperitoneally with the G-4260 strain

Changes	Days after inoculation							
	1	3	5	7	9	14	21	28
Interstitium								
Lymphocytic infiltration	–	+	++	++	+++	++	++	+
Swelling of capillary endothelium	–	+	++	++	+++	++	–	–
Proliferation of fibroblasts	–	–	+	++	+++	++	++	++
Follicular lymphoid hyperplasia	–	–	+	+	++	+++	++	+
Granular degeneration of epithelium of proximal convoluted tubules	–	+	+++	++	+	+	–	–
Swelling of glomerular epithelium	–	+	++	+++	+++	++	+	+

Severity of the changes: –, negative; =, mild; ++, moderate; +++, severe.

Discussion

Avian encephalomyelitis virus is classified as a picornavirus that has a pathogenicity for chicks[1]. In the present studies, the newly isolated picornavirus could be distinguished from avian encephalomyelitis virus in its pathogenicity on CKC culture and antigenicity was also distinct from that virus in clinical signs, in distribution in the body and in histopathological changes.

The virus produced histopathological changes only in the kidney (nephritis) when inoculated into chicks by the intraperitoneal route. Imada *et al.*[2] revealed that young specific-pathogen-free chickens were infected easily with the virus by the oral route of inoculation or by contact with infected chickens, and that the same nephritis was produced. The authors (unpublished data) demonstrated that fluorescent antigen was present only in the kidneys of chick embryos infected with the virus by the yolk-sac route, and that the same nephritis could be produced. Taking those results and the results of the present studies into consideration, it is presumed that the target organ of the virus may be the kidney. Therefore, it is proposed that the virus should be called "avian nephritis virus".

Renal lesions in the chicks infected with the picornavirus started as focal lymphocytic inflammatory lesions with granular degeneration of the renal tubular epithelium in the glomerular area of the cortex, 3 days after inoculation. The focal lesions extended toward the area of intralobular venules until 14 days after inoculation. Later, the lesions regressed into focal sclerotic lesions. It is quite plausible to consider that the granular degeneration of epithelial cells of the proximal convoluted tubules was due to the propagation of the virus in the cells, as demonstrated also by electron-microscopic observation and direct fluorescent-antibody technique.

Nephrosis-nephritis was reported by Pohl[3], who infected chicks with the Australian T-strain of infectious bronchitis virus. The changes described started as an inflammation of the ureters and medullary pyramids, and spread to the cortex. Granular degeneration, observed in the present studies, was not mentioned in Pohl's nephrosis-nephritis. Sato[4] reported another nephrosis-nephritis in chicks on a high-calcium diet. The changes first appeared as a severe degeneration of epithelial cells of the renal tubules and collecting tubules due to calcareous deposits, and they resulted in nephron collapse accompanied by glomerulitis and interstitial fibrosis.

Cortical focal nephritis in this picornavirus infection thus seems to differ from both kinds of nephrosis-nephritis from the pathological point of view.

Distribution of the virus and economic losses in the field are unknown. Of 673 chicken sera collected from 70 flocks and submitted for diagnosis to the authors' laboratory from 1972 to 1977, however, 69 sera from 29 flocks were positive for antibodies when examined by the indirect fluorescent-antibody method[5]. It is thus presumed that the virus may be prevalent among field chicken flocks in Japan.

Summary

The pathogenicity of a newly isolated picornavirus for day-old chicks was studied by intraperitoneal inoculation. No clinical signs were observed. A mild yellowish-tan discoloration of the kidneys was noticed at necropsy 7 to 21 days after inoculation. Mean body weight was significantly lower ($P<0.01$) in inoculated groups than in control groups 7 days after inoculation. In a chronological study on the distribution of the virus in organs, the virus was recovered from various organs, except for the brain and trachea. The virus titer was higher in the kidneys, jejunum, rectum, and bursa of Fabricius than in any other organ. Fluorescent antigens were seen predominantly in the epithelia of the renal tubules. Histologically, focal lesions were observed in the cortex of the kidneys from 3 to 21 days after inoculation. The lesions were characterized by interstitial lymphocytic infiltration and degeneration of epithelial cells of the proximal convoluted tubules. The degenerated cells contained acidophilic granules in their cytoplasm. Electron-microscopic examination of the cytoplasm revealed electron-dense amorphous areas, phagosomal areas with viral particles, and isolated crystal arrays of the virus particles, about 30 nm in size.

References

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Discussion

Ogata M. (Japan): How many specimens did you examine and how many flocks were positive?

Answer: We examined 673 chicken sera collected from 70 flocks during the period 1972-1977. Twenty-nine out of 70 flocks were positive for antibodies to the G-4260 strain of avian nephritis virus. Latent infection is often observed among chicken flocks in Japan. The role of the virus in the field is not known.