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ISOLATION OF HERPES TYPE VIRUS FROM CHICKENS WITH MAREK'S DISEASE USING DUCK EMBRYO FIBROBLAST CULTURES[1]

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SUMMARY Using duck embryo fibroblasts (DEF), CPE agents were isolated from chickens diagnosed clinically and histologically to have Marek's disease (MD). In monolayer cultures of DEF, the agents formed foci, consisting of refractile rounded or shrunken spindle cells and syncitial type, giant cells. In preparations stained with hematoxyline-eosin, most of the cells in the foci showed typical herpes type-intranuclear inclusions. Intracytoplasmic eosinophilic inclusions were also seen in these cells. The cells in these foci showed specific fluoresence by the fluorescent antibody technique. Autoradiography using ^{3}H-thymidine showed that DNA synthesis occurred in localized areas of the nuclei of the cells in these foci. Naked particles like those of herpes virus and occasional particles with an additional membrane were seen in thin sections of these cells examined with an electron microscope. Typical naked particles were also seen in negatively stained preparations of concentrated culture fluid. The morphogenesis and morphology of the virus were similar to those of herpes-type viruses (HTV). The agents were maintained in DEF cultures for 311 days. For induction of CPE by the agents in the cell culture inoculum containing intact cells was required. No evidence was obtained that the agents had transforming activity. Similar agents were also isolated from apparently healthy chickens and bantams. The HTV was readily recovered after its inoculation into day old chicks. However, the DEF passaged-HTV (Biken C strain) did not show any pathogenicity to chickens. Infectivity of HTV to various cultured mammalian cells was not recognized.

Most of these characteristics of the HTV strains isolated are identical with those of herpes type viruses isolated from chickens with Marek's disease by previous investigators.

INTRODUCTION

Several investigators have isolated herpes type virus (HTV) from chickens with Marek's

1 This work was presented at the 28th General meeting of the Japanese Cancer Association, Kanazawa, in October 1969 and at the 69th Meeting of the Japanese Society of Veterinary Science, Kanagawa in April 1970.

disease (Churchill and Biggs, 1967; Solomon et al., 1968; Nazerian et al., 1968; Churchill, 1968; Sharma et al., 1969; Yuasa et al., 1969; Rispens et al., 1969). There now seem good reasons for believing that a herpes virus of a cell-associated type is the etiological agent of Marek's disease (Biggs et al., 1968). There have been outbreaks of Marek's disease at the Poultry Center of the Agricultural Cooperative Association of Kaibara in Hyogo Prefecture since 1968. Since 1969 our laboratory has established contact with the Poultry Center. This disease is now a major problem of the poultry industry in Japan. This disease is also interesting from the standpoint of oncogenesis of avian herpes type virus as a model of an EB virus, thought to be related with human cancer.

This paper reports the isolation and some virological characteristics of the agents from chickens in duck embryo fibroblasts.

MATERIALS AND METHODS

1. *Inoculum obtained from chickens*

Chicken blood collected in heparin (20 units per ml) was used for inoculation of cell cultures. Bone marrow cells were dispersed mechanically with sterile scissors, and the resulting suspension was aspirated with a pipette and centrifuged for 5 min at 1,000 rev/min. The sediment was then resuspended in growth medium. Kidney cells obtained by trypsinization were also used as inoculum.

2. *Duck embryo fibroblast (DEF) culture*

Fibroblasts were prepared by the method of Solomon et al. (1968) by trypsinization of decapitated 12 to 13 day-old duck embryos. The suspension was diluted with growth medium, transferred to large prescription bottles and incubated at 37 C. The growth medium consisted of Eagle's Minimum Essential Medium (MEM) with 0.5% lactalbumin hydrolysate and 5% calf serum and 100 units of penicillin and 100 μg of streptomycin per ml. For subculture, primary cultures were dispersed with 0.025% trypsin in phosphate buffered saline and the mixture was centrifuged at about 400 $\times g$ for 5 min. Then the cells were resuspended in growth medium and plated at a concentration of 1×10^6 cells/ml.

Cultures were inoculated with whole blood or other materials at least 1 day after subculture to permit attachment of the cells. The volume of inoculum per bottle varied from 0.1 to 1.0 ml for blood and from 0.2 to 1.0 ml for bone marrow and kidney cells (about 10^5 cells/ml). After 48 hours, the cultures were washed 3 times with Hanks solution to remove excess blood cells. Subcultures were made at 3–5 day intervals by the method described above. When CPE was detected in infected DEF cultures, 0.5 ml of suspension of infected cells ($1\text{–}5 \times 10^4$ cells/ml) was inoculated onto noninfected DEF. The morphology of unstained cells was noted at each passage and coverslip preparations obtained from selected passages were fixed with Bouin's fluid or methanol and stained with hematoxylin-eosin or Giemsa solution.

3. *Plaque assay in cell culture*

Monolayers of cultured DEF were prepared in 70 ml prescription bottles and used on the day they became confluent. After removal of the growth medium, 0.2 ml volumes of serial two fold dilutions of the suspension of infected cells to be assayed were inoculated onto plates and incubated at 37 C for 24 hrs. Then infected cultures were overlaid with nutrient medium containing either 0.8% or 1% final concentration of Noble's agar (Difco). Five days later the cultures were again overlaid with the agar overlay medium containing 1/100,000 neutral red. Two plates were used for each dilution, and titers in plaque-forming units (PFU) were calculated from the average of the counts for the two plates on the 8th day after infection.

4. *Concentration of agents*

Growth fluids were collected from infected cultures 48 hours after the appearance of cytopathic effects and centrifuged for 60 min at $112,000 \times g$. The pellets were then suspended in 1/20 of the original volume of PBS, frozen and thawed twice, and clarified by low speed centrifugation. Negatively stained preparations of the resulting suspensions were made for electron microscopy with 2% potassium phosphotungstate, pH 7.0 and 0.01%, sucrose in distilled water.

5. *Electron micrography*

Monolayers of infected and control duck embryo fibroblast cultures were scraped from the bottles and the suspended cells were sedimented by centrifugation at 1,000 rev/min for 10 min. The resulting

pellet was fixed for 1 hr in 1% glutaraldehyde, washed well and fixed for 30 min in 1% osmium tetroxide. All specimens were dehydrated in ethyl alcohol, embedded in Epon 812, sectioned with an LKB ultramicrotome, and stained with uranyl acetate and lead citrate. Preparations were examined with a Hitachi 11 B type electron microscope.

6. *Fluorescent antibody technique (FAT)*

Direct FAT was done by the method of Naito et al. (1969). Sera were obtained from chickens No. 72, 73 and 76 which contained no anti-RAV antibody or COFAL factor. The standard ammonium sulfate method was used for separation of globulins from these sera. After labeling with fluorescein isothiocyanate (FITC), unreacted dye was removed from the crude conjugates by gel filtration on Sephadex G-25. A fraction was separated by chromatography on DEAE-cellulose, eluting with 0.005 M phosphate buffer, pH 7.0, in 0.5 M NaCl.

7. *Autoradiography of ^3H-thymidine*

Infected DEF showing typical cytopathic effects were kept in medium containing ^3H-thymidine (5 μc/ml, specific activity 5 c/mM) for 1 hr. Then the cells were fixed in Bouin's fluid for 2 hrs and treated with 2% perchloric acid for 40 min at 4 C. Then autoradiography was carried out by the dipping method using Sakura NR-M2 nuclear emulsion. After 5 days exposure, the preparations were developed, fixed and stained with hematoxylin-eosin.

8. *Assay of infectivity in chickens by recovery of virus*

The presence of the agent in DEF cultures, their supernatants and extracts was tested by intra-abdominal inoculation of specimens into day-old chicks. The chicks were bled to death at intervals. Blood samples were inoculated onto DEF. These DEF cultures were examined by hematoxylin-eosin staining and the fluorescent antibody technique 15 days after the inoculation.

RESULTS

1. *Clinical and histological diagnosis of MD chickens*

Thirty five chickens (about 100 day old) showing typical clinical symptoms of MD were bled to death and autopsied. The clinical symptoms noted were paralysis of the legs or wings (Fig. 1). MD chickens were often characterized by the presence of a swollen

FIGURE 1. *A 100 day old chick (no. 80) showing typical paralysis of the legs and wings.*

FIGURE 2. *Ovarian tumor in a 140 day old chick (no. 73).*

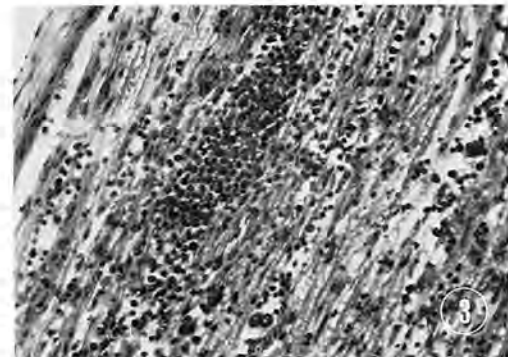


FIGURE 3. *Section of a nerve lesion of chick no. 80 showing a lymphoproliferative area. Stained with hematoxylin-eosin.*

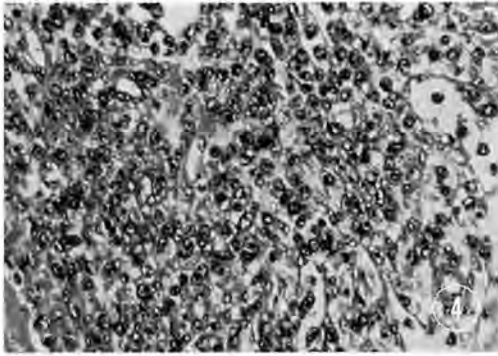


FIGURE 4. *Section of the ovarian tumor of chick no. 73 composed of many lymphoid cells. Stained with hematoxylin-eosin.*

peripheral nerve and ovarian tumor (Fig. 2). Fourteen chickens were further diagnosed as showing typical histological symptoms of MD, by the presence of lymphoid cells infiltrating the peripheral nerves (sciatic, brachial, celiac and abdominal vague nerves) (Fig. 3). The ovarian tumor also consisted of numerous lymphoid cells (Fig. 4).

2. *Relationship between virus isolation and evidence of infection with avian leucosis virus in MD chickens*

The presence of anti-RAV1 and RAV2 antibody and COFAL factor in the blood samples obtained from the 14 MD chickens was examined. As shown in Table 1, anti-RAV1 antibody, anti-RAV2 antibody and COFAL factor were found in 4, 3 and 4 out of 14 chickens respectively. CPE agents were readily isolated from all the chickens using either blood, bone marrow or kidney samples regardless of the presence of anti-RAV antibody and COFAL factor. The failure to isolate CPE agent from a blood sample from chicken No. 71 was probably due to mishandling of the sample.

3. *Characteristics of CPE*

The cytopathic effect was characterized by the appearance of foci of refractile rounded or shrunken cells (Fig. 7). These foci also

TABLE 1. *Relationship between virus isolation and evidence of infection with avian leukosis virus in MD chickens*

Chicken	Anti-RIF		COFAL	Virus isolation		
	RAV1	RAV2		Blood	Bone marrow	Kidney
No. 68	+	+	−	+	+	+
No. 69	+	−	−	+	NT	+
No. 70	+	−	−	+	NT	+
No. 71	+	+	−	−	+	+
No. 72	−	−	−	+	+	+
No. 73	−	−	−	+	+	+
No. 74	−	+	−	+	+	+
No. 76	−	−	−	+	+	+
No. 80	−	−	−	+	NT	NT
No. 81	−	−	−	+	NT	NT
No. 82	−	−	+	+	NT	NT
No. 83	−	−	+	+	NT	NT
No. 84	−	−	+	+	NT	NT
No. 85	−	−	+	+	NT	NT

NT: Not tested.

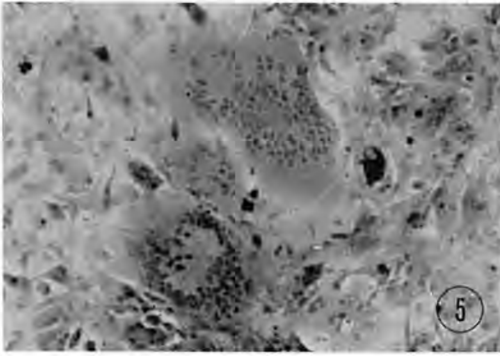


FIGURE 5. *Giant cells with many nuclei in a focus of DEF 5 days after inoculation with Biken C strain at the 32nd passage level. Stained with hematoxylin-eosin.*

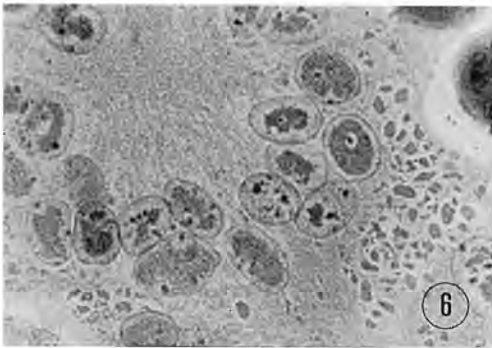


FIGURE 6. *Herpes type nuclear inclusions and eosinophilic cytoplasmic inclusions in a giant cell found in a focus of DEF 5 days after inoculation with Biken B strain at the 18th passage level. Fixed with Bouin's fluid, stained with hematoxylin-eosin.*

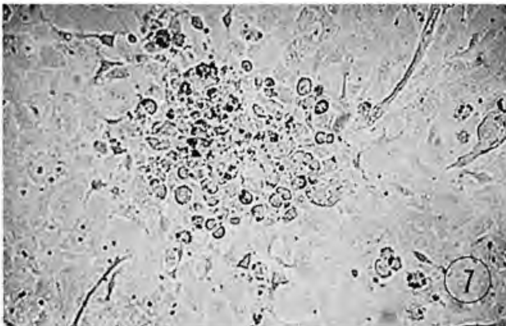


FIGURE 7. *Focal area of CPE 3 days after infection of DEF with Biken C strain. The infected cells are refractile and rounded.*

contained multinucleate giant cells (Fig. 5). Cytopathic effects were detected about 3 days after inoculation. Tissue culture transfer of the cytopathic agent using cell free supernatants was unsuccessful. Therefore, the agent must be transmitted from one culture to another with cellular material. Infected cells were fixed with Bouin's fluid and stained with hematoxylin-eosin. The cells in the foci were seen to have many eosinophilic, cytoplasmic inclusions as well as herpes type nuclear inclusions, as reported by Ono et al. (1970) (Fig. 6). Neither cytoplasmic nor nuclear inclusions were seen in uninfected DEF cultures or in DEF cultures inoculated with the supernatant of the infected DEF culture. The size and frequency of incidence of multinucleate giant cells in foci gradually increased in successive passages.

FIGURE 8. *Microplaques in DEF monolayer cultures with agar overlay, 8 days after inoculation with Biken C strain.*

These characteristics were similar in DEF infected with all the strains isolated and passaged, which are shown in Table 1.

4. *Autoradiography of ^3H-thymidine*

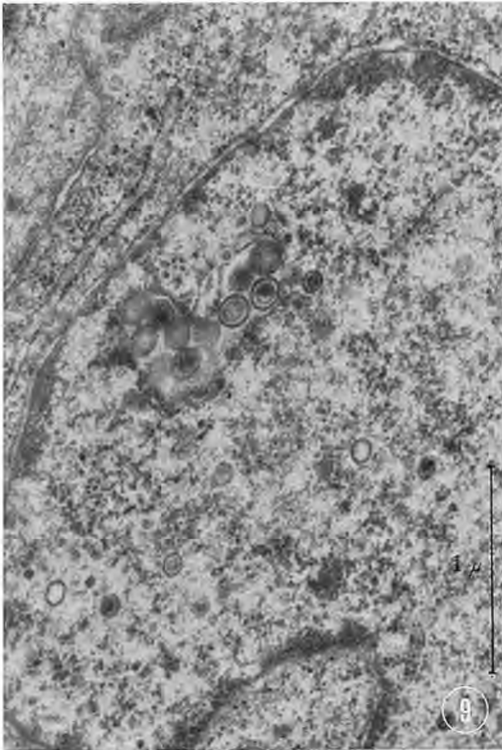
As reported previously (Ono et al., 1970), there were various shaped accumulations of silver grain in the nuclei of most of the cells in the foci. Some nuclei which had not yet developed morphological changes detectable by ordinary staining, already showed one or a few aggregations of silver grains. In these foci, there were few mitotic figures or nuclei showing diffuse distribution of silver grains, indicating the normal S phase of the cell cycle. These observations indicate that the cells in the foci were not transformed cells and did not multiply, but degenerated.

5. *Association of the agent with cells*

Supernatant medium removed from heavily infected cultures at various passage levels up to the 45th was freed from cells by 2 cycles of centrifugation at $600\times g$ for 20 min. No agent was found in the supernatant by inoculation of cell cultures. No cell-free infectivity for DEF cultures was observed for at least 30 days when an infected DEF suspension capable of inducing CPE within 7 days was disrupted by ultrasonic disintegration (150 Watts, 5 min.) or rapid freezing and thawing (3 cycles).

6. *Electron micrography*

Virus particles were found in the nuclei of cells from DEF cultures showing CPE (Fig. 9, 10, 11). These particles had a hexagonal



FIGURES 9, 10. *Electron micrograph of part of a DEF infected with Biken A strain. Viral particles similar to herpes viruses are seen. Some particles have envelopes.*

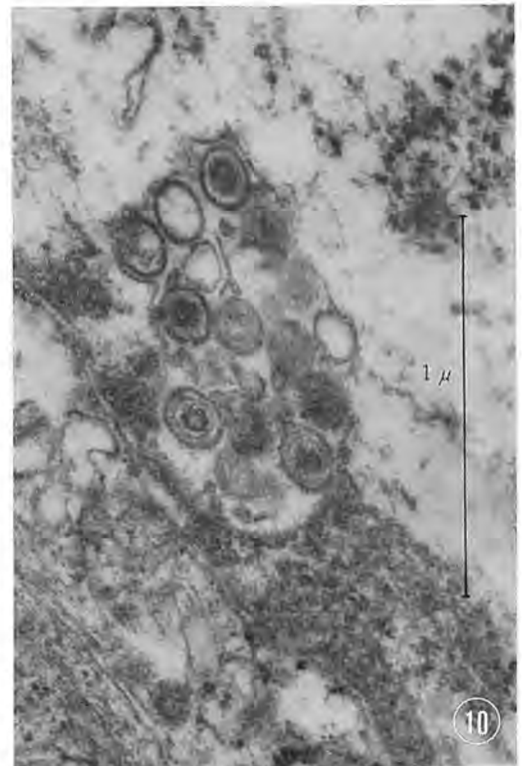


FIGURE 10.

capsid with a diameter of about 100 mμ and a central core, about 65 mμ in diameter. Empty capsids were also present. A few mature virions were close to the nuclear membrane and seemed to obtain an envelope by budding through this membrane. Enveloped particles measured about 140 mμ in diameter. Virus particles were also observed in the cytoplasm.

The extracellular particles examined by the negative staining technique were similar to the capsids of typical herpes type virions (Fig. 12, 13).

The intranuclear origin and morphological features of these particles suggested that they were herpes type virus (HTV).

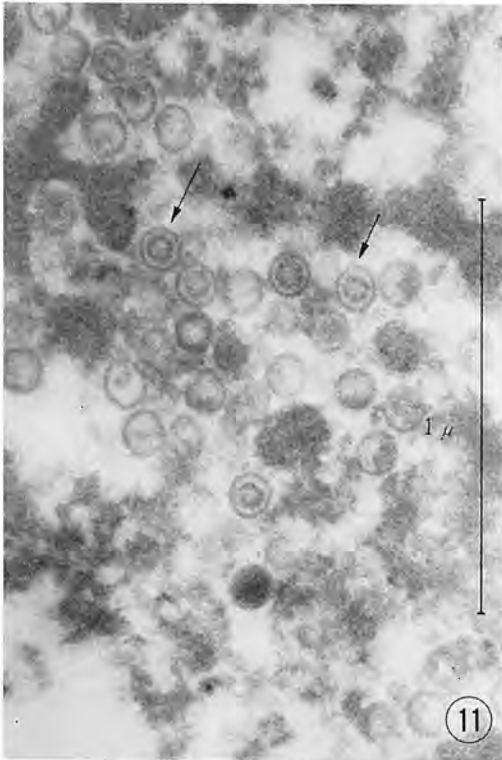
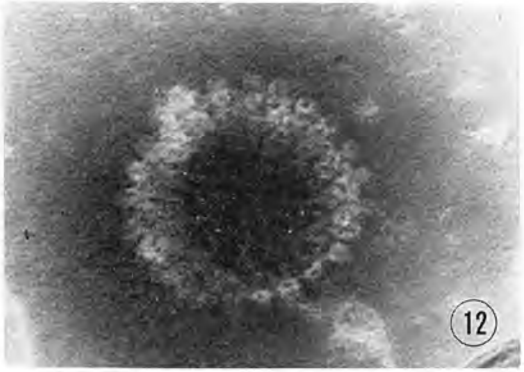
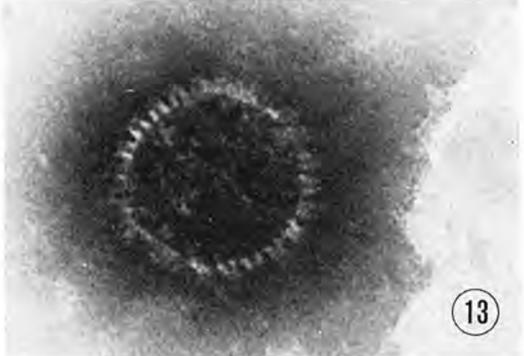


FIGURE 11. *Electron micrograph of a degenerating nucleus of DEF infected with Biken C strain. Many naked herpes type virus particles are seen. Notice the hexagonal profile of the capsid (arrows).*





FIGURES 12, 13. *Details of structure are seen in these negatively stained particles. Nucleocapsids composed of hollow capsomeres are seen but no envelopes are visible.*

7. *Plaque assay*

The relationship between the concentration of inoculum and the number of microfoci was examined. As shown in Fig. 14, a linear relationship was found between the number of foci formed and the number of dilutions of the inoculum of Biken C strain. The typical morphological appearance of the plaques is shown in Fig. 8. The infectivity of HTV in cultures at various passage levels was $1 \sim 3 \times 10^4$ PFU/ml.

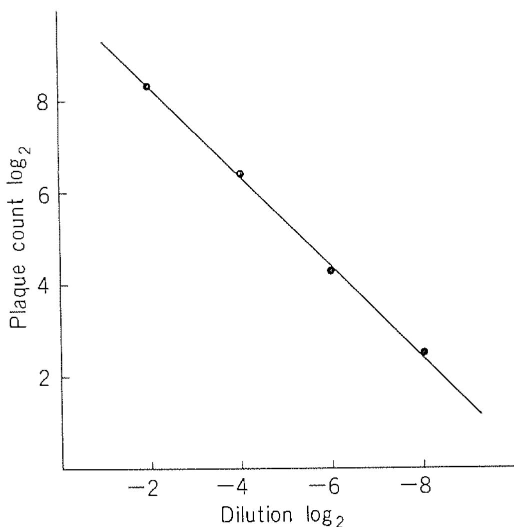


FIGURE 14. *Dose-response curve. Serial two-fold dilutions of cells were inoculated onto cultures. Each point represents the mean of the focus counts of pairs of plates.*

8. *Attempts to isolate virus from the blood of various birds*

Blood samples were obtained from 20 chickens of strain De Kalb 161, 11 adult bantam chickens, 6 adult pheasants and 5 adult pigeons. All these birds were apparently healthy. Each blood sample was inoculated onto a DEF culture, and observed for 30 days. The number of birds with viremia is shown in Table 2.

TABLE 2. *Trials of virus isolation from the blood of various birds*

Birds		Number of birds examined	Number of birds with viremia
Chickens	♀ (100 day old)	10	4
	♂ (140 day old)	10	6
Bantams	♀ (adult)	8	8
	♂ (adult)	3	1
Pheasants	♀ (adult)	3	0
	♂ (adult)	3	0
Pigeons	(adult)	5	0

The incidence of isolation of virus was high in adult chickens and bantams. No virus was obtained from pheasants or pigeons. The characteristics of the CPE and the antigenicity demonstrated by the FA technique with fluorescent MD serum (No. 73) of these isolated specimens were similar.

9. *Passages of strains of the agents*

Ten strains of the agent were passaged. Biken A, B, C, D and E strains were isolated from chickens No 72, 73, 76, 80 and 81 respectively shown in Table 3, Biken A1, B1 and C1 substrains were separated from the original strains at passage levels 2, 9 and 6, respectively. These substrains were then passaged independently. Strains Ban-3 and Hlth-18 were isolated from a bantam and from an apparently healthy chicken respectively. Strains Biken A and C1 were passaged the most (75 times, for 311 days after their isolation). Biken B and B1 strains were stocked in a deep freezer at the passage levels shown in Table 3.

TABLE 3. *Number of passages of strains isolated (June 2nd, 1970)*

Strain	Number of passages	Days
Biken-A	75	311
Biken-A1	73	311
Biken-B	20	71
Biken-B1	21	71
Biken-C	73	311
Biken-C1	75	311
Biken-D	52	264
Biken-E	52	264
Ban-3	24	137
Hlth-18	24	137

COFAL tests were carried out on each strain at several passage levels, but no incidence of contamination with avian leucosis virus was observed.

10. *Immunological relationships between the strains isolated*

As shown in Table 4, the strains passaged all showed specific fluorescence with 3 MD sera conjugated with FITC. The fluorescence in these strains also appeared similar.

TABLE 4. *Antigenic relationship between 8 strains, using 3 fluorescent MD sera*

Strain	Immunofluorescent antibody		
	MD No. 72	MD No. 73	MD No. 76
Biken–A	+	+	+
Biken–B	+	+	+
Biken–C	+	+	+
Biken–D	NT	+	+
Biken–E	NT	+	+
Ban–3	NT	+	+
Hlth–18	NT	+	+
DEF (noninfected)	–	–	–

NT: Not tested.

11. *Recovery of HTV from chickens inoculated with DEF passaged-HTV*

Cultured DEF infected with DEF passaged-HTV (Biken A1 strain) between the 10th and 13th passage was inoculated into 1 day old chicks by the intra-abdominal route. Table 5 shows the frequency of recovery of HTV from the blood of these chicks. The chicks inoculated with HTV showed evidence of viremia throughout the period of observation (from 1 week up to 10 weeks after inoculation). However, HTV was not recovered from the blood of chicks inoculated with live cell-free inoculum during a 7 week period after inoculation.

12. *Pathogenicity of DEF passaged-HTV to chickens*

DEF passaged-HTV (Biken C strain) at the 43rd passage level was inoculated to 1 day old 20 chickens (a White Leghorn strain) intraperitoneally. As a positive control, blood

TABLE 5. *Recovery of HTV from blood of chickens inoculated with HTV (Biken A1 strain).*

	Chickens	Age (weeks) / Inoculum	1	2	3	4	5	6	7	8	9	10
Expt. 1.	Pilch × Peterson 1 day old	HTV–DEF (at the 13th passage level)	$2^a/2^b$	2/2	4/4	2/2	2/2	2/2	2/2	1/2	—	2/2
	Pilch × Peterson 1 day old	Supernatant of HTV–DEF (at the 13th passage level)	—	0/1	0/4	0/2	0/2	0/2	1/2	2/2	—	—
	Pilch × Peterson 1 day old	Freeze-thawed HTV–DEF (at the 13th passage level)	—	0/2	0/2	0/2	0/2	0/2	0/2	1/2	—	2/2
Expt. 2.	White Leghorn 1 day old	HTV–DEF (at the 10th passage level)	—	—	—	—	12/12	—	—	—	—	—
	Hy-Line 1 day old	HTV–DEF (at the 12th passage level)	—	—	—	—	12/14	—	—	—	—	—
	Hy-Line 1 day old	Sonicated HTV–DEF (at the 12th passage level)	—	—	—	—	1/12	—	—	—	—	—
	Hy-Line 1 day old	Uninfected DEF	—	—	—	—	0/10	—	—	—	—	—

a Numerator: Number of chickens from whose blood HTV was recovered.
b Denominator: Number of chickens autopsied at various ages.

sample taken from a typical MD chicken which had been inoculated with chick passaged virulent strain was also inoculated to 1 day old 20 chickens intraperitoneally. All these chickens were observed for 140 days. As shown in Table 6, chickens inoculated with DEF passaged-HTV did not show any incidence of MD, while chickens inoculated with blood of MD chicken showed high incidence of MD.

13. *Inoculation of DEF passaged-HTV onto cultured mammalian cells*

Human embryonal lung cells, human embryonal kidney cells, the FL and HeLa strains, derived from human cells, the BSC1 and Vero strains, derived from simian cells, the RK 13 strain, derived from rabbit cells, and the BHK 21 strain, derived from hamster cells, were inoculated with DEF passaged-HTV (Biken A and C strains) and observed for 40 days. These samples were also subjected to the FA test. Neither CPE nor virus antigen was observed in any of these cultures.

DISCUSSION

Using duck embryo fibroblasts (DEF), cell associated herpes type virus (HTV) could be isolated from chickens with Marek's disease, apparently healthy chickens and bantams. DEF was used instead of chick kidney cells because it is easy to cultivate and is resistant to the A and B groups of avian leucosis viruses (Duff and Vogt, 1969). The virological and immunological characteristics of the strains isolated were similar. Most of these characteristics were also identical with those of herpes type virus of Marek's disease described by previous authors (Churchill and Biggs, 1967; Solomon et al., 1968; Nazerian et al., 1968; Yuasa et al., 1969; Rispens et al., 1969). However, the direct evidence that chick HTV isolated by us is a causative agent of MD, has not been obtained, since DEF passaged-HTV tested has not shown any pathogenicity to chickens. Probably this will be explained by the attenuation of DEF passaged-chick HTV (Biken C strain). As shown by Naito et al. (1970), the virus seems highly contagious and to prevent chickens from being infected with HTV they must be kept in vinyl isolators. Experiments on the pathogenicity of various strains of HTV in chickens without anti-HTV antibody in vinyl islators are in progress.

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TABLE 6. *Pathogenicity of DEF passaged-HTV (Biken C strain) to chickens*

Group	Inoculum \ Week-old	0~2	3~4	5~6	7~8	9~10	11~12	13~14	15~20	Total	Percentage of mortality due to MD
1 day old 20 chicks	MD blood (0.2 ml)	0[a]	0	0	5	4	2	3 (2)[b]	0	14 (2)	70%
1 day old 20 chicks	DEF passaged-HTV at 43rd passage level (0.2 ml)	0 (2)	0	0	0	0	0	0	0	0 (2)	0%

a Numbers of chickens which died of MD were shown.
b Numbers in parentheses showed the number of chickens which died of other than MD.

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