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STUDIES ON THE CHARACTERISTICS OF A HERPES-TYPE VIRUS ISOLATED FROM A CHICKEN WITH MAREK'S DISEASE

TETSUO ONODA, KUNIAKI KOYAMA, TAKEO KONOBE and KEISUKE TAKAKU

Kanonji Institute of the Research Foundation for Microbial Diseases of Osaka University, Kanonji, Kagawa

KOICHI ONO and SHIRO KATO

Department of Pathology, Research Institute for Microbial Diseases, Osaka University, Yamada-kami, Suita, Osaka

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SUMMARY The characteristics of the plaques, infectivity in different kinds of host cells and pathogenicity to chickens of a herpes-type virus (HTV) (Biken C strain) isolated from a chicken with Marek's disease were examined. The rate of development of plaques of HTV gradually increased on passage in either quail embryo fibroblasts (QUEF) or duck embryo fibroblasts (DEF) cultures. By approximately the 100th passage in either DEF or QUEF, large plaques were produced. Syncytia were larger in DEF than in QUEF. QUEF were less susceptible than DEF to DEF passaged-HTV, as shown by the number and size of the plaques formed. However, DEF and QUEF were equally susceptible to QUEF passaged-HTV. The characteristics of QUEF passaged-HTV were stable and were not lost when the virus was passaged 5 times in DEF cultures. No pathogenicity of either DEF or QUEF passaged-HTV was demonstrated during long term cell culture. The virus was reisolated from chickens 13 weeks after inoculation with either QUEF or DEF passaged-HTV, although the frequency of isolation after inoculation of DEF passage-HTV was rather less than after inoculation of virulent HTV (Biken V1 strain). The characteristics of the reisolated-HTV examined, did not show any evidence of reversion to the original type. These characteristics of HTV seem to be good virological markers.

INTRODUCTION

Previous investigators indicated the existence of differences in the characteristics of samples of herpes-type virus (HTV) isolated from chickens with Marek's disease (MD), particularly with respect to pathogenicity, antigenic composition, and cultural features in vitro (Churchill et al., 1969a; Nazerian, 1970; Churchill et al., 1969; Mikami et al., 1970;

Kato et al., 1970). Chick-HTV (Biken C strain) was isolated from an MD chickens using duck embryo fibroblasts (DEF) by Kato et al. (1970). Onoda et al. (1970) found that DEF passaged-HTV propagated well in quail embryo fibroblasts (QUEF). They also noticed differences in the plaque morphology and infectivity to DEF and QUEF of DEF passaged-HTV and QUEF-passaged-HTV.

This paper reports further characteristics of chick-HTV on passage in cell culture with reference to pathogenicity to chickens.

MATERIALS AND METHODS

1. *Cell cultures*

The preparations of duck and quail embryo fibroblasts (DEF and QUEF), and the techniques for the passage and assay of infected cells, were described previously (Kato et al., 1970 ; Onoda et al., 1970).

2. *Virus*

DEF passaged-HTV (Biken C strain) isolated by Kato et al. (1970) was used throughout. As a subline of Biken C strain, the virus was propagated in QUEF, at the 12th passage level in DEF, and then again in QUEF, and was cloned at the 48th passage level. A virulent chick-HTV (Biken V1 strain) was isolated from a chicken with Marek's disease and maintained by passage from chick to chick in vinyl isolators.

3. *Assay of pathogenicity to chickens*

One day old chicks were obtained from RIF free flocks in this Institute. The chicks were inoculated intraperitoneally with 0.2 ml of cultured material containing about 2×10^3 infected cells. The disease was diagnosed from clinical signs and the gross and histological appearance of lesions found at necropsy.

4. *Reisolation of virus from chickens inoculated with chick-HTV*

A volume of 0.25 ml of heparinized blood (100 units of heparin per ml) from chickens inoculated with chick-HTV was inoculated onto normal DEF or QUEF. These cultures were observed for at least 18 days.

5. *Plaque assay*

Monolayer cultures of DEF and QUEF were prepared in 60 mm Falcon plastic dishes and used on the day they became confluent. Infected cells were inoculated into the cultures. These cultures were incubated at 37 C under CO_2. On the 4th day after infection, cultures were fixed with methanol and stained with Giemsa solution. Some infected cultures were overlaid with growth medium containing a final concentration of 0.9% Bacto-agar (Difco). On the 8th day, cultures were fixed with methanol and stained with Giemsa solution. The size of plaques was measured under a light microscope.

RESULTS

1. *Characteristics of HTV in tissue culture*

1) DEF passaged-HTV

At a low passage level (18th passage level) DEF cultures, HTV produced plaques composed of round refractile cells and some multinucleated giant cells. The size and frequency of incidence of multinucleated giant cells in plaques gradually increased on successive passage, as shown by Kato et al. (1970). At higher passage levels (more than about 70) the virus produced a syncytial type of large plaque (Fig. 1). One or more large syncytia developed in the center of the plaque by 72–96 hr after inoculation and within one to two days the syncytia became detached from the monolayer. In contrast, the plaques produced by HTV at a low passage level were still small after 8 days. By the 120th passage all infected centers gave rise to large plaques which attained 1.0 to 2.5 mm in diameter (Table 1).

2) QUEF passaged-HTV

DEF passaged-HTV at the 12th passage level was transferred to QUEF and passaged through QUEF thereafter. DEF passaged-HTV and HTV after few passages in QUEF produced small plaques appearing as discrete cytopathic areas in QUEF monolayers (Fig. 2). These plaques were composed of refractile cells which were mainly round. High-passage (more than 100 passages) of HTV through QUEF results

in development of medium sized plaques in QUEF monolayers containing numerous small syncytia (Fig. 3). Plaques attained a diameter of 0.6 to 1.5 mm. HTV after numerous passages in QUEF also produced large plaques of 1.0 to 2.5 mm diameter in DEF cultures (Fig. 4).

Syncytia were larger in DEF than in QUEF cultures (Table 1).

3) Virulent HTV (Biken V1 strain)

Biken V1 strain was grown in DEF and passaged through DEF. At the 5th passage level, infected DEF were inoculated onto DEF and QUEF monolayers. In both very small plaques of about 0.3 mm diameter were formed (Table 1).

4) Stability of the characteristics of plaques

TABLE 1. *Changes of plaque morphology on cell culture passage of HTV*

Inoculum	Plaque size		Giant cell formation	
	QUFF	DEF	QUEF	DEF
DEF 120[g]	S[c]	L[a]	±	+(LG)[f]
DEF 12-QUEF 108[g]	M[b]	L	+(SG)[e]	+(LG)
DEF 12-QUEF 110[g] -DEF 5	M	L	+(SG)	+(LG)
DEF 5 (Biken VI)	VS[d]	VS	−	−

a large size 1.0—2.5 mm.
b medium size 0.6—1.5 mm.
c small size 0.3—0.5 mm.
d very small size 0.2—0.4 mm.
e small giant cell.
f large giant cell.
g Biken C strain.

TABLE 2. *Pathogenicity of QUEF passaged-HTV (Biken C strain) to chickens*

Number of 1 day old chicks in group	Weeks / Inoculum	0~2	3~4	5~6	7~8	9~10	11~12	13~14	15~20	Total	Percentage mortality due to MD
20	Biken VI (MD blood) (0.2 ml)	0[a] (1)[b]	0	0	5	4 (2)	3	3	0	15 (3)	75%
32	Biken VI (DEF 3)[c]	0 (3)	0	0	4	1	7	4	3	19 (3)	59.4%
20	QUEF passaged-HTV at 65th passage level (5.8×10^3 PFU/chick)	0	0 (2)	0	0	0	0	0 (1)	0	0 (3)	0%
25	Uninfected QUEF	0 (1)	0	0	0 (1)	0	0	0	0	0 (2)	0%

a Numbers of chickens which died of MD.
b Numbers in parentheses showed the number of chickens which died of other causes than MD.
c Number of passages in DEF.

of QUEF passaged-HTV

QUEF passaged-HTV at the 110th passage level was inoculated onto a DEF culture, and passaged through DEF 5 times. Then the HTV was inoculated onto DEF and QUEF monolayers to see the stability of the characteristics of plaques of QUEF passaged-HTV. As shown in Table 1, after repassage in DEF it still formed plaques with the same morphology as QUEF passaged-HTV (Fig. 5a, b).

DEF passaged-HTV formed at least 4 times more plaques on DEF monolayers than on QUEF monolayers while QUEF passaged-HTV formed equal numbers on DEF and QUEF monolayers (Onoda et al., 1970). The infectivity of QUEF passaged-HTV after back-passage 5 times through DEF was examined in DEF and QUEF monolayers. The plaque counts of HTV were essentially the same in QUEF and DEF monolayers (Fig. 8).

2. *Pathogenicity of QUEF passaged-HTV to chickens*

QUEF passaged-HTV (Biken C strain) at the 65th passage level was inoculated intraperitoneally into 20 one day old chickens (a White Leghorn strain). As positive controls, 20 one day old chickens were inoculated intraperitoneally with blood from a typical MD chicken which had been inoculated with a chick passaged virulent strain (Biken V1 strain) of virus. The blood sample containing the Biken V1 strain was also inoculated onto DEF cultures and passaged through DEF 3 times. This low DEF passaged-HTV was then inoculated intraperitoneally into 32 one day old chickens. As a negative control uninfected QUEF were inoculated intraperitoneally into 28 one day old chickens. All these chickens were observed for 140 days. As shown in Table 2, chickens inoculated with HTV which had been highly passaged in QUEF or with uninfected QUEF did not develop MD, while chickens inoculated with either blood from a MD chicken, or with HTV after 3 passages in DEF showed a high incidence of MD.

3. *Frequency of reisolation of HTV from chickens inoculated with HTV*

Three groups of 1 day old chickens were inoculated with DEF passaged HTV (at the 110th passage level), QUEF passaged-HTV (at the 104th passage level) and DEF passaged-Biken V1 strain (at the 3rd passage level) respectively (Table 3). A 4th group of chickens served as controls. Blood was collected from the birds in each of groups after 13 weeks. Then 0.25 ml of each blood sample was inoculated onto QUEF or DEF cultures. The presence of HTV was detected by the appearance of typical cytopathic effects of HTV and by the immunofluorescent technique. Five of the 6 chickens in group III inoculated with virulent HTV showed difinite clinical signs of MD after 13 weeks and HTV was reisolated from their blood on DEF. The other 2 groups (I and II) appeared normal with no clinical signs of MD. However, HTV was reisolated from the blood of 4 of the 10 chickens in group 1 and 9 of the 20 chickens in Group 2. HTV

TABLE 3. *Recovery of HTV from chickens inoculated with QUEF and DEF passaged-HTV*

Group of Chickens	Inoculum	Dose PFU/birds	Virus isolation		Pathogenicity to chickens
			QUEF	DEF	
I	DEF 110[a]	2.6×10^3	2/10	4/10	−
II	DEF 12–QUEF 104[a]	2.2×10^3	8/20	9/20	−
III	DEF 3 (Biken VI)	2.0×10^3	0/5	5/6	+
IV	uninfected control	none	0/5	0/5	−

a Biken C strain.

was reisolated less frequently using QUEF cultures than using DEF cultures and the susceptibility of QUEF to virulent Biken V1 strain was especially low. No HTV was isolated from uninoculated control chickens.

4. *Characteristics of reisolated HTV*

As shown in Table 4 the HTV recovered from chickens inoculated with HTV after numerous passages in DEF or in QUEF formed plaques with the same morphology in QUEF and DEF cultures as the original DEF- and QUEF-passaged HTV. Fig. 6a and 6b showed a large plaque with large syncytia on a DEF monolayer and a small plaque with round cells on a QUEF monolayer respectively, produced by HTV isolated from a chicken inoculated with DEF passaged-HTV. Figs. 7a and 7b show a large plaque with large syncytia on a DEF monolayer and a medium sized plaque with many small syncytia on a QUEF monolayer respectively, produced by HTV isolated from a chicken inoculated with QUEF passaged-HTV. The morphologies of these plaques are the same as those of plaques formed by the original QUEF passaged-HTV.

HTV reisolated from chickens inoculated with DEF passaged-HTV was inoculated onto DEF and QUEF monolayers. As shown in Fig. 9, at least three times more plaques were formed in DEF monolayers than in QUEF monolayers. When HTV reisolated from chickens inoculated with QUEF passaged-HTV was inoculated onto DEF and QUEF monolayers, it formed the same number of plaques on both (Fig. 10).

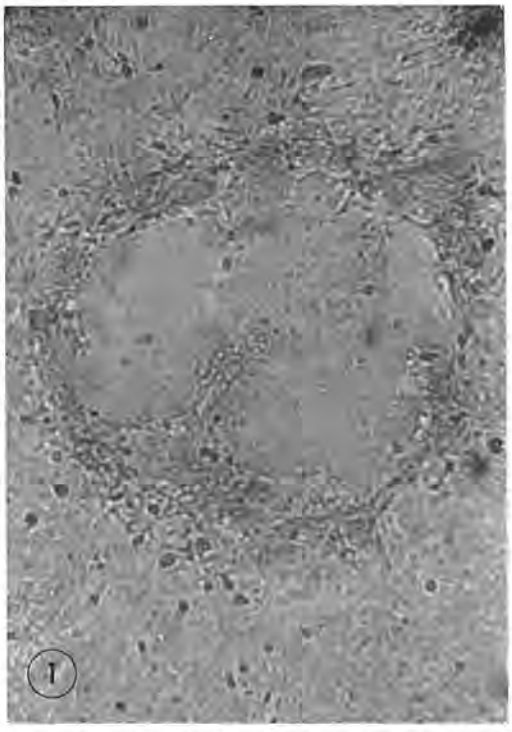
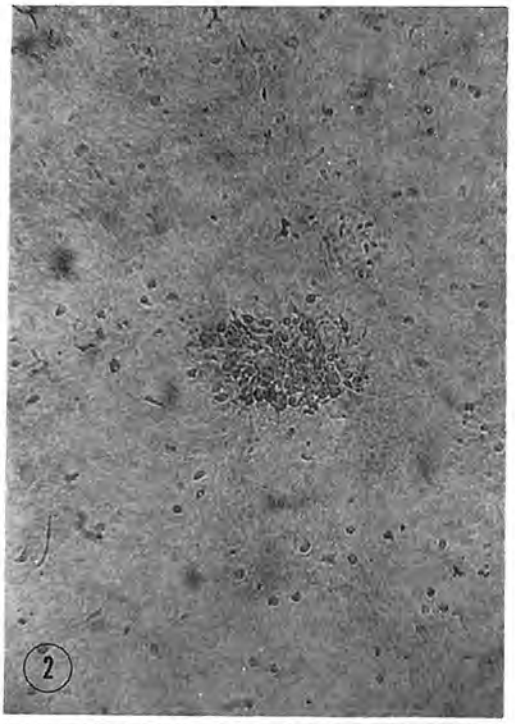


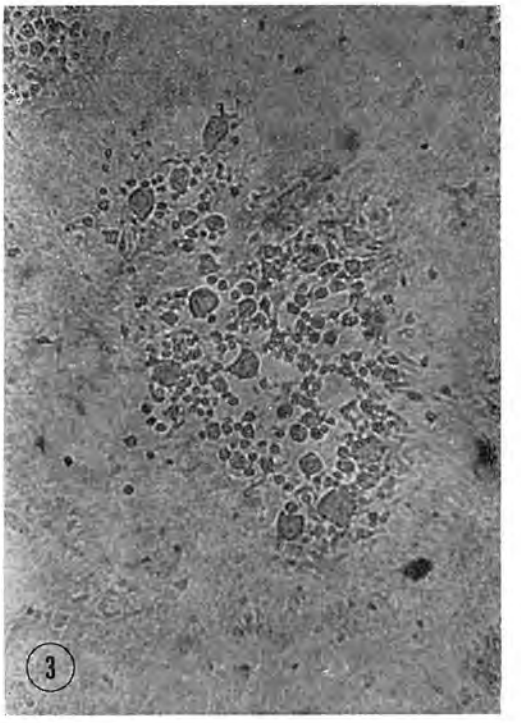
FIGURE 1. *A large plaque 4 days after infection of a DEF monolayer with DEF passaged-HTV (at the 105th passage level). Large syncytia are seen.*

TABLE 4. *Plaque morphology of HTV reisolated from chickens inoculated with DEF and QUEF passaged-HTV*

Inoculum in chickens	Properties of HTV isolated from blood of chickens			
	Plaque size		Giant cell formation	
	QUEF	DEF	QUEF	DEF
DEF 110	S	L	±	+ (LG)
DEF 12-QUEF 104	M	L	+ (SG)	+ (LG)
DEF 3 (Biken VI)	VS	VS	−	−

Abbreviations are the same as for Table 1.





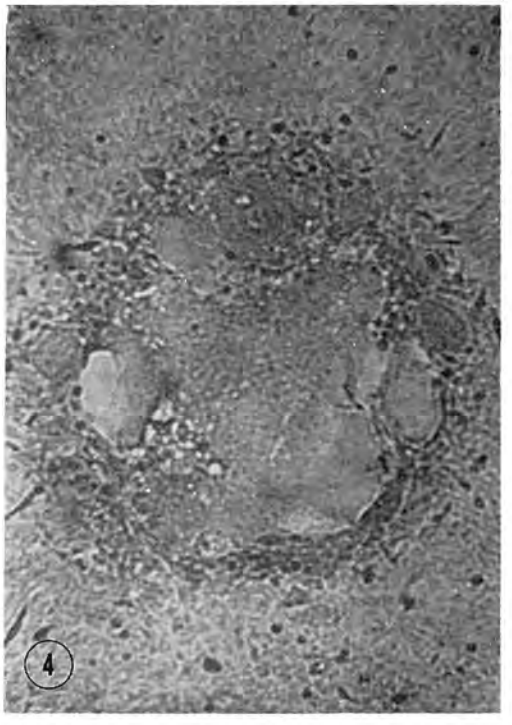


FIGURE 2. *A small plaque 4 days after infection of a QUEF monolayer with DEF passaged-HTV (at the 120th passage level). The infected cells are refractile and round.*

FIGURE 3. *A moderately large plaque 4 days after infection of a QUEF monolayer with QUEF passaged-HTV (at the 112th passage level). Many small syncytia are seen in the plaque.*

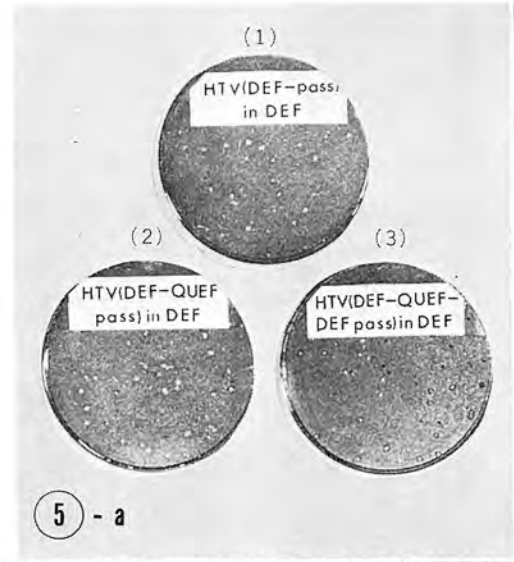
FIGURE 4. *A large plaque 4 days after infection of a DEF monolayer with QUEF passaged-HTV (at the 108th passage level). Large syncytia are seen.*

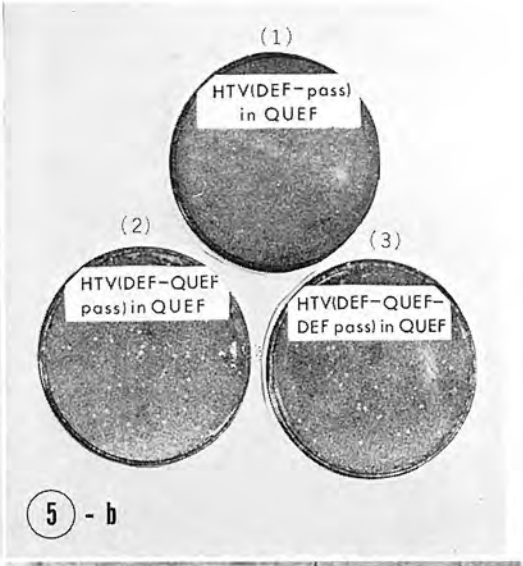
▶

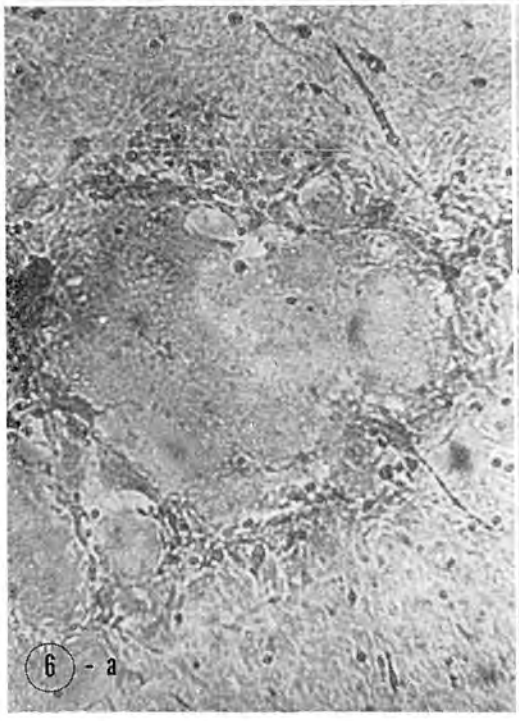
FIGURE 5. *Comparison of plaque sizes of HTV in DEF and QUEF monolayers.*

(a) *DEF monolayers showing large plaques 8 days after infection with 1) DEF passaged-HTV (at the 118th passage level), 2) QUEF passaged-HTV (at the 105th passage level), 3) QUEF passaged-HTV (at the 110th passage level) backpassaged through DEF 5 times.*

(b) *QUEF monolayers showing plaques 8 days after infection with HTV. 1) Small plaques of DEF passaged-HTV (at the 118th passage level). 2) Medium sized plaques of QUEF passaged-HTV (at the 105th passage level). 3) Medium sized plaques of QUEF passaged-HTV (at the 110th passage level) backpassaged through DEF 5 times.*







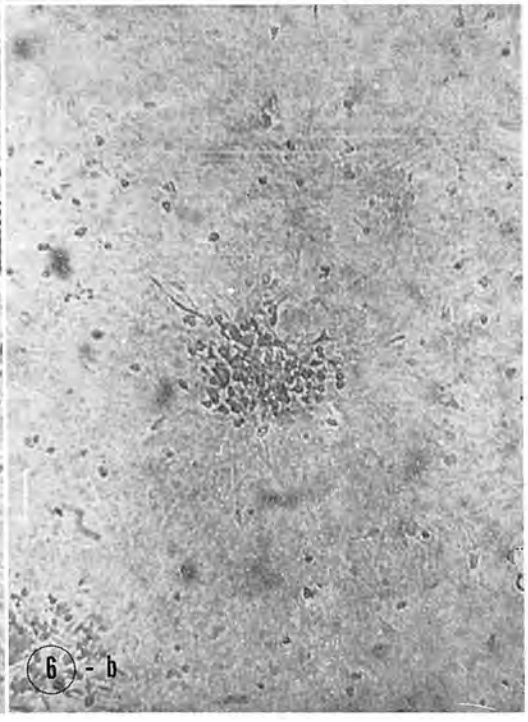


FIGURE 6 a. *A large plaque 4 days after infection of a DEF monolayer with HTV reisolated from chickens inoculated with DEF passaged-HTV.*

FIGURE 6 b. *A small plaque 4 days after infection of a QUEF monolayer with HTV reisolated from chickens inoculated with DEF passaged-HTV.*

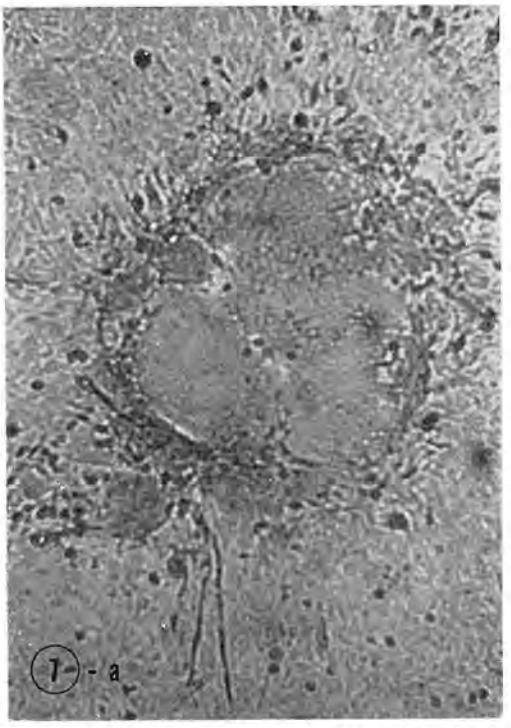


FIGURE 7 a. *A large plaque 4 days after infection of a DEF monolayer with HTV reisolated from chickens inoculated with QUEF passaged-HTV.*

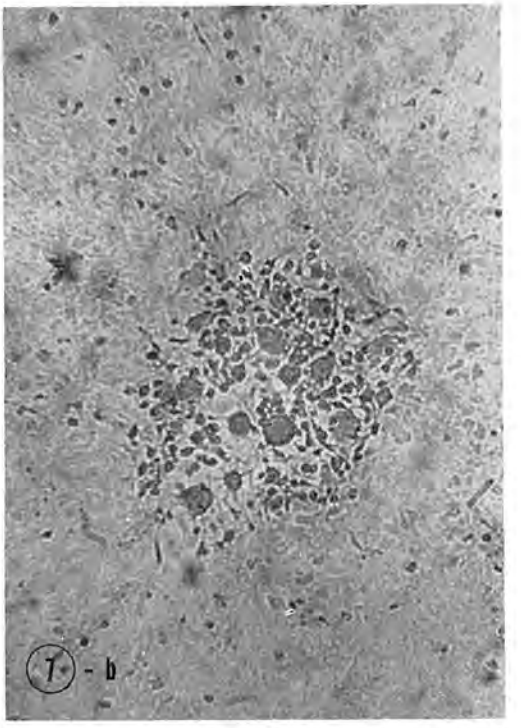


FIGURE 7 b. *A medium sized plaque with many small syncytia on a QUEF monolayer 4 days after infection with HTV reisolated from chickens inoculated with QUEF passaged-HTV.*

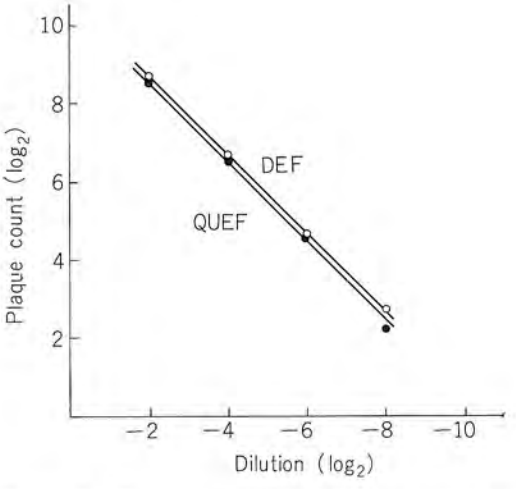


FIGURE 8. *Dose-response curves of plaque counts on DEF and QUEF monolayers inoculated with QUEF passaged-HTV 8 days after inoculation.*

DISCUSSION

Churchill et al. (1969a, b) demonstrated that a herpes-type virus of Marek's disease when serially passaged in chick kidney cell cultures showed marked changes in plaque morphology, growth, antigenicity and pathogenicity to chickens. These changes were progressive and by the 60th passage macroscopic plaques of about 1.5 mm diameter were produced after 6 days under liquid overlay. Nazerian (1970) also reported that the cytopathology of Marek's disease virus changed on passage in cell cultures and by approximately the 60th passage plaques

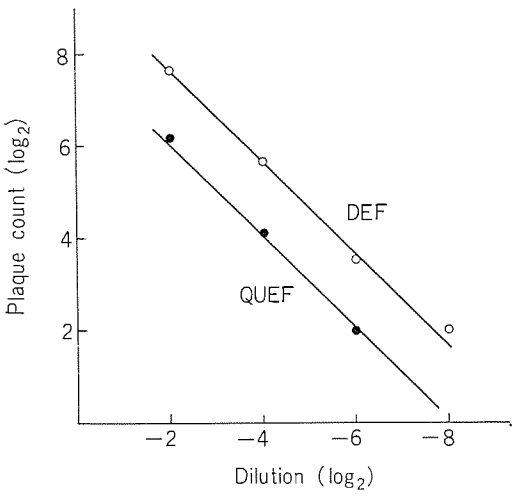


FIGURE 9. *Dose-response curves of plaque counts on DEF and QUEF monolayers inoculated with HTV recovered from chickens inoculated with DEF passaged-HTV.*

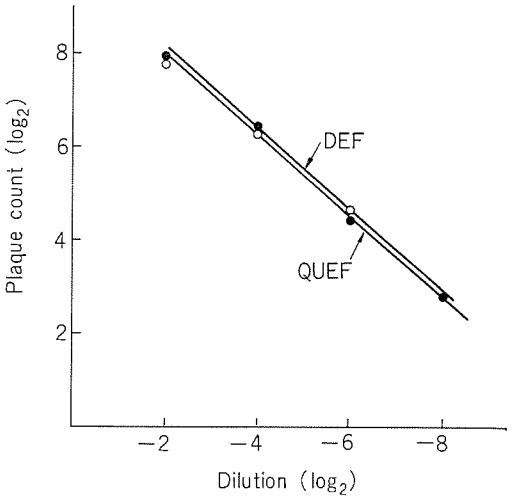


FIGURE 10. *Dose-response curves of plaque counts on DEF and QUEF monolayers inoculated with HTV recovered from chickens inoculated with QUEF passaged-HTV.*

developed and expanded faster than at low passage levels (up to the 23rd), and that pathogenicity decreased on prolonged cell culture. No pathogenicity of DEF passaged-HTV (Biken C strain) from an MD chicken was demonstrated when HTV at the 43rd passage level was inoculated into 1 day old chickens (Kato et al., 1970). Mikami et al. (1970a, b), reported that the Cal-1 strain of Marek's disease agent produced two types of plaque in chick kidney cell cultures. One type was small (0.3–0.8 mm in diameter) and the other large (1.0–2.5 mm in diameter). When virus samples from the two different types of plaques, were inoculated into chickens, the virus recovered from the chickens formed the same type of plaque as that of the inoculated sample.

The present results demonstrate that during passage of HTV in cell cultures, two change occur in the properties of the virus, namely change in the type of cytopathology in cell culture and loss of pathogenicity to chickens. DEF passaged-HTV (at a low passage level) produced very small plaques composed of round and refractile cells and few if any multinucleated giant cells in either DEF or QUEF monolayers, and when inoculated into chickens caused MD. When HTV was highly passaged through DEF, the virus produced large plaques with large syncytia on DEF monolayers, and small plaques with few syncytia on QUEF monolayers, and did not show any pathogenicity to chickens. When DEF passaged-HTV was transferred to QUEF and passaged repeatedly through QUEF thereafter, moderately large plaques with many small syncytia were observed in QUEF cultures, and large plaques with large syncytia developed in DEF cultures. QUEF passaged-HTV produced the same number of plaques in QUEF monolayers as in DEF monolayers, while DEF passaged-HTV produced fewer plaques in QUEF monolayers than in DEF monolayers. HTV after numerous passages in QUEF also caused no MD in chickens. The characteristics of QUEF passaged-HTV were not affected by backpassage of the virus through DEF 5 times. Despite the loss of pathogenicity of HTV to chickens,

HTV replicated in chickens and after inoculations was reisolated from their blood. The reisolated HTV showed the same properties as HTV before inoculation. These results indicate that passage of HTV through a chicken did not result in reversion of the virus to the original type. Change in the plaque morphology and loss of pathogenicity may be caused by adaptation of the virus to these cell cultures. The morphology and size of the plaques of HTV and its infectivity to DEF and QUEF seem to be useful markers of HTV. Studies on the stability of the characteristics of HTV after repeated passage in chickens are in progress.

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