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SHORT COMMUNICATION

MAREK'S DISEASE VIRUS AND HERPESVIRUS OF TURKEY NONINFECTIVE TO CHICKENS, OBTAINED BY REPEATED IN VITRO PASSAGES[1]

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The relation between the passage level of Marek's disease virus, C2 strain, and of herpesvirus of turkey (HVT), O1 strain, in cell culture and the level of the serological response of chickens to these viruses was examined. In both cases the immune response of chickens to these viruses decreased with increase in the number of in vitro passages of virus.

Virus was not recovered from chickens inoculated with HVT highly passaged in vitro, which had become a high producer of cell-free virus in vitro, and grew equally well at 37 C and 41 C.

Marek's disease (MD) is a lympho-proliferative disease of chickens caused by MD virus (MDV). Multiple passages of pathogenic MDV in cultured cells results in a number of changes in the properties of MDV (Churchill et al., 1969; Nazerian, 1970; Eidson and Anderson, 1971; Onoda et al., 1971b; Phillips and Biggs, 1972). These authors reported that during successive passages the rate of spread of the virus in chick or duck embryo cell cultures increases, resulting in formation of larger plaques, but that the rate of spread in chickens decreases, resulting in lower levels of infectivity.

Herpesvirus of turkey (HVT) has been shown to be serologically related to MDV (Witter et al., 1970) and has been used effectively as live virus for vaccination of chicken against MD (Okazaki et al., 1970; Purchase et al., 1971). However, its protective efficacy became less with continued passage in chick embryo cells (Witter and Offenbecker, 1979). Recently, we analyzed the DNAs of a vaccine strain of HVT, O1 strain (Ono et al., 1974),

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and a variant of the strain highly passaged in duck, quail and chick embryo fibroblasts, and we could not detect any difference between these DNAs with respect to their sedimentation behaviors in neutral glycerol and alkaline sucrose gradients, their buoyant densities in a CsCl density gradient, or the restriction enzyme-cleavage patterns of their DNAs (Hirai et al., 1979).

This paper reports studies on the serological responses of chickens to MDV and HVT at different passage levels in cell culture.

The C2 strain of MDV (Kato et al., 1970) and the O1 strain of HVT (Ono et al., 1974) were used. Primary cultures of duck embryo fibroblasts (DEF) and quail embryo fibroblasts (QEF) were prepared as described previously (Onoda et al., 1970). Primary cultures of chick embryo fibroblasts (CEF) were prepared from 10-day-old embryos in the same way. MDV and HVT were grown on these primary cultured cells at 37 C.

Four preparations of MDV, C2 strain, with the following passage levels in tissue culture were used: 12 times in DEF and 79 times in QEF ($D_{12}Q_{79}$); 12 times in DEF and 89 times in QEF ($D_{12}Q_{89}$); 12 times in DEF and 135 times in QEF ($D_{12}Q_{135}$); and 12 times in DEF and 153 times in QEF ($D_{12}Q_{153}$). The infective titers of these viruses were assayed by plaque-formation on QEF as described previously (Onoda et al., 1970). Inocula of 3–5×10^3 PFU of these viruses were injected intramuscularly into specific pathogen-free chickens (White Leghorn, one-day-old). Four weeks later, the antibody titer of the serum in the chickens was assayed by the indirect immunofluorescence test as described previously (Naito et al., 1969). Figure 1 shows the relationship between the number of in vitro passages of the virus and the immune response of the chickens to the viruses. Viruses $D_{12}Q_{79}$ and $D_{12}Q_{89}$ induced antibody to MDV in all chickens examined. However, $D_{12}Q_{135}$ induced antibody in only two in ten chickens examined, and $D_{12}Q_{153}$ did not induce antibody in any chickens examined. The chickens with no detectable antibody after four weeks still did not have any detectable antibody after eight weeks, like uninoculated chickens.

Four preparations of HVT, O1 strain, were examined in the same manner. These preparations had the following passage levels in

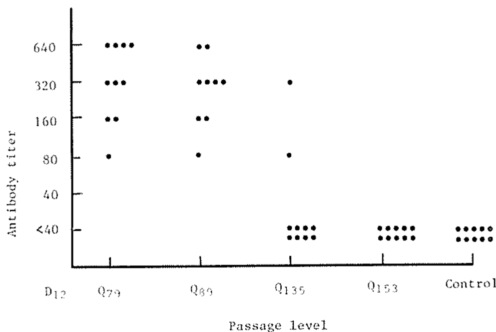

FIGURE 1. Relationship between the number of in vitro passages of MDV and the induction rate of an immune response in infected chickens. 3–5×10^3 PFU of each virus were injected intramuscularly into one-day-old chickens. After four weeks, the serum was prepared, and the titer of MDV-specific antibody was assayed by the indirect immunofluorescence test. As a control, MEM was inoculated in the same way.

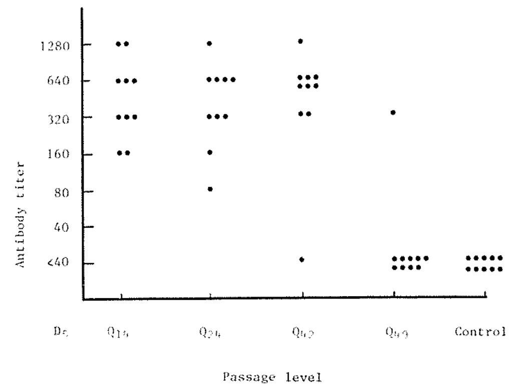

FIGURE 2. Relationship between the number of in vitro passages of HVT and the induction rate of the immune response in infected chickens. One-day-old chickens were infected with each virus in the same manner as for Fig.1. The titer of HVT-specific antibody was assayed by the indirect immunofluorescence test. As a control, MEM was inoculated in the same way.

tissue culture: 5 times in DEF and 14 times in QEF (D_5Q_{14}); 5 times in DEF and 24 times in QEF (D_5Q_{24}); 5 times in DEF and 42 times in QEF (D_5Q_{42}); and 5 times in DEF and 49 times in QEF (D_5Q_{49}). As shown in Fig. 2, two viruses, D_5Q_{14} and D_5Q_{24}, induced antibody to HVT in all chickens examined; D_5Q_{42} induced antibody in nine of ten chickens, and D_5Q_{49} induced antibody in one of ten chickens.

These results in Figs. 1 and 2 show that induction of an immune response in infected chickens decreases with increase in the number of in vitro passages of the virus in quail cells. No viremia was detected in chickens infected with multiple-passaged HVT, D_6Q_{48}, although it was observed in chickens infected with D_6Q_{15} virus (Table 1). These results indicate that during passage in QEF culture HVT, O1 strain, lost the ability to grow in chickens.

TABLE 1. *Absence of viremia in chickens infected with high passaged virus of HVT*

Virus	PFU of inoculum	Number of chickens examined	Number of chickens showing viremia[a]
D_6Q_{48}	1×10^4	5	0
	1×10^3	4	0
D_6Q_{15}	3×10^3	5	5

[a] Cultures of DEF were inoculated with buffy coat cells separated from whole blood cells, to observe plaques.

TABLE 2. *Comparison of infectious cell-free virus in culture fluid of four virus preparations of HVT*

Virus[a]	Cell-free virus titer (PFU/0.2 ml) in culture fluid		
	24 hr[b]	48 hr	72 hr
D_6Q_9	1.0×10^1	1.8×10^2	7.5×10^1
D_6Q_{15}	5.0×10^0	1.0×10^2	7.0×10^1
D_6O_{25}	4.5×10^1	6.8×10^3	2.0×10^2
D_6Q_{50}	1.0×10^2	3.6×10^4	3.8×10^2

[a] The passage numbers in DEF and QEF are shown.

[b] The time after infection is shown.

The titers of infectious virus in the culture fluids of the four preparations of HVT, O1 strain, were assayed as described above. Results showed that the titer of cell-free virus in the fluid increased with increase in the number of passages in QEF (Table 2). Especially, at 48 hr after infection, the titer of high-passaged virus was about 200 times that of low-passaged virus.

Witter and Offenbecker (1979) demonstrated that after many passages in CEF, HVT, FC 126 strain, did not show temperature-sensitivity, although the high-passaged variant of MDV, JM strain, showed temperature-sen-

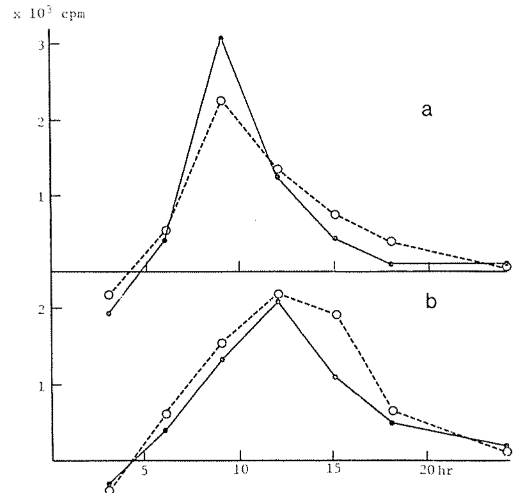

FIGURE 3. Comparison of induction rates of DNA synthesis of high-passaged virus of HVT in CEF cells at 37 C and 41 C. Monolayer cells of CEF were infected with high-passaged virus ($D_6Q_{58}C_7$) or with low-passaged virus ($D_6Q_9C_{10}$), or mock-infected, and adsorbed for 2 hr at 37 C. Then the medium was changed and the infected cells were cultured at 37 C (b) or 41 C (a), and labeled with [^{3}H]-thymidine for 3 hr at appropriate times after adsorption as shown in figure. The radioactivities incorporated into cells infected with $D_6Q_{58}C_7$ virus (●——●) and with $D_6Q_9C_{10}$ virus (O- - -O) were calculated by subtracting the counts in mock-infected cells from those in infected cells.

sitivity. We examined whether high-passaged HVT, O1 strain, showed temperature-sensitivity by comparing the induction rates of viral DNA synthesis at 37 C and at 41 C. Monolayer cells of CEF were infected with cell-free virus, obtained from infected CEF by sonication in the presence of sucrose, or were mock-infected with cell extract, obtained from uninfected CEF by the same way. The high-passaged virus ($D_6Q_{58}C_7$) and the low-passaged virus ($D_6Q_9C_{10}$) of HVT, O1 strain, were used. After adsorption for 2 hr at 37 C, the medium was changed and the infected cells were incubated at 37 C or 41 C. At appropriate times (see legend to Fig. 3), the infected or mock-infected cells were pulse-labeled for 3 hr with [^{3}H]-thymidine (56.4 Ci/mmol; New England Nuclear). Figure 3 shows the induction rates of DNA synthesis by HVT infection at 37 C and 41 C. The rates of incorporation of [^{3}H]-thymidine in infected CEF at 41 C and 37 C were nearly similar, although the peak of DNA synthesis at 41 C was earlier than that at 37 C. Moreover, on plaque assay of this high-passaged virus of HVT, O1 strain, at 37 C and 41 C, the plating ratio at 37 C to that at 41 C was about 1. These results indicate that the high-passaged HVT, O1 strain, does not show temperature-sensitivity.

A temperature-sensitive mutant of MDV was obtained by Onoda et al. (1971a). This mutant did not replicate in vivo (Onoda, personal communication). If all high-passaged viruses showed temperature-sensitivity, this could well be the explanation for its inability to replicate in vivo, because the body temperature of the chicken (41 C) is about the restrictive temperature in vitro. However, since the high-passaged virus of HVT, O1 strain, did not show temperature-sensitivity, there must be another reason for the poor replication of this virus in vivo.

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