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PERSISTENCE OF GENOMES OF BOTH HERPESVIRUS OF TURKEYS AND MAREK'S DISEASE VIRUS IN A CHICKEN T-LYMPHOBLASTOID CELL LINE

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SUMMARY A cell line tentatively designated as MDCC-BOl(T), was established from spleen cells of an apparently healthy chicken inoculated with herpesvirus of turkey (HVT). BOl(T) cells were T lymphoblastoid cells and the more than 95% of them had Marek's disease (MD) tumor-associated surface antigen (MATSA). However, no viral internal antigens or membrane antigens could be demonstrated in them by immunofluorescence tests using chicken anti-HVT and -MD virus (MDV) sera. The virus could be rescued from BOl(T) cells by co-cultivation with chick embryo fibroblasts (CEF). The DNA of the rescued virus was characterized as HVT DNA by its sedimentation profile in a neutral glycerol gradient and its endonuclease Hind III cleavage-pattern. Ultrastructural studies on CEF infected with the rescued virus revealed the presence of HVT-like virions. However, DNA-DNA reassociation kinetics showed that the BOl(T) cells contained a few copies of HVT and also MDV genomes.

INTRODUCTION

Marek's disease (MD) is a highly contagious malignant lymphoma of chickens caused by a herpesvirus named MD virus (MDV). Herpesvirus of turkeys (HVT) was shown to be antigenically related to MDV and has been used for vaccination against MD. Recently, MD tumor-associated surface antigen (MATSA), which was supposed to be expressed only on MDV-transformed cells (Witter et al., 1975; Matsuda et al., 1976b), was demonstrated on spleen and peripheral blood lymphocytes of chickens vaccinated with HVT (Schat and Calnek, 1978; Powell and Rennie, 1978; Kitamoto et al., 1979). Moreover, chickens infected with HVT were found to develop transitory lymphoproliferative lesions at an early stage of infection (Witter et al., 1976). These results suggest that neoplastic trans-

formation of lymphocytes may occur in vaccinated chickens. However, no HVT-associated cell line has yet been established from HVT-vaccinated chickens. This paper reports the establishment of a T-lymphoblastoid cell line from a chicken vaccinated with HVT and presence of both HVT and MDV genomes in the cells.

MATERIALS AND METHODS

1. *Virus and chicken*

The HVT used for inoculation was the O1 strain (Ono et al., 1974), propagated in primary chick embryo fibroblasts (CEF). Three chickens from a specific pathogen-free (SPF) flock were inoculated intra-abdominally with 5,000 plaque-forming units (PFU) of HVT/0.2 ml/chicken on days 1 and 60 after birth. The MDV used for characterization of MDV genome was the C2 strain (Kato et al., 1970).

2. *Cultures*

Primary spleen cells from chickens 7 days after the second inoculation were used as starting material. The spleen, which had no gross lesions, was chopped into small pieces with scissors, suspended in growth medium (RPMI-1640 supplemented with 20% fetal calf serum and 10% chicken serum) and passed through a stainless-steel wire sieve. The cell suspension was layered on Lymphoprep (density, 1.077 g/ml; Nyegaard & Co., Oslo, Norway), centrifuged at 1,500 rpm for 20 min, and washed three times with the growth medium. The cell pellet was resuspended in growth medium at a final density of 1×10^7 cells/ml and seeded into petri dishes (100×20 mm). The dishes were incubated at 41°C in a humidified atmosphere of 5% CO_2 in air.

3. *Immunofluorescence tests*

Direct immunofluorescence tests to detect virally induced intracellular antigen (ICA) and cell surface antigen (CSA) were performed by the methods of Naito et al. (1969) and Ishikawa et al. (1972), respectively. Chicken anti-MDV serum and anti-HVT serum were used. The rabbit and chicken anti-MATSA sera used in the indirect immunofluorescence test were prepared as described by Matsuda et al. (1976b). In tests for T- and B-surface determinants by the indirect immunofluorescence technique, rabbit anti-normal thymus cell (anti-T) serum and anti-normal bursa cell (anti-B) serum were used (Matsuda et al., 1976a). The antisera were extensively cross-absorbed before use.

4. *Chromosomal analysis*

Chromosome preparations were made by the method of Owen (1965). The cells were fixed on a glass slide by drying them over a flame after arresting mitosis by treatment with colcemide (0.1 μg/ml) for 2 hr. The dried preparations were stained with Giemsa.

5. *Isolation of virus from the line*

Monolayers of CEF cells (1×10^7) in 100×200 mm petri dishes were inoculated with 1×10^7 established line cells in minimum essential medium (MEM) containing 5% calf serum and the dishes were kept at 37°C. Two days after inoculation the liquid medium was replaced by MEM. Cells were observed every day.

6. *Analysis of the virus rescued from the line*

The viruses rescued from the line were passaged five times in CEF and then subcultured in phosphate-free MEM, and 20 μCi/ml of ^{32}P was added to the cultures. The cells were harvested when about 50% of them showed CPE. The virions were isolated from the cytoplasm of cells treated with 1% Nonidet P-40 as described previously (Hirai et al., 1979). The virions were lysed overnight at 37°C in 0.1 *M* Tris-HCl, pH 9.0, 0.1 *M* NaCl, 0.001 *M* EDTA, 1% sodium dodecyl sulfate (SDS) with 1 mg/ml of pronase. The lysates were layered on linear gradients of 10 to 30% glycerol in 0.01 *M* Tris-HCl, pH 7.4, 0.01 *M* EDTA, 1% Sarkosinate and centrifuged in a Beckman SW41 rotor at 175,000 *g* for 4 hr at 18°C. The ^{32}P DNA from the rescued viruses was found in the same position in the gradient as ^{32}P HVT DNA. The ^{32}P viral DNA isolated by velocity sedimentation in neutral glycerol gradients was digested with Hind III restriction endonuclease and subjected to electrophoresis on a 0.5% horizontal agarose gel at 60 V for 20 hr at 22°C as described previously (Hirai et al., 1979). The gel was dried and placed on Sakura-X-ray film for autoradiography.

7. *DNA-DNA reassociation kinetics*

HVT and MDV DNAs were isolated and labeled

with ^{3}H-TTP in vitro using *Escherichia coli* DNA polymerase I as described previously (Hirai et al., 1979). ^{3}H-HVT DNA (0.023 μg, 3.9×10^6 cpm/μg) or ^{3}H-MDV DNA (0.021 μg, 4.2×10^6 cpm/μg) was mixed with 2 mg of sonicated cell DNA in 0.6 ml of 0.1 mM EDTA. The mixture was denatured by heating it at 100°C for 10 min and then chilling in ice. Then 0.4 ml of 5 *M* NaCl was added to the mixture. Hybridization was carried out at 66°C and the fraction of reassociated ^{3}H-DNA was analysed by differential digestion with nuclease S1 (Hirai et al., 1979). The following equation was used for analysis of the data: $(Co/C)^{1/0.55}=1+kCot$, where C and Co are the concentrations of single stranded ^{3}H-viral DNA at time t and t=0, and k is the reassociation constant (Britten and Davidson, 1976).

RESULTS

1. *General properties of the cell line*

One culture that grew for over 150 days was designated as MDCC-BOl(T). Table 1 summarizes the characteristics of the MDCC-BOl(T) cells in comparison with those of the MDCC-MSB-1 line, which was derived from an MD lymphoma (Akiyama and Kato, 1974). The cells grew mainly singly, with a few small

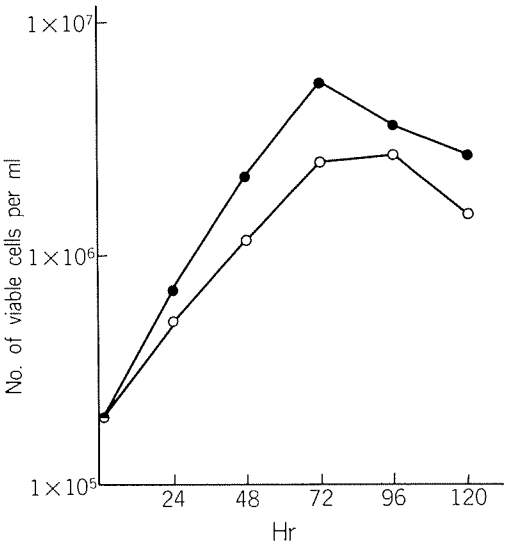

FIGURE 1. Growth curves of MDCC-BOl (T) and MDCC-MSB-1 line cells. Symbols: ○, BOl (T); ●, MSB-1.

TABLE 1. *Properties of the MDCC-BO1(T) and MDCC-MSB-1 lines.*

Characteristics	BO1(T)	MSB-1
Morphology	Lymphoblastoid	Lymphoblastoid
Doubling time	18 hr	10 hr
Cells with MATSA[a]	>95%	>95%
Cells with ICA[b]	—	0.5% or less (MDV)
Cells with CSA[c]	—	0.5% or less (MDV)
T- or B-cell determinant	T	T
Herpes-type capsid	—	+
C-type particle	—	—
Karyotype	Female	Female
Chromosomal aberration	+[d]	+
MDV viral genome	3.5 copies/cell	50 copies/cell
HTV viral genome	1.6 copies/cell	<0.1 copy/cell
Cloning efficiency	High	High
Isolation of virus by co-cultivation with CEF	HVT	MDV

[a] Marek's disease tumor-associated surface antigen.
[b] MDV- or HVT-induced intracellular antigen.
[c] MDV- or HVT-induced cell surface antigen.
[d] Chromosomal aberration of No. 1 and two submetacentrics were present.

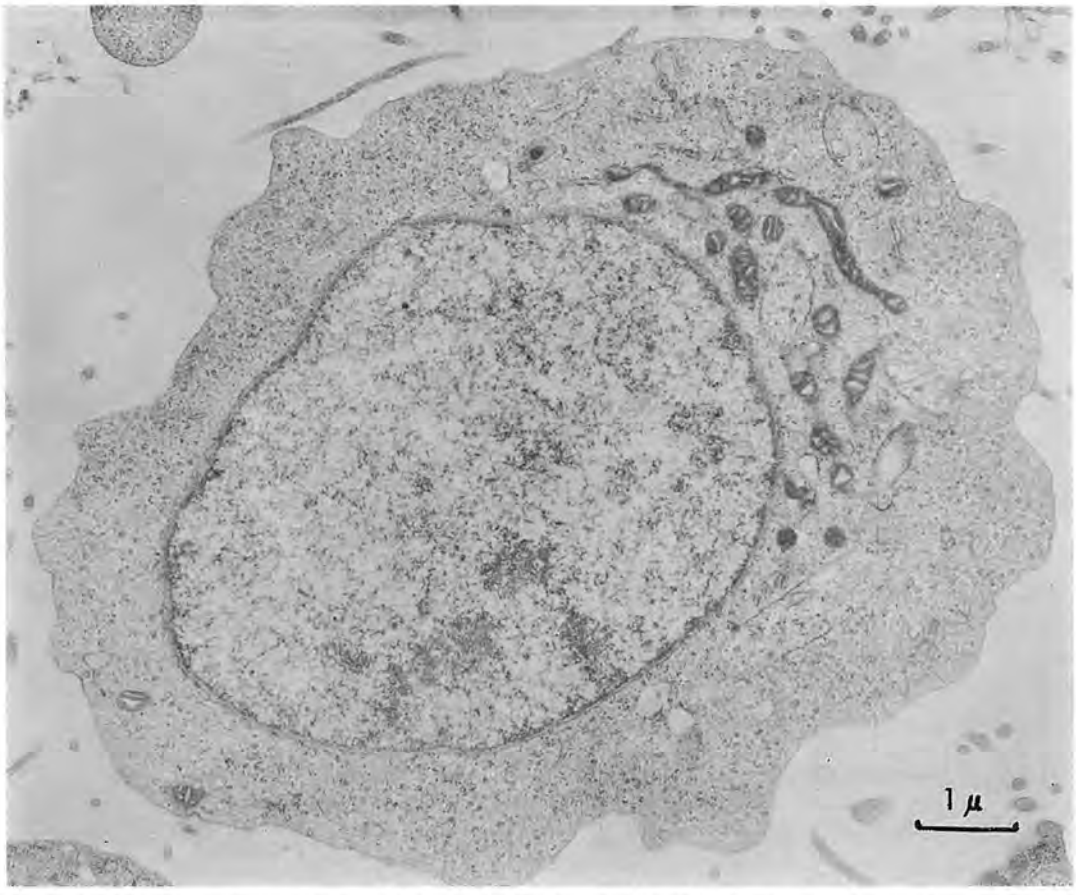

FIGURE 2. Electron micrograph of an MDCC-BOl (T) lymphoblastoid cell. Neither herpesvirus nor C-type particles are detectable.

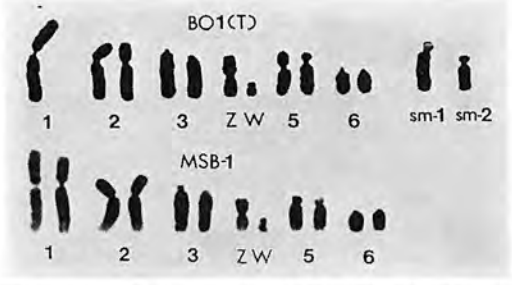

FIGURE 3. Chromosomes of MDCC-BOl (T) and MDCC-MSB-1 line cells.

clumps, and did not become attached to the glass. They grew well at 41°C with a doubling time of 18 hr (Fig. 1). Neither herpesvirus nor C-type particles were detectable in the cells by electron microscopy (Fig. 2). Morphologically, the MDCC-BOl(T) cells resembled those of established MD lymphoma lines, such as the MDCC-MSB-1 line. Karyotype analysis confirmed the origin of the cell line from a female chicken (Fig. 3). The karyotype of BOl(T) cells was different from those of other established cell lines from MD lymphomas.

2. *Immunofluorescence tests*

The presence of HVT- and MDV-induced intracellular antigens (ICA) and cell surface antigen (CSA) in MDCC-BOl(T) cells was

examined by the direct immunofluorescence technique using FITC-conjugated chicken anti-MDV and anti-HVT sera. Neither ICA nor CSA for either virus was observed in any preparation. However, tests on living MDCC-BOl(T) cells by the indirect immunofluorescence technique demonstrated the presence of MATSA on more than 95% of the cells, as found on MD lymphoma cells. MDCC-BOl(T) cells were tested for T- and B-surface determinants by the indirect immunofluorescence technique using anti-T and anti-B sera. The cells were identified as T cells, like all cell lines of MD lymphoma.

3. *Isolation of virus from the line*

To rescue herpesvirus from MDCC-BOl(T) cells, monolayer cultures of CEF cells (1×10^7) in 100×20 mm petri dishes were inoculated with 1×10^7 MDCC-BOl(T) cells. Only a few foci per dish were observed 9–10 days after inoculation, and these were subcultured for detection of herpesvirus particles by electron microscopy and of viral ICA by the direct immunofluorescence technique. The electron micrograph in Fig. 4 shows herpesvirus particles in a thin section of the CEF cells inoculated with viruses isolated from MDCC-BOl(T) cells. Several particles had distinctive cross-shaped internal structure, as indicated by long arrows in Fig. 4. This structure is characteristic of HVT (Nazerian et al., 1971; Nii et al., 1973), but not of MDV. CEF cells inoculated with viruses isolated from MDCC-BOl(T) cells stained more intensely for HVT ICA than MDV ICA using FITC-

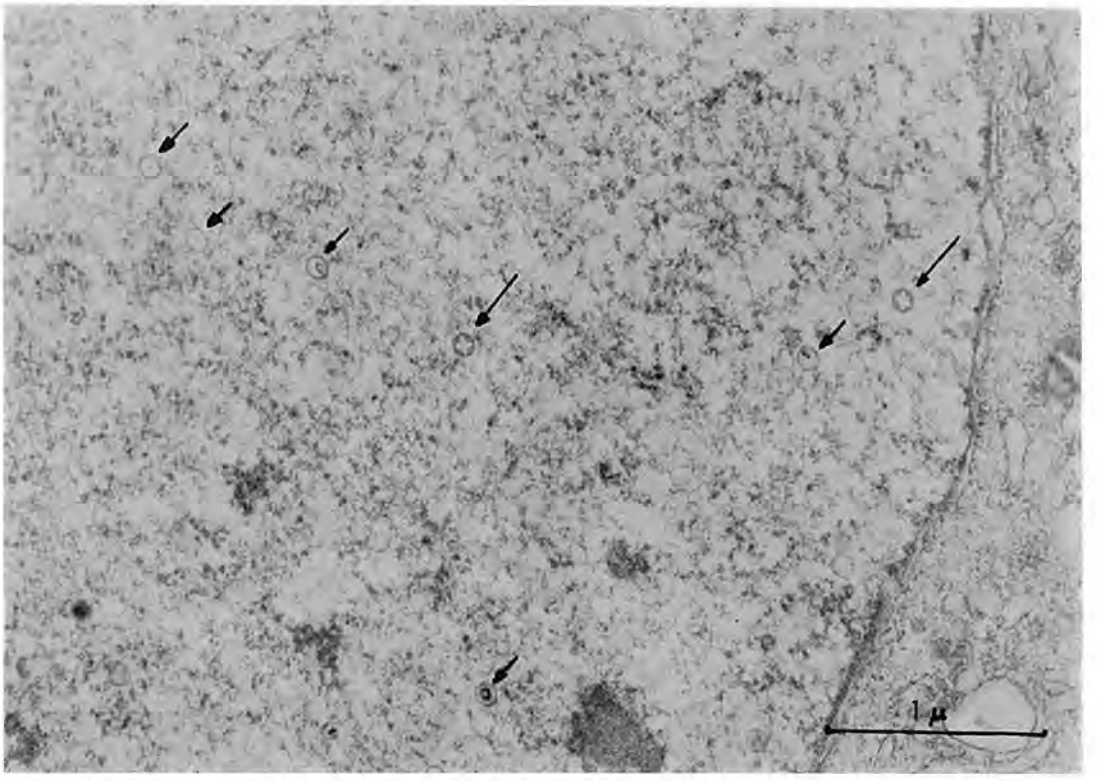

FIGURE 4. Thin section electron micrograph of chick embryo fibroblasts (CEF) after co-cultivation with MDCC-BOl (T) line cells. Several herpes-type capsid structures (short arrows) and cross-shaped capasids (long arrows) are observed in the nucleus.

conjugated chicken anti-MDV serum and anti-HVT serum. These findings indicates that most of the virus rescued from MDCC-BOl(T) cells and passaged in CEF is HVT, although MDV may be present in CEF co-cultivated with MDCC-BOl(T) cells and may be eliminated by passages in CEF.

Comparison of the electrophoretic patterns of the digestion products of MDV and HVT DNAs with restriction endonuclease Hind III and EcoRl showed similarities in the cleavage patterns between strains, but not between those of MDV and HVT (Hirai et al., 1979). Therefore, these patterns can be used for comparison of these herpesvirus DNAs. As an example, an autoradiograph of the products of the DNAs with Hind III is shown in Fig. 5. The Hind III-cleavage pattern of the rescued viral DNA was in general similar to that of HVT DNA, but clearly different from that of MDV DNA. The fact that a few bands of the rescued viral DNA did not correspond with those of HVT DNA may be due to the conditions used for digestion with the restriction enzyme. The results support the conclusion that most of the rescued virus was HVT.

FIGURE 5. Hind III digestion products of DNA of the virus rescued from MDCC-BOl (T) cells. Track 1, HVT DNA; track 2, DNA of the virus rescued from MDCC-BOl (T) cells; track 3, MDV DNA.

4. *DNA-DNA reassociation kinetics*

The number of virus genomes per cell in

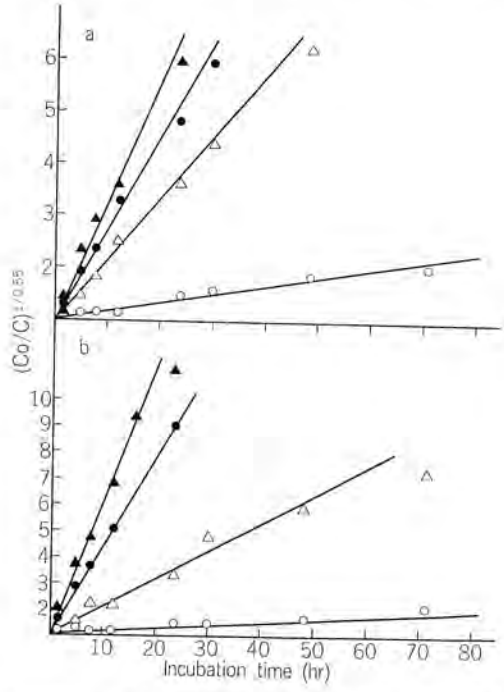

FIGURE 6. DNA-DNA reassociation kinetics of MDCC-BOl (T) cell DNA and ^{3}H-HVT DNA or ^{3}H-MDV DNA.

Symbols: (a), ○, normal chicken blood (CB) cell DNA and ^{3}H-HVT DNA; △, cold HVT DNA (1 genome/cell, 0.142 μg), CB cell DNA and ^{3}H-HVT DNA; ▲, cold HVT DNA (2 genomes/cell, 0.282 μg), CB cell DNA and ^{3}H-HVT DNA; ●, MDCC-BOl (T) cell DNA and ^{3}H-HVT DNA. (b), ○, CB cell DNA and ^{3}H-MDV DNA; △, cold MDV DNA (1 genome/cell, 0.157 μg), CB cell DNA and ^{3}H-MDV DNA; ▲, cold MDV DNA (5 genomes/cell, 0.785 μg), CB cell DNA and ^{3}H-MDV DNA; ●, MDCC-BOl (T) cell DNA and ^{3}H-MDV DNA.

MDCC-BOl(T) cells was examined by DNA-DNA reassociation kinetics. It has been shown by DNA-DNA reassociation kinetics that MDV does not have any detectable homology with HVT (Lee et al., 1979; Hirai et al., 1979). Figures 6a and b show that MDCC-BOl(T) cells at passage 45 contained 1.6 HVT genome equivalents and 3.5 MDV genome equivalents per cell. Both HVT and MDV genomes were found in the cells even at passage 60, more than 200 days after the line was established.

DISCUSSION

As far as we know, this is the first time that the HVT genome has been found persistently in a lymphoblastoid cell line derived from the apparently normal spleen of an apparently healthy chicken vaccinated with HVT.

The presence of MDV genomes in MDCC-BOl(T) cells raises the questions of whether HVT plays any part in establishment of the cell line and expression of MATSA. The origin of MDV genomes in this cell line is not clear. Non-SPF chickens happened to be kept with these experimental chickens for about 20 days before the second HVT-vaccination, and the MDV genome detected in BOl(T) cells might thus have been derived from these non-SPF chickens.

Since MDCC-BOl(T) cells have no detectable HVT ICA, it appears that this cell line is a nonproducer of HVT and contains latent HVT genomes. The latent state of DNA of oncogenic herpesviruses such as Epstein-Barr virus (Nonoyama and Pagano, 1972; Adams et al., 1973), MDV (Tanaka et al., 1978), and herpesvirus saimiri (Werner et al., 1977), has been well documented. We are now examining the state of HVT genomes in MDCC-BOl(T) cells. The characters of the MDCC-BOl(T) cell line suggest that neoplastic transformation of lymphocytes by either MDV or HVT may occur even in HVT-vaccinated chickens.

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