BIKEN JOURNAL Vol. 17, 105-116, 1974

TWO CELL LINES FROM LYMPHOMAS OF MAREK'S DISEASE[1]

YOKO AKIYAMA and SHIRO KATO

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

(Received April 9, 1974)

SUMMARY Two cell lines were obtained from an ovarian lymphoma and a splenic lymphoma, respectively of chicks with Marek's disease (MD). They were designated as line MOB-1 and MSB-1, respectively. They were grown in nutrient mixture RPMI 1640 supplemented with 10 to 20% fetal calf serum at 41 C in a humidified atmosphere of 5% CO_2 in air. Both lines consisted of lymphoblastoid cells. The cells gave a negative peroxidase reaction and, did not adhere to the surface of the culture vessel. The MOB-1 line degenerated after 320 days, while the MSB-1 line has so far grown well for over 240 days. Recultivation of cells of the MOB-1 line from an early culture which had been kept in a deep freeze, is under way. Immunofluorescence tests were performed on these lines to detect viral antigen (VA) and cell surface antigens (CSA) of MDV. Cultures consistently contained some VA positive cells but very few CSA positive cells. Electronmicroscopic examination showed that some cells contained several particles regarded as nucleocapsids and empty capsids of herpes virus in their nuclei, but no mature viral particles were observed in any cells. No cell-free virus could be isolated from either culture fluids or disrupted cells of these lines, but MDV could be reisolated after cocultivation with susceptible chick cells. A chromosomal aberration was observed in MSB-1 line cells. C type particles were not observed in any cells. Both lines gave negative reactions in the COFAL test and RIF tests for avian leucosis virus (ALV). The gs antigen of ALV was present in MOB-1 line cells, but not in MSB-1 line cells. Ten cloned sublines derived from the MSB-1 line also contained some VA positive cells. This is the first time that MDV associated lymphoma cell lines have been obtained and they should be useful in comparative research on herpes associated lymphocytic cell lines.

INTRODUCTION

Marek's disease virus (MDV) has many advantages over other oncogenic herpesviruses for experimental purposes, since much information is available on its pathology and viro-

1 Parts of this work were presented at the 5th International Symposium on Comparative Leukemia Research in Nagoya, September, 1973, at the 32nd Annual Meeting of the Japanese Cancer Association in Tokyo, October, 1973 and at the 21st Annual Meeting of the Society of Japanese Virologists in Tokyo, November, 1973.

logy, on the prevention of the disease and on determinants of the incidence of the disease of MDV. However one of the most serious disadvantages of using MDV is that no cell line derived from an MD lymphoma is available (Klein, 1972). Akiyama et al. (1973) succeeded in obtaining long term culture cell lines, named MOB, derived from an ovarian lymphoma of an MD chick, as stated in a preliminary report. This paper describes 2 cell lines, MOB-1 and MSB-1, derived from MD lymphomas and the characteristics of these lines.

MATERIALS AND METHODS

1. *Culture of cell lines*

1) Medium

Nutrient mixture RPMI 1640 medium supplemented with 10 to 20% fetal calf serum, and 100 units of penicillin and 100 μg of streptomycin per ml was used through out (RPMI growth medium).

2) Initiation of culture

Cultivation of cells from 22 MD lymphomas obtained from 20 chicks was attempted, as shown in Table 1. The 14 lymphomas in Group A were obtained by inoculating one day old chicks with blood from chickens infected with the MDV BC-1 strain, kindly supplied by Dr. A. Okaniwa (Tanabe Pharmaceutical Co., Ltd.). The 6 lymphomas in Group B were obtained by inoculating one day old chicks with 2×10^7 cells of the MOB-1 line. Two of these lymphomas were splenic tumors, and the rest were ovarian tumors. These tumors were minced into very small fragments with surgical knives. The minced materials were gently pipetted in Hanks' balanced salt solution and then passed through 6 layers of gauze and centrifuged at 1,000 rpm for 5 min. The packed cells were resuspended in RPMI growth medium at a final count of 5×10^6 cells/ml and 15 ml of the cell suspension were seeded into a Falcon Petri dish ($100\times$ 20 mm).

3) Maintenance of cultures

Cultures were maintained at 41 C in a humidified atmosphere of 5% CO_2 in air. Floating cells were removed with the medium and 15 or 30 ml of fresh medium were added. Then the suspended cells were dispersed in 2 or 3 new dishes. Subcultivation was carried out every 2 or 3 days.

2. *Immunofluorescence tests for MDV antigen*

Direct immunofluorescence tests to detect viral antigen (VA) and cell surface antigens (CSA) of MDV were performed by the methods of Naito et al. (1969) and Ishikawa et al. (1972) respectively.

3. *Cloning in soft agar*

RPMI growth medium was used for the cloning, with addition of 0.48% and 0.3% acetone washed Bacto-agar for the base and seed layers, respectively. Conditioned media were collected from cultures of the MOB-1 and MSB-1 lines in the logarithmic phase of growth, passed through a membrane filter of 450 mμ pore size and stored at 4 C, and were added at concentrations of 5 to 10% to both the base and seed layers. Three ml of seed agar, containing MSB-1 line cells, were layered onto 3 ml of the base agar in a Falcon Petri dish (60×15 mm). Then dishes were incubated in a CO_2 incubator at 41 C. Within 7 to 10 days after plating, well isolated colonies were picked up with a Pasteur pipette, transferred to RPMI growth medium containing 5 to 10% conditioned medium and incubated in the CO_2 incubator at 41 C.

4. *Resistance inducing factor (RIF) test*

The RIF test was made on the MOB-1 and MSB-1 line cells. The medium was removed from the cells and used to measure RIF activity, by the method of Rubin (1960). Chick embryo fibroblast cultures were made from 9-day embryonated eggs of RIF-free breeder chickens of the C/E type and used for detection of subgroups A, C and D. Fibroblast cultures made from 10-day embryonated eggs of Japanese quails were used for detection of subgroup E. The Rous sarcoma viruses used were gifts from Prof. Toyoshima in this Institute. RIF tests were performed in inoculated and control cultures at the third cell transfer level to determine resistance to superinfection with Rous sarcoma virus. The Eagle growth medium used consisted of 90 parts of Eagle's medium, 5 parts of inactivated calf serum, and 5 parts of Difco tryptose phosphate broth, with 100 units of penicillin, 100 μg of streptomycin and 1 μg of Fungizone per ml. The viruses used were the Schmidt-Ruppin strain of Rous sarcoma virus of subgroup A and subgroup D, avian sarcoma virus B77 of subgroup C and pseudotype Bryan high titer strain of Rous sarcoma virus of subgroup E.

5. *Agglutination assay with concanavalin A*

The method of Inbar and Sachs (1969) was used. Cells of the MOB-1 and MSB-1 lines and normal spleen lymphocytes were washed one or three times with Ca- and Mg-free phosphate buffered saline (PBS), pH 7.2 and then diluted to a concentration of $1-3\times10^6$ cells per ml with PBS. To test for agglutination, 0.5 ml volumes of different concentrations of concanavalin A in distilled water were mixed with 0.5 ml of the cell suspension in a 35-mm dish, and the time required for agglutination was observed with a binocular microscope. Agglutination was considered to be positive when over 75% of the cells agglutinated and one aggregate contained at least 5 cells.

6. *Cultures of chick kidney cells (CK), chick embryo fibroblasts (CE) and duck embryo fibroblasts (DE)*

Kidneys from 1- to 10-day old RIF free chicks were washed in PBS (pH 7.2), to removed blood, minced with surgical knives, and rewahed; the cells were collected after treating the mince with 0.25% trypsin at 37 C at 20-minute intervals. The trypsinized cells were passed through 6 layers of gauze and centrifuged at 1,000 rpm for 5 minutes. The packed cells were resuspended in growth medium, consisting of medium 199 containing 10% calf serum and 100 units of penicillin and 100 μg streptomycin per ml and adjusted to a final count of 1.5 to 2.0×10^6 cells per ml. 100-mm dishes were each seeded with 10 ml of the cell suspension and incubated at 38.5 C in a humidified atmosphere in 5% CO_2 in air. Confluent monolayers developed in 3-4 days. CE and DE cultures were prepared from decapitated 9- to 10-day RIF embryos and decapitated 10-day duck embryos. Procedures for culture were as for CK cultures, except that trypsinization was done at 5-minute intervals. The packed cells were resuspended in the same growth medium and adjusted to a final count of 1×10^6 cells per ml. Volumes of 4 and 10 ml of cell suspension were distributed in 60-mm and 100-mm dishes, respectively and coverslips (11×40 mm) were placed in some dishes to obtain cells for histological observations and for immunofluorescence tests.

7. *Virus isolation from the lines*

The growth medium of monolayers of CK cultures or CE cultures in 100-mm dishes was replaced by maintenance medium. Then 1×10^6 to 3×10^6 cells of the MOB-1 or MSB-1 line were inoculated and the dishes were kept at 37 C. Two days after inoculation the liquid medium was replaced by maintenance medium. Cells were observed every day. When CPE was detected, coverslip cultures were fixed in Bouin's solution and stained with hematoxylin and eosin. For detection of MD VA, coverslip cultures were fixed in cold acetone and stained with FITC conjugated MD chicken γ-globulin. The maintenance medium consisted of medium 199 containing 2% calf serum and 100 units of penicillin and 100 μg streptomycin per ml.

To isolate cell-free virus, 5×10^7 MOB-1 cells or MSB-1 cells were suspended in one ml of culture medium and disrupted by sonic vibration for 5 min at 9kHz (Insonator model 200 M, Kubota Instruments Incorp., Tokyo). Then the preparation was centrifuged at 5,000 rpm for 15 min and 0.2 ml of the supernatant was inoculated onto monolayers of CE cells in dishes (60×15 mm). After adsorption for two hr, 5 ml of maintenance medium were added and cultures were observed every day for 7 to 10 days.

8. *Complement-fixation tests*

The complement-fixation (CF) test for detection of avian leukosis group-specific antigen (gs antigen) was performed with the microtiter technique, overnight fixation at 4 C and 2 units of complement (Sarma et al., 1964). Four units of antibody were used for all antigen detection tests. Samples of 5×10^7 cells of the MOB-1 or MSB-1 line were suspended in 0.5 ml of PBS, homogenized gently with a teflon homogenizer, subjected to one cycle of freezing and thawing and centrifuged at 2,000 rpm for 5 min. The supernatant was stored at -70 C until use. Control gs antigen and anti-gs pigeon sera were gifts from Prof. K. Toyoshima in this Institute. Reactions of 3+ to 4+ in the absence of positive reactions in normal control cultures and in the absence of anti-complementary reactions were considered evidence of gs antigen.

RESULTS

1. *General progress of the cell lines*

After cultivation for one day live cells decreased rapidly in number. Some large lymphoid cells survived and floating single cells

began to grow actively after 4 to 5 days' cultivation. Since fibroblastic cells also grew on the bottom of the culture plates, only culture fluids containing floating cells were transferred to new dishes. In only two of 22 trials was continuous cultivation of cells successful (Table 1). When cultures were kept at 37 C instead of 41 C, cells rapidly degenerated. Floating cells survived longer at 38.5 C. However, trials at this temperature were not successful. The two cell lines obtained from the ovarian tumor of chicken No. 4 and the splenic tumor of chicken No. 15 were designated as the MOB-1 line and the MSB-1 line, respectively.

TABLE 1. *Trials to cultivate cells obtained from lymphomas of MD chicks*

Group[a]	Chicken No.	Days after inoculation	Lesions[b]	Cultured tumor[c]	Cell line
A	1	40	V, N	Ovarian t.	MOB-1
	2	42	V, N	Ovarian t.	
	3	44	V, N	Ovarian t.	
	4	44	V, N	Ovarian t.	
	5	45	V, N	Ovarian t.	
	6	45	V, N	Ovarian t.	
	7	48	V, N	Ovarian, splenic t.	
	8	48	V, N	Ovarian t.	
	9	48	V, N	Ovarian t.	
	10	50	V, N	Ovarian t.	
	11	51	V, N	Ovarian, splenic t.	
	12	52	V, N	Ovarian t.	
	13	56	V, N	Ovarian t.	
	14	60	V, N	Ovarian t.	
B	15	56	V, N	Splenic t.	MSB-1
	16	42	V, N	Ovarian t.	
	17	46	V, N	Ovarian t.	
	18	48	V, N	Ovarian t.	
	19	48	V, N	Ovarian t.	
	20	56	V, N	Ovarian t.	

[a] A, each chick inoculated intramuscularly with 0.2 ml of blood of chickens infected with BC-1 strain of Marek's disease. B, each chick inoculated intramuscularly with 2×10^7 cells of MOB-1.

[b] V, visceral lesion: The gonads and spleen were most consistently involved, although, in many cases, almost all visceral organs were involved. N, neural lesion: Sciatic nerves were examined.

[c] All cultures were kept at 41 C.

2. *Mode of cell growth*

The MOB-1 and MSB-1 line cells did not become attached to the glass but floated free in the medium, as single cells or sometimes as transient clumps of about 10 to 20 cells. Large clumps were frequent in cultures of the MSB-1 line. The rate of growth of MOB-1 cells was determined in three dishes (100×20 mm) containing 15 ml of the same cell suspension after 10 and 100 days of culture. The results in Fig. 1 show that the doubling time of cells was about 24 hr and that cells grew actively at 41 C but poorly at 37 C. The cells were usually maintained at the concentration of about 8×10^5 to 3×10^6 cells/ml by addition of fresh

medium, and the growth rate at cell concentrations below 3×10^5 cells/ml was not good. But growth of cells at an initiation concentration of 1×10^5 cells/ml was markedly increased by addition of 10 to 20% conditioned medium to the fresh medium (Fig. 2). Addition of over 40% conditioned medium did not increase growth. Unfortunately the MOB-1 line cells became contaminated with fungi at the third crisis after cultivation for 320 days and died (Fig. 8). Recultivation of the line with cells from an early stage of culture is under way. During these crises small, irregular-shaped cells became predominant, and the division time of cells was prolonged, even though the medium was changed. The MSB-1 line grew more actively than the MOB-1 line. The results in Fig. 3 show that the doubling time was only 10 hr at 41 C and cells could even grow at 37 C with a doubling time of 28 hr. After 9 weeks of culture, the MSB-1 line cells were cultivated with 10% calf serum in place

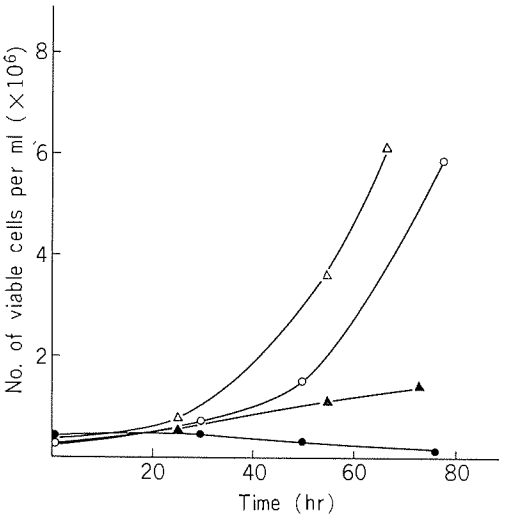

FIGURE 1. *Effect of temperature on the growth rate of MOB-1 line cells.* ○, *41 C, after 10 days cultivation;* ●, *37 C, after 10 days cultivation;* △, *41 C, after 100 days cultivation;* ▲, *37 C, after 100 days cultivation.*

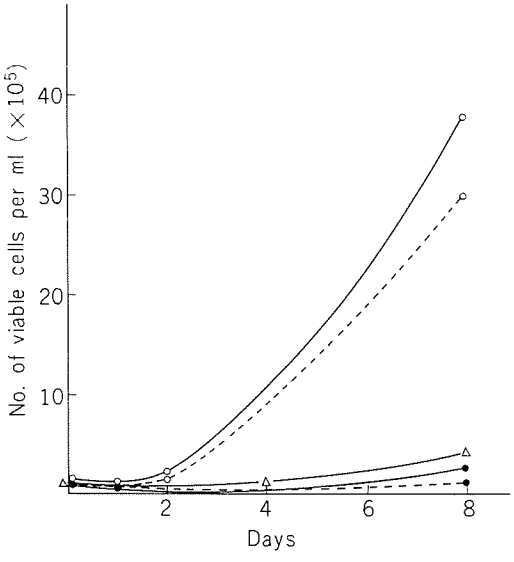

FIGURE 2. *Effect of conditioned medium on the growth rate of MOB-1 line cells.*
△——△, *0%;* ○——○, *10%;* ○·····○, *20%;* ●——●, *40%;* ●·····●, *60%.*

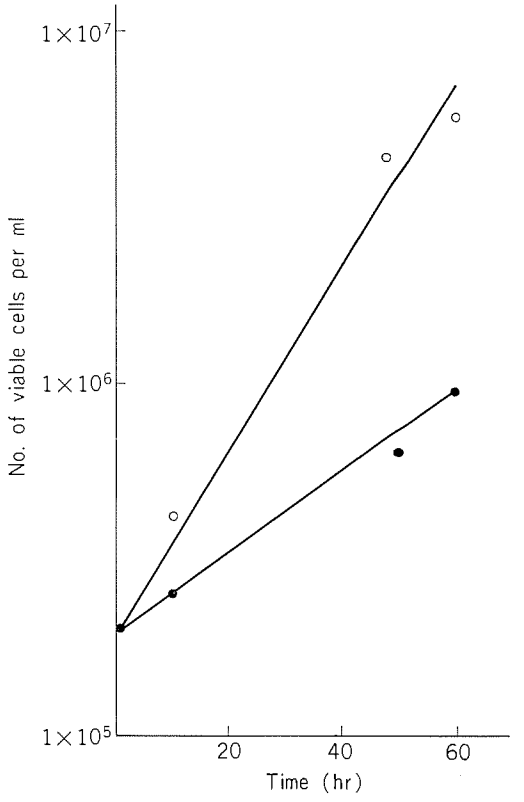

FIGURE 3. *Effect of temperature on the growth rate of MSB-1 line cells.* ○, *41 C.;* ●, *37 C.*

of fetal calf serum. After poor growth for the first 2 months, cells began to grow actively, with a division time of 15 hr. The growth rate of MSB-1 line cells was prolonged at a cell concentration of over 2×10^6 cells/ml. The optimum cell concentration for MSB-1 cells for growth ranged from 2×10^5 to 8×10^5 cells/ml. The MSB-1 line cells has had no crisis, although it has been cultured for 240 days

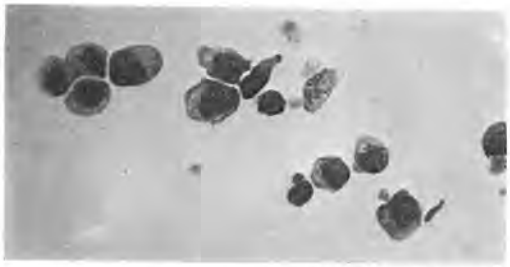

FIGURE 4. *Smear preparation of MOB-1 line cells after 30 days' cultivation. Fixed with methanol, stained with Giemsa solution. The line consists of lymphoblastoid cells.*

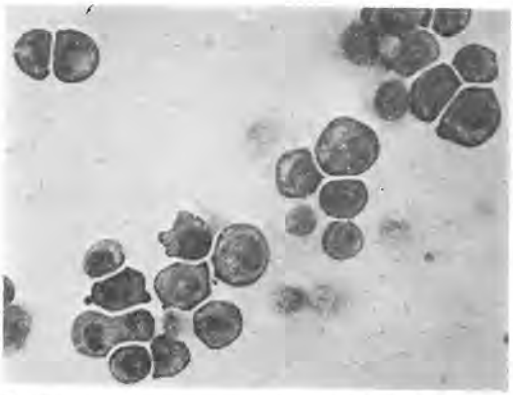

FIGURE 5. *Smear preparation of MSB-1 line cells after 240 days' cultivation. Giemsa stain.*

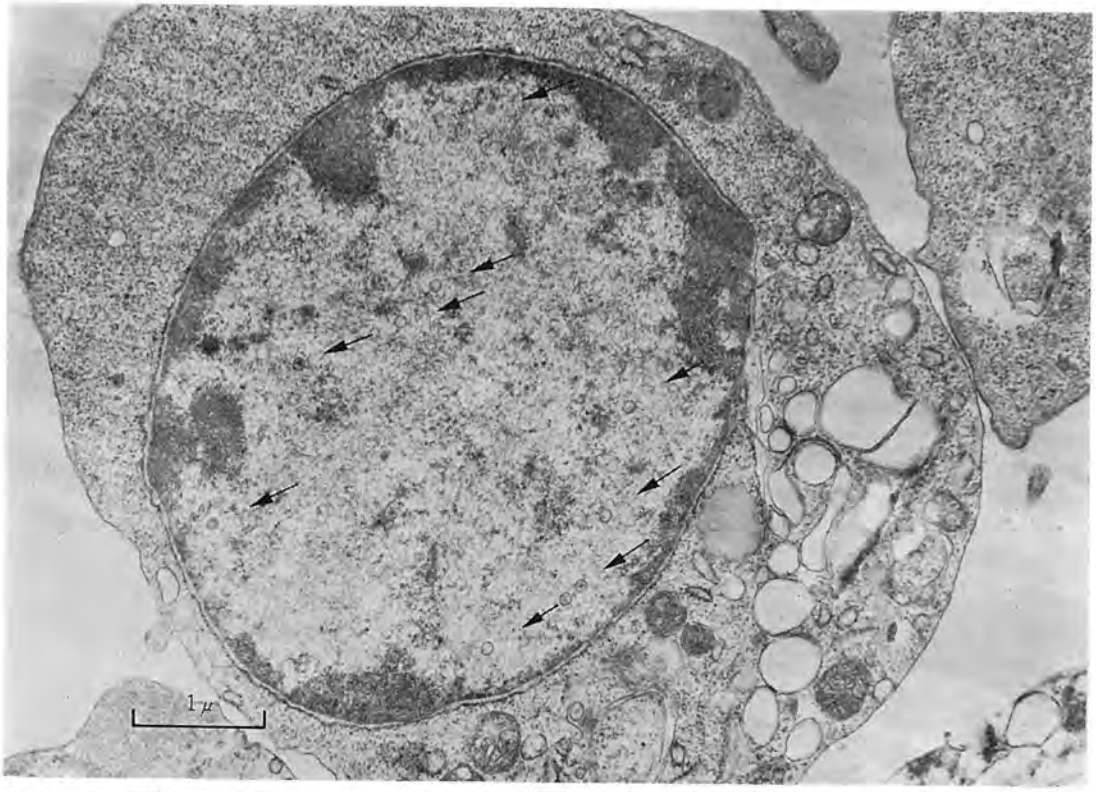

FIGURE 6. *Thin section electron micrograph of a lymphoblastoid cell of the MOB-1 line. Several intranuclear herpes-type capsid structures are observed (arrows).*

(Fig. 9).

3. *Cell morphology*

1) Light microscopy

Cultures of both lines always consisted entirely of free-floating cells. Most of the MSB-1 line cells were round and very few cells were irregular-shaped, but the latter were fairly frequent in cultures of the MOB-1 line. Cells of both lines were about 8 to 15 μ in diameter in the floating state. MSB-1 line cells were somewhat larger than MOB-1 line cells. No difference could be detected between the free single cells and cells in clumps. In preparations of the MOB-1 line cells stained with Giemsa about half the cells were round and half irregular-shaped. Nucleoli of cells in advanced passages were not distinct. As shown in Figs. 4 and 5, the cells were usually smaller than MSB-1 line cells. Fig. 4 represent MOB-1 line cells cultured for 30 days. In preparations of MSB-1 line cells stained with Giemsa most cells were round, and frequently contained one or two nucleoli. Fig. 5 represents MSB-1 line cells cultured for 240 days. Both MOB-1 and MSB-1 line cells gave a negative peroxidase reaction

2) Electronmicroscopy

Electron microscopy showed that cells of the MOB-1 and MSB-1 lines were 8 to 15 μ in diameter and had a fine structure typical of undifferentiated lymphocytic cells. Characteristic lymphoblasts were seen with a relatively extensive cytoplasm packed with ribosomes arranged in clusters, with round or kidney-shaped or irregular nuclei, and with a smooth cell surface (Fig. 6, 7). The MSB-1 line cells had more abundant mitochondria than MOB-1

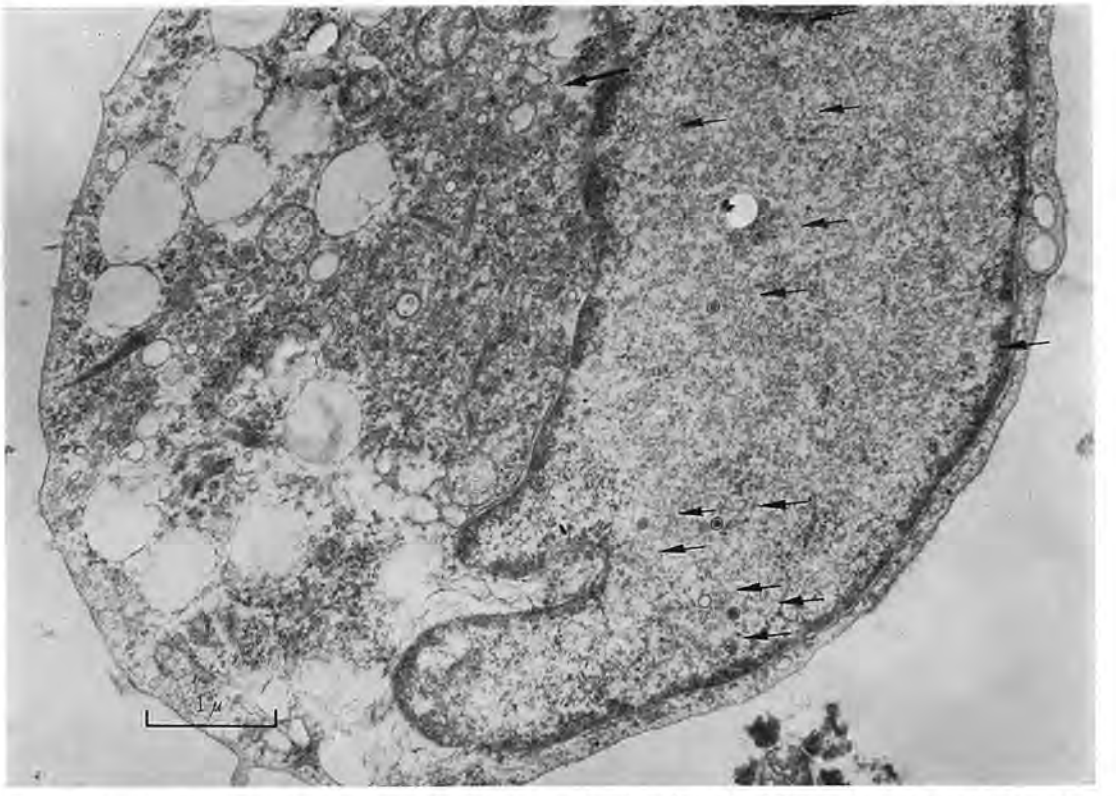

FIGURE 7. *Thin section electron micrograph of a lymphoblastoid cell of the MSB-1 line. Several herpes-type capsid structures are observed in both the nucleus and cytoplasm (arrows).*

line cells. The nuclei of some cells of both lines contained several particles which seemed to be nucleocapsids and empty capsids of herpes type virus, but no enveloped particles have yet been observed in any cells. Some cells with herpes capsids showed degenerative changes. Generally the nuclei of MSB-1 line cells contained more capsids than those of MOB-1 line cells. Some cells of the MSB-1 line contained many capsids even in the cytoplasm (Fig. 7). The percentages of cells containing capsids corresponded well to the percentages of cells having MDV antigen, demonstrated in immunofluorescence tests. No C-type particles were observed in any cells.

4. *Direct immunofluorescence tests*

Smears of MOB-1 and MSB-1 line cells on coverslips were dried and fixed with cold acetone, and stained with fluorescein isothiocya nate-conjugated γ-globulin obtained from MD chickens for detection of MD VA.

A small proportion of the MOB-1 and MSB-1 line cells were consistently VA positive, while the percentage of VA positive cells varied (0.1 to 10%) from passage to passage and from clone to clone derived from the MSB-1 line (Fig. 8, 9). Both lines also consistently contained a small proportion of cells with MDV-induced CSA. However, the percentage of

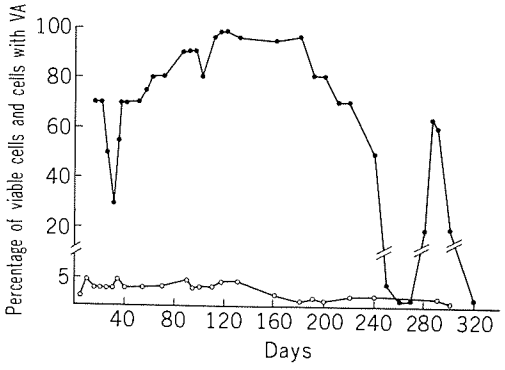

FIGURE 8. *Percentages of viable cells (●——●) and cells with VA (○——○) in cultures of MOB-1 line cells during cultivation for 320 days.*

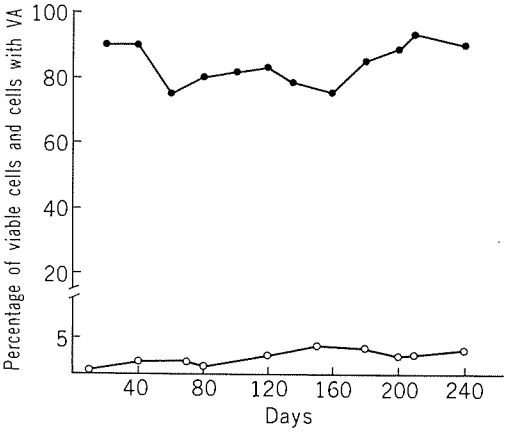

FIGURE 9. *Percentages of viable cells (●——●) and cells with VA (○——○) in cultures of MSB-1 line cells during cultivation for 240 days.*

TABLE 2. *Percentages of VA and CSA positive cells in clones derived from MSB-1 line cells*

Clone	Colony efficiency (%)	VA (%)			CSA (%)		
Uncloned	57	1.8	1.3	0.3	0.4	0.1	0.1
C 2	20	1.2	1.5	1.0	0.6	0.3	0.1
C 4	13	10.4	6.9	7.5	1.5	1.6	3.0
C 7 C	55	2.5	1.8	0.7	1.0	0.7	0.3
C 17	54	2.0	1.0	1.5	1.0	0.6	0.1
C 31	50	5.0	8.3	1.3	1.2	2.3	0.8
C 73	100	1.8	1.3	0.6	1.2	0.8	0.05
C 81	52	2.8	2.0	2.0	1.0	0.8	0.5
C 82	11	1.0	1.9	2.0	0.5	0.2	0.2
C 83	36	3.0	3.5	2.0	1.1	0.6	0.3
C 91	50	12.0	7.5	2.2	1.0	1.1	0.9

CSA positive cells was always smaller than that of VA positive cells. Some cloned lines derived from the MSB-1 line had high percentages of CSA positive cells (Table 2).

5. *Attempts to isolate virus*

CE cultures were inoculated with cell free samples prepared as described in the MATERIALS AND METHODS. However, no CPE or VA positive cells were observed during an observation period of 10 days. CK cultures and CE cultures were inoculated with either MOB-1 line cells or MSB-1 line cells. Typical foci of MDV were observed 4 to 6 days after inoculation. Cells constituting the foci showed typical intranuclear inclusion formation of the herpes type. The number of foci was very few and did not correspond to the number of cells inoculated into the dishes. In direct immunofluorescence tests, these foci showed specific fluorescence of MD VA. When virus was inoculated onto DE cultures, typical virulent type foci appeared, as mentioned previously (Kato et al., 1970; Onoda et al., 1971). That is, small foci consisting of a few round cells were observed and on staining with hematoxylin-eosin after fixation with Bouin scattered cells with intranuclear inclusions were seen and small syncytia with a few nuclei were observed 5 days after inoculation (Fig. 10).

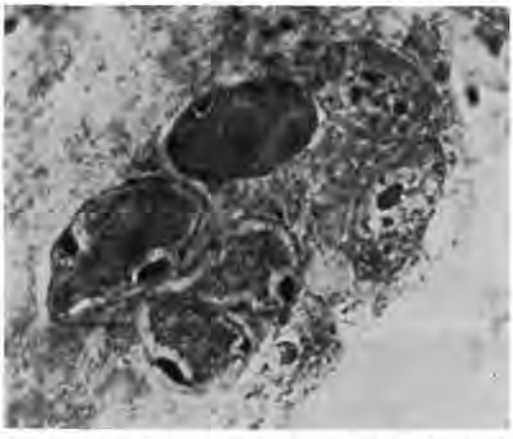

FIGURE 10. *Formation of intranuclear inclusions in DE culture 5 days after inoculation of MD virus isolated from the MSB-1 line.*

6. *Cloning in soft agar*

All efforts to obtain clones from individual MOB-1 line cells failed. However, individual cells of the MSB-1 line grew into colonies seven to ten days after plating. These colonies were approximately 0.5 to 3 mm in diameter (Fig. 11). As shown in Table 2, the cloning efficiency of the cells ranged from 10 to 100%. The cloning efficiency decreased with a high initial cell density of over 500 cells per dish because of decrease in the medium. The optimal initial cell density was found to range from 10 to 100 cells per dish. As shown in Table 2, all clones which had been cloned three times from MSB-1 line cells, contained about 1 to 10% VA positive cells and about 0.1 to 2% CSA positive cells.

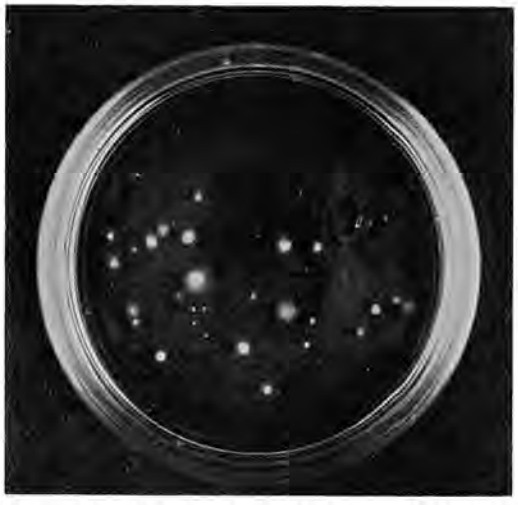

FIGURE 11. *Colonies of MSB-1 line cells 10 days after plating.*

7. *Agglutination by concanavalin A*

The MOB-1 line and MSB-1 line cells were both agglutinated by the carbohydrate-binding protein, concanavalin A (Con A). The times required for positive agglutination with aggregates of at least 5 to 10 cells, at different concentrations of Con A, are shown in Fig. 12.

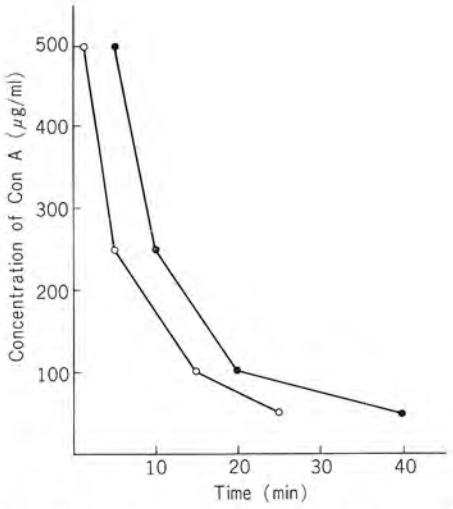

FIGURE 12. *Time required for agglutination by Con A of MSB-1 line cells (○——○) and MOB-1 line cells (●——●). Agglutination was considered to be positive when over 75% of the cells were agglutinated and one aggrrgate contained at least 5 cells. No agglutination of normal chicken lymphocytes from spleen was observed even after 40 min with 500 μg Con A/ml.*

The degree of agglutinability of MSB-1 line cells was more than that of MOB-1 line cells. Aggregates of MSB-1 line cells contained about 10 to 50 cells after treatment with 500 μg Con A/ml for 20 min, whereas under the same conditions aggregates of MOB-1 line cells contained fewer cells. These cells did not agglutinate on incubation for 40 min with the same concentration of NaCl without Con A, and no agglutination of normal chicken lymphocytes from the spleen was observed on treatment with 500 μg Con A/ml for 40 min.

8. *Karyotype*

Chromosome analysis was kindly performed by Dr. N. Takagi, Chromosome Research Unit, Hokkaido University, Sapporo. Chromosome preparations were made according to a routine air-drying technique after arrest of mitosis by treatment with colcemid (0.1 μg/ml) for 2 hr. The modal chromosome number of the MOB-1 line cells was about 78, the normal diploid number of the chicken (Owen, 1965).

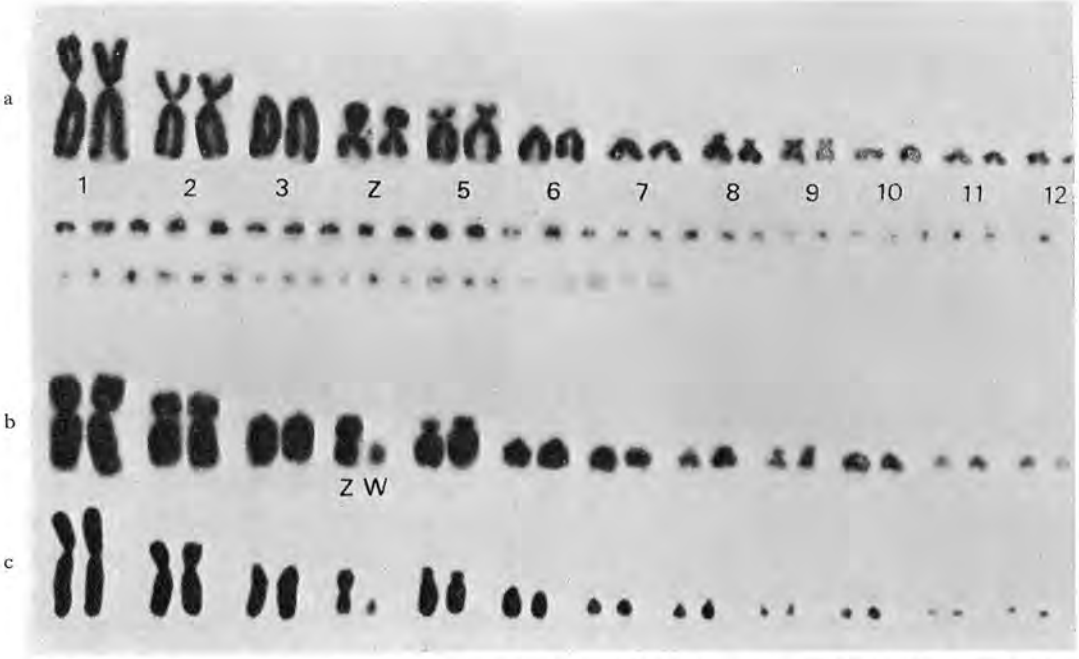

FIGURE 13. *Chromosomes of a normal male chick cell (a), a MOB-1 line cell (b) and a MSB-1 line cell (c).*

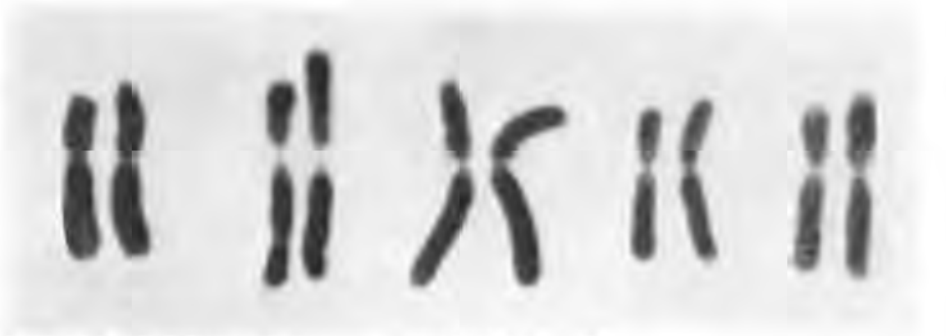

FIGURE 14. *No. 1 pairs of chromosomes of MSB-1 line cells.*

Karyotype analysis confirmed the female origin of the cell line, and revealed no apparent structural change so far as the 12 largest pairs were concerned (Fig. 13b). The karyotype of the MSB-1 line was similar to that of the MOB-1 line, except that No. 1 pair was heteromorphic (Fig. 13c, 14). The short arm was much longer in one of the homologs than in the other. Translocation rather than pericentric inversion appeared responsible for the abnormality, since the lengths of the long arms were comparable in both normal and abnormal homologs. However, the origin of the extra segment could not be determined. It remains unknown whether MDV infection caused this aberration.

9. *Tests for avian leukosis virus (ALV)*

The involvement of these line cells with ALV was examined as follows. No C-type particles were observed in any line cells by electron microscopy as described above. RIF tests on both MOB-1 and MSB-1 line cells for subgroups A, C, D and E of ALV and COFAL tests were consistently negative. Complement-fixation tests for gs antigen of ALV were negative in the MSB-1 line cells, while MOB-1 line cells only gave a reaction of 3+ at a dilution of 1:1. The extent of this reaction is indicated as ±. These results are summarized in Table 3.

TABLE 3. *Tests for avian leukosis virus*

Test	Line	
	MOB-1	MSB-1
C particles	−	−
COFAL	−	NT[a]
RIF	−	−
gs antigen	±	−

[a] Not tested.

DISCUSSION

The present paper reports the first successful long term cultivations of cells derived from Marek's lymphomas. One of the reasons for successful cultivation seems to be the temperature of the cultivation, since the optimal temperature for growth of both lines is 41 C. However, other unknown factors seem important, since the rate of success (2 out of 22 trials) was rather low. The MOB-1 line cells grew for 320 days and then died. The last culture was unfortunately contaminated with fungi, so we could not determine whether about 320 days is the life span of the line or not. Recultivation of cells from an early culture which had been kept in a deep freeze, is under way.

The MSB-1 line cells were derived from an MD splenic lymphoma of a chick which had been inoculated with MOB-1 line cells. Although chicks inoculated with MOB-1 line cells develop lesions, the cellular continuity of the line has not been determined yet. However, judging from their growth rate, lack of gs anti-

gen, cloning efficiency and chromosomal aberration, MSB-1 cells were probably not derived from MOB-1 cells, but from splenic lymphoma cells by transformation with virulent viruses released from the inoculated MOB-1 cells. Studies on the oncogenicity and characteristics of the virus isolated from these two line cells are also in progress.

These 2 cell lines are closely associated with MDV, as demonstrated by the immunofluorescence technique and electronmicroscopy. All ten cloned cells derived from MSB-1 line cells also contained some MDV antigen positive cells. It is unlikely that cell free viruses were transmitted to cloned cells, because cell free viruses were rarely obtained from the line. It seems likely that MSB-1 line cells are intrinsically involved with the MDV genome. Application of the complement-fixing immunofluorescence technique for detection of a nuclear antigen, analogous to the nuclear antigen EBV (Reedman and Klein, 1973) and MDV nucleic acid hybridization, such as that carried out by Nazerian et al. (1973) could clarify this point.

ACKNOWLEDGEMENTS

Chromosome analysis was kindly performed by Dr. N. Takagi, Chromosome Research Unit, Hokkaido University, Sapporo. We thank Prof. K. Toyoshima of this Institute for advice on the RIF test. Electronmicrography was kindly carried out by Mr. T. Naito, Osaka Medical College and the COFAL test by Mr. O. Tanishita, Kanonji Institute, The Research Foundation for Microbial Diseases of Osaka University, Kanonji, Kagawa. This work was supported in part by a Scientific Research Grant from the Ministry of Education.

REFERENCES

Akiyama, Y., N. Iwa, and S. Kato. 1973. Continuous cell culture from lymphoma of Marek's disease. Biken J. 16: 177–179.

Inbar, M., and L. Sachs. 1969. Interaction of the carbohydrate-binding protein concanavalin A with normal and transformed cells. Proc. Natl. Acad. Sci. U.S.A. 63: 1418–1425.

Ishikawa, T., M. Naito, S. Osafune, and S. Kato. 1972. Cell surface antigen on quail cells infected with herpesvirus of turkey or Marek's disease virus. Biken J. 15: 215–222.

Kato, S., K. Ono, M. Naito, T. Doi, N. Iwa, Y. Mori, and T, Onoda. 1970. Isolation of herpes type virus from chickens with Marek's disease using duck embryo fibroblast cultures. Biken J. 13: 193–203.

Klein, G. 1972. A summing up. Oncogenesis and herpesvirus. p. 501–515. I. A. R. C. Scientific Publication No. 2. International Agency for Research on Cancer, Lyon.

Naito, M., K. Ono, S. Tanabe, and S. Kato. 1969. Immunofluorescent studies using fractionated immunoglobulin of sera of fowls with Marek's disease. Biken J. 12: 257–261.

Nazerian, K., T. Lindahl, G. Klein, and L. F. Lee. 1973. Deoxyribonucleic acid of Marek's disease virus in virus-induced tumors. J. Gen. Virol. 12: 841–846.

Onoda, T., K. Koyama, T. Konobe, and K. Takaku. 1971. Studies on the characteristics of a herpes-type virus isolated from a chicken with Marek's disease. Biken J. 14: 167–176.

Owen, J. J. T. 1965. Karyotype studies on *Gallus domesticus*. Chromosoma 16: 601–608

Reedman, M., and G. Klein. 1973. Cellular localization of an Epstein-Barr virus (EBV)-associated complement-fixing antigen in producer and non-producer lymphoblastoid cell lines. Int. J. Cancer 11: 499–520.

Rubin, H. 1960. A virus in chick embryos which induces resistance in vitro to infection with Rous sarcoma virus. Proc. Natl. Acad. Sci. U.S.A. 46: 1105–1119.

Sarma, P. S., H. C. Turner, and R. J. Huebner. 1964. An avian leucosis group-specific complement fixation reaction. Application for the detection and assay of non-cytopathogenic leucosis viruses. Virology 23: 313–321.