In Vitro Cultivation of MN-Lymphosarcoma Cells

Shigeki MORI, Yoshio HARADA, and Ikuo YAMAOKA

The Institute for Constitutional and Diathetic Medicine, Yamagushi Medical School

SYNOPSIS

Fundamental conditions suitable for the cultivation of MN-lymphosarcoma cell were assayed and the results were presented as follow:

1) Static culture was a more suitable method than roller tube culture.

2) The culture in initial pH 7.6 was found to be optimal.

3) Continuous cell multiplication was obtained with the inoculum size of over 100,000 nuclei/ml/tube.

4) 0.4% Lactalbumin hydrolysate in the medium was found to be optimal.

5) The optimal concentration of yeast extract was determined as 0.08% in the medium.

6) Vigorous propagation of the cells was obtained with a medium containing 40% bovine serum.

7) The cell multiplication was not sustained in the medium in which dialysed bovine serum was substituted for nondialysed one.

8) The cell growth was accelerated markedly in the medium supplemented with dialysed bovine serum after the addition of hexestrol, a synthetic estrogen, to it.

INTRODUCTION

MN-Lymphosarcoma was found in 1953 by Mori and his associates in NA_2 strain of female mouse injected with a synthetic estrogen.[21, 23, 25] Since Lacassagne reported his distinguished work concerning the correlation between estrogen and lymphoid sarcoma, references to this subject have appeared in many papers.[7, 19, 24]

By the prolonged subcutaneous administration of a solution of acrylonitrile derivatives in oil or as pellets into uniform strain NA_2 female mice for over 6 months, lymphatic leukemia and lymphosarcoma were induced in them. In addition, T-strain of the tumor was established in a mouse treated with subcutaneous injections of hexestrol for over 6 weeks. Fragments of such a tumor were inoculated into the same strain of female mice and their transplantation was effected by the intra-

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peritoneal, subcutaneous, and intravenous routes. In the first route, the ascites tumors were obtained in a pure culture from the peritoneal cavity 5～7 days after the inoculation and has undergone passage for more than 6 years.

When transplanted intraperitoneally with approximately 10 million tumor cells, the life span of inoculated mouse was usually within 2～3 weeks. In the T-strain of the tumor employed in this experiment, metastases of the cells to lymph nodes, and swelling of the liver and spleen were prominent at autopsy.

Since the establishment of MN-lymphosarcoma *in vivo*, many investigations on the rôle of steroidal hormones in relation to cancer were conducted by Mori and his collaborators histologically, cytologically, and immunologically[10, 11, 17, 27, 29, 34, 35]

In order to obtain permanent cell strains of the tumor in tissue culture, fundamental conditions suitable for cultivation were examined in the primary culture. The conditions examined were culture method, pH of the medium, inoculum size, and concentration of lactalbumin hydrolysate, yeast extract, and bovine serum. Substitution of bovine serum with dialysed serum and the response of cell proliferation rate to estrogen were also assayed.

MATERIALS AND METHODS

Animals and Tumor Cell Uniform strain NA_2 female mice raised in our laboratory were used for the experiments. The T-strain of MN-lymphosarcoma cell converted to ascites type was obtained from the Department of Pathology in this medical school in 1960. Since then, the tumor has been maintained by weekly serial passage in NA_2 female mice and has been neither frozen nor cloned. During the routine passage, each mouse received an intraperitoneal inoculation of about 10 million tumor cells. Prior to the present experiments MN-lymphosarcoma was passaged for 6 months in our laboratory.

Characterization of tumor Cells *in vivo* Observations were made with a phase-contrast microscope and by routine Giemsa staining.

The tumor cell in an ascites was round and in it existed a large round nucleus with one or two round nucleoli. Many mitochondria were scattered around the nuclear membrane. The ground cytoplasm of the cell was relatively narrow and homogeneous, and took intensive basophilia. Golgi body was not observable. On supravital staining with Neutral Red, only one or two Neutral Red vacuoles were seen in the cytoplasm. Polynuclear cells were seldom observed in the ascites.

Culture Medium The medium used throughout the work reported here was made up of the following materials. Hanks balanced salt solution, containing lactalbumin hydrolysate (Enzymatic, Nutritional Biochemicals Corp., U.S.A.) in 0.5% and yeast extract (Difco) in 0.1%, was sterilized by filtration through Seitz filter and stored in a refrigerator at 4°. Bovine serum was removed from the cooled blood obtained

from a local abattior, inactivated by heating at 56° for 30 minutes, sterilized by Seitz filter, and was stored at −20°. At the time of experiment Hanks solution described above and bovine serum, used as the medium, were mixed in a ratio of 80 : 20.

Penicillin and dihydrostreptomycin were added to the medium in a final concentration of 50U/ml, and 50γ/ml, respectively. Initial pH of the medium was estimated with a pH-meter Model HM-5A, (Toa Dempa Kogyo Co.) and adjusted to 7.6 with *N* HCl or NaOH.

Culture Method In order to obtain an accurate proliferation rate, the simplified replicate tissue culture method[12] was employed. Short test tubes (15 × 110mm) were used as the culture vessel.

The medium was renewed at two-day interval by pipetting off the cell-free supernatant. Each experimental group consisted of 10 tubes and on the 2nd, 4th, and 7th day of cultivation, three of the tubes were taken out to count the nucleus number.

Ascites was removed aseptically by a glass capillary from the intraperitoneal cavity of mice inoculated 5～7 days previously and the number of tumor cells was counted with a hemocytometer. Usually two drops of this ascites were added to an Erlenmeyer flask containing 30 ml of culture medium and the mixture was pipetted throughly so as to obtain a homogeneous suspension. Following cell counting 2 ml of the suspension was poured into each short test tube.

Tubes were sealed with double rubber stoppers and incubated stationarily at an angle of 5° from the horizontal and the temperature of 37°.

Estimation of the multiplication rate was made by counting the number of cell nuclei in the culture tubes.

Enumeration of Cell Nuclei The cultured mixture was treated with the 0.1 *M* citric acid solution in distilled water, containing 0.02% of Crystal Violet and a few drops of formaldehyde solution. The number of cell nuclei was counted in a hemocytometer and the average of three tubes was recorded.

Dialysis of Bovine Serum Thirty ml of bovine serum was dialysed through a Cellophane membrane against distilled water (6,000 ml in total) at 4° for 48 hours. After dialysis, the fluid inside the Cellophane bag was centrifuged at 3,000 r.p.m. for 15 minutes and the precipitate was discarded. Nine volumes of the dialysed bovine serum was mixed with one volume of ten-fold concentrated Hanks balanced salt solution and the mixture was sterilized by passing through Seitz filter.

Hormone A synthetic estrogen, hexestrol (Teikoku Hormone Mfg. Co.), was dissolved in absolute ethanol for use in this experiment.

Results

1) Comparison between Roller Tube and Static Culture The two culture methods were compared to obtain the most suitable method in the following assays.

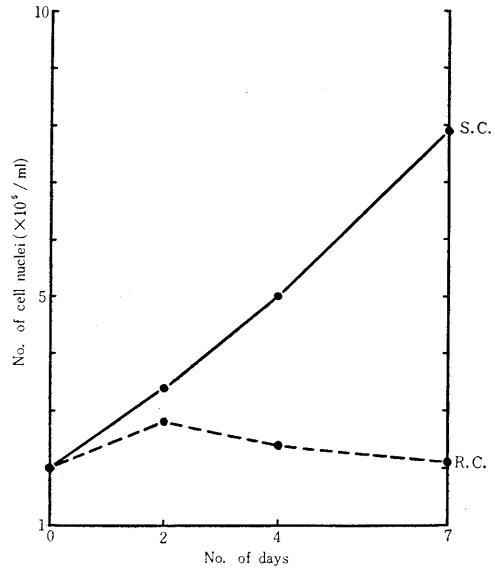

Fig. 1. Comparison of cell multiplication between roller tube culture (R. C) and static culture (S. C)

In both static and roller tube methods, short test tubes employed were placed at an angle of 5°. Tubes in roller tube method were rotated at 12 r.p.h. The medium consisted of 20% bovine serum and 80% Hanks solution containing 0.4% lactalbumin hydrolysate and 0.08% yeast extract. The number of cell nuclei in the inoculum was 200,000/ml. As illustrated in Fig. 1, the number of cell nuclei multiplied continuously during the week in static culture and contrarily decreased in the other method. MN-Lymphosarcoma cells *in vitro* scarecely formed cell aggregates and preserved their round shape, as was observed *in vivo*. A round nucleus was seen and it contained a few round nucleoli. There was no definite morphological difference between the cells *in vivo* and *in vitro*, as shown in Photos 1 and 2. The cells grow without adhering to glass surface and floating at the bottom during cultivation, which is similar to the behavior of Yoshida sarcoma cells.[14] From this result static culture method was employed routinely in the following experiments.

2) Effect of pH of the Medium Experiment was carried out to find the optimal pH of the culture medium among 6. 7, 7. 3, 7. 6, and 7. 9. The pH was adjusted with *N* HCl or *N* NaOH. The medium contained 20% bovine serum and 80% Hanks solution described above.

Until the 2nd day of cultivation, the number of cell nuclei increased in all of the four pH values. Cells in pH 7.6 showed continuous growth without any sign of degeneration until the end of the experiment and acquired the highest number of cells. In pH 7.9, the number of cultured cells showed neither increase nor decrease after two days of cultivation and cells in pH 6.7 and 7.3 decreased gradually in number. From this result, pH 7.6 was selected for the medium for the following experiments (Fig. 2).

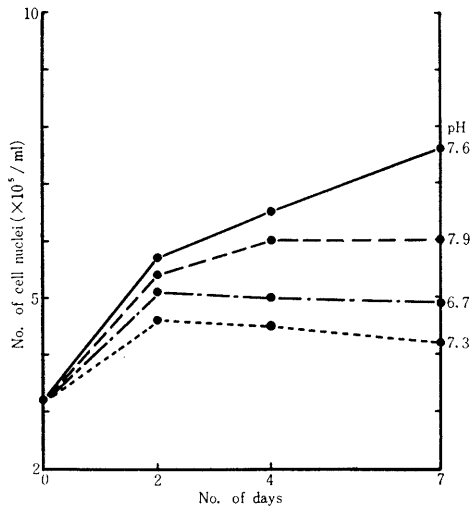

Fig. 2. Effect of pH of the culture medium on cell proliferation

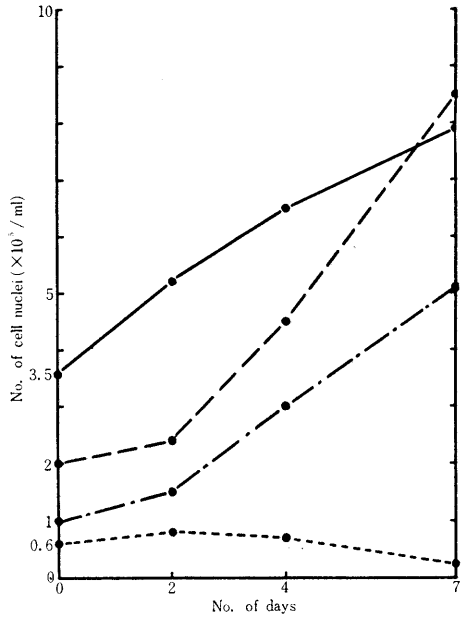

Fig. 3. Effect of the inoculum size

3) Effect of Inoculum Size The optimal inoculum size of cells to be added into culture tubes was examined in four levels of 350,000 2000,00, 100,000, and 60,000 cells/ml/tube. The medium consisted of 20% bovine serum and 80% Hanks solution containing 0.4% lactalbumin hydrolysate and 0.08% yeast extract in a final concentration. The cultivation of cell number above 1000,000, i.e., 350,000, 200,000, and 100,000/ml, resulted in marked increase throughout the week and that of 200,000

showed the highest increase. On the contrary, 60,000 cell nuelei/ml did not effect any propagation of the cells. From these results, it was thought that the optimal inoculum size should be over 100,000 cell nuclei/ml in primary culture and this size was adopted in the following experiments (Fig. 3).

4) Effect of the Concentration of Lactalbumin Hydrolysate In order to determine the optimal concentration of lactalbumin hydrolysate in the medium final concentrations of 0.6, 0.4, 0.2 and 0% were examined. The medium consisted of 20% bovine serum and 80% of the Hanks solution containing yeast extract, in a final concentration of 0.08%. The inoculum size was 200,000 cell nuclei/ml/tube. This result is illustrated in Fig. 4. Except for absence of lactabumin hydrolysate in the medium, continuous cell proliferations were attained in any of the concentrations tested. Among them, final concentration of 0.4% of lactalbumin hydrolysate gave the highest value. From this result, 0.4% was taken as the optimal concentration.

5) Effect of the Concentration of Yeast Extract Five kinds of media containing yeast extract in a final concentration of 0.12, 0.08, 0.04, 0.02, and 0% were com pared. Composition of the medium was 20% of bovine serum and Hanks solution containing 0.4% lactalbumin hydrolysate. The inoculum size was 300,000 cell nuclei/ml/tube. In all assayed groups, the cell multiplied throughout the 7 days of cultivation. The medium with 0.08% concentration of yeast extract showed the highest rate of cell propagation. The most suitable concentration of yeast extract seemed to be 0.08% in the medium (Fig. 5).

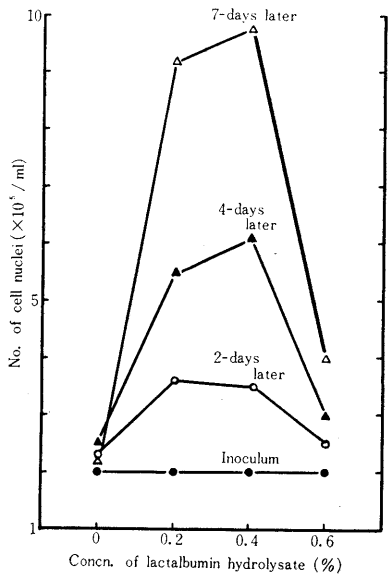

Fig. 4. Effect of the concentration of lactalbumin hydrolysate in the medium

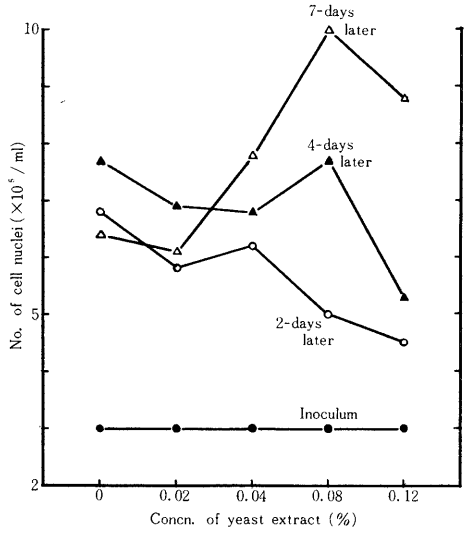

Fig. 5. Effect of the concentration of yeast extract in the medium

6) Effect of the Concentration of Serum Effect of the concentration of bovine serum in the medium on the multiplication of MN-lymphosarcoma cell was examined. Bovine serum concentrations examined were 60, 40, 20, 10, 5, and 0%. In all of these groups, the final concentration of lactalbumin hydrolysate was adjusted to 0.4% and yeast extract to 0.08%. The inoculum size was 200,000/ml/tube.

In the media containing more than 40% bovine serum, marked multiplication of cell nuclei was observed and a moderate increase was obtained in a group with 20%

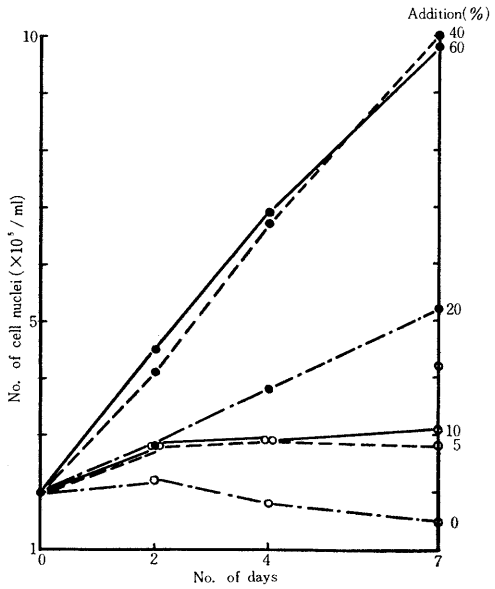

Fig. 6. Effect of the concentration of serum in the medium

bovine serum, during 1 week. Without bovine serum, the number of cell nuclei decreased gradually and in the groups with 10 and 5% bovine serum, their growth rate ceased after a slight increase in the early stage. The medium supplemented with 40% bovine serum was finally adopted for the following studies and for long-term tissue culture of MN-lymphosarcoma cells (Fig.6).

7) a) Comparison of Effects between Bovine Serum and Dialysed Bovine Serum in the Medium Whether bovine serum in the medium could be substitute with dialysed serum or not was examined.

Dialysis of bovine serum was described earlier and components of the medium were the same as that in (6). The inoculum size was 200,000 cell nuclei/ml. In the medium supplemented with dialysed bovine serum, cell proliferation rate was moderately less than that in the medium containing bovine serum (Fig. 7).

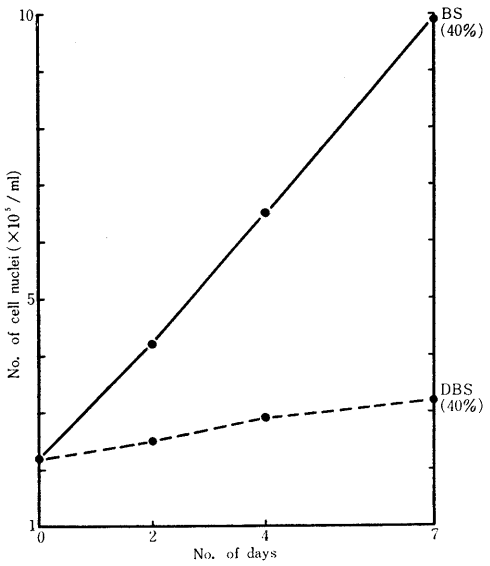

Fig. 7. Comparison of effects between bovine serum (BS) ane bialysed bovine serum (DBS) in the medium

b) Effect of Hexestrol in the medium supplemented with DBS According to Szego and Roberts,[31] estrogen associated with blood protein, as well as the hormone not bound to protein, could readily be dialyzed quantitatively past a collodion membrane. In addition to their result, it may be thought that low molecular substances contained in whole serum could also be dialyzed by the present method described herein.

Rather than attempting to fractionate the material isolated by dialysis of the serum to identify the missing growth factors, hexestrol, one of synthetic estrogens, was examined for possible growth-promoting activity when added to the medium containing the dialyzed serum.

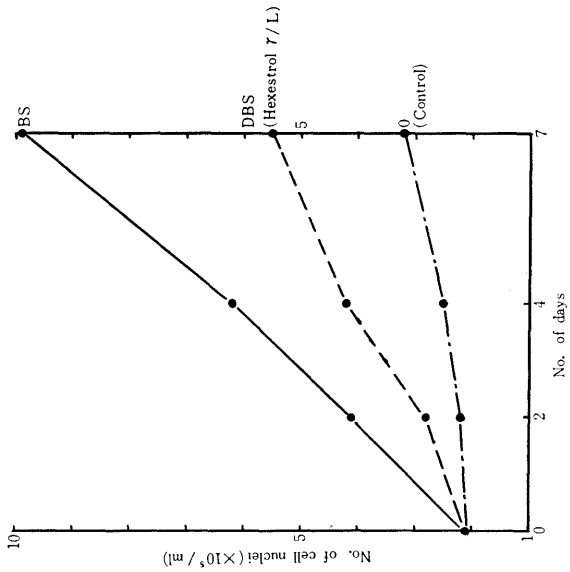

Fig. 8. Effect of Hexestrol (synthetic estrogen) in the medium supplemented with DBS on the cell multiplication rate

According to biological estimation, the concentration of blood estrogen was 2 to 5.8γ/L of blood during the normal menstrual cycle.[16] From this fact, hexestrol was dissolved in absolute ethanol and added to the medium supplemented with the dialysed bovine serum in a final concentration of 5γ/L. The inoculum size was 210,000 cell nuclei/ml.

As shown in Fig. 8, cell proliferation rate was accelerated markedly in the medium containing hexestrol and the retarded rate of proliferation in the medium supplemented with dialysed serum recovered to approximately 35% within a week.

This experiment indicated that MN-lymphosarcoma cell preserved the property of estrogen-dependency after *in vitro* cultivation and that estrogen stimulated the mitosis of lymphatic cells.

Summary and Conclusion

For much advantages *in vitro* condition in evaluating some biological behavior of cancer cell, cultivation of cancer tissues has been attempted but many strains of lymphomas and leukemias of laboratory mice have been little reported.[2,3,6,8] We attempted the *in vitro* cell culture of MN-lymphosarcoma in suspension type without the use of feeder system, because the method was useful for the investigations of cytotoxic effect of chemical agents including hormones, and interaction between tumor cells and their milieu under moderately uniform conditions.

It was demonstrated that a static culture was a more suitable method than roller tube culture. Although the adherence to the glass surface might be necessary for

the cell division of some cells such as HeLa and fibroblasts, it would be of less significance in the case of lymphocytic tumor. It could be concluded that MN-lymphosarcoma cell also might need a more anaerobic condition for their multiplication, as mentioned in the cultivation of Yoshida sarcoma cells.[14, 32] As MN-lymphosarcoma cells grow without adhering to glass surface but float at the bottom during cultivation, cells were dispersed by several pipetttings and made into a homogeneous suspension. Therefore, subculture of the cells to the following generation has been carried out without treatment with trypsin digestion or ethylenediaminetetraacetate.

Culture in initial pH of 7.6 was found to be optimal for multiplication during 1 week and this result was quite similar to the cultivations of HeLa[9] or Yoshida sarcoma cells.[14] According to Hotchin,[9] however, Novikoff rat hepatoma cell was intolerant to pH above 7.4.

MN-Lymphosarcoma cells showed continuous propagation with the inoculum size over 100,000 nuclei/ml in a primary culture experiment. Earle *et al.*[4] and Katsuta *et al.*[15] reported that the seeding of a moderate number of cells was necessary in a primary culture in order to obtain continuous proliferation. These might be discussed with the hypothesis of "conditioned medium" introduced by Sanford *et al.*[28]

The effect of the concentration of lactalbumin hydrolysate in the medium was examined and final concentration of 0.4% was found to be the optimal.

The optimal concentration of yeast extract was determined as 0.08% in the final medium. Yeast extract, which had been reported as being growth-inhibiting in the cultivation of Yoshida sarcoma and ascites hepatoma cells[13, 14, 15] but not toxic or rather, essential, to strain L cell, Walker carcinoma 256,[20] and Novikoff hepatoma,[9] was found to be necessary for the culture of MN-lymphosarcoma cell. The cause of such different behaviors among cell types is not known yet.

The optimal concentration of bovine serum for the vigorous propagation of MN-lymphosarcoma cells was found to be 40% in the medium. MN-Lymphosarcoma cells could maintain multiplication in a simple medium consisting of 40% bovine serum, 0.4% lactalbumin hydrolysate, 0.08% yeast extract, and Hanks saline. Substitution of dialysed serum for the whole one in the medium failed to sustain the growth of MN-lymphosarcoma.

On dialysis of serum against distilled water, low molecular-weight substances including estrogen passed through the Cellophane membrane.[31] In the method of dialysis employed in this experiment, the euglobulin fraction of serum with which estrogen was bound in circulating blood[26] was also discarded. Therefore, it might be assumed that the supernatant in the Cellophane sack was free of estrogen after prolonged dialysis.

Hexestrol, a synthetic estrogen, was added to the medium supplemented with dialysed bovine serum and the cell multiplication rate was accelerated markedly. From the result of this experiment, it might be considered that factors essential for

promoting the cell growth of MN-lymphosarcoma existed not only in euglobulin fraction or low molecular-weight substances dialyzable through a Cellophane membrane but also in the small amount of estrogen in the serum. In other words, MN-lymphosarcoma cell *in vitro* seemed to be still dependent on estrogen.

Many reports concerning the effect of estrogen on the propagation of normal cells and tumor cells have been published so far and estrogen has been found to produce some stimulation of mitosis *in vitro*.[1, 5, 18, 30, 33] The stimulating effect of estrogen on mitosis of tumor cells was also obvious from this experiment.

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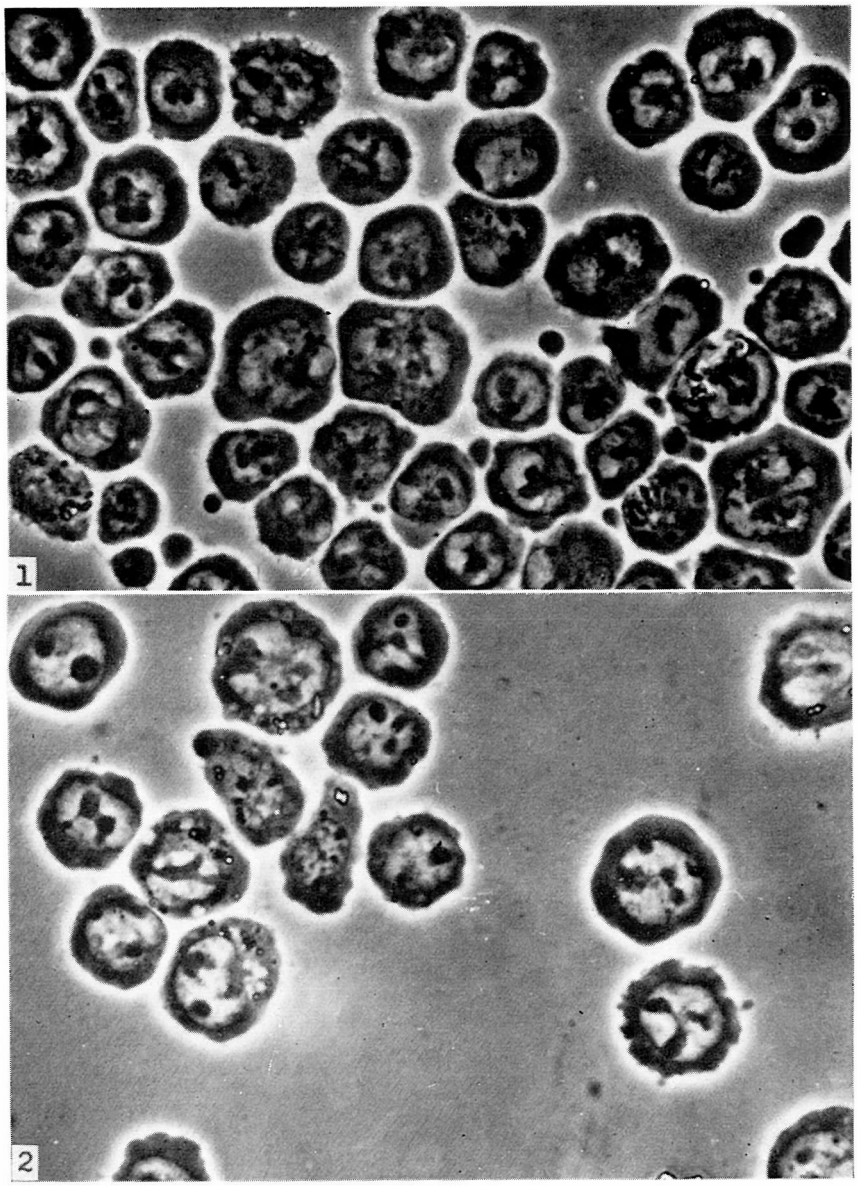
EXPLANATION OF PLATE

General morphological characteristics of the T-strain of MN-lymphosarcoma cells *in vivo* and *in vitro*. All photographs taken under phase contrast microscope. ×850.

Photo 1. The T-strain of MN-lymphosarcoma cells, 7 days after inocuiation.

Photo 2. The sarcoma cells grown in the medium consisting of 40% bovine serum, 0.4% lactalbumin hydrolysate, 0.08% yeast extract, and Hanks saline. The cell is round in shape and there is a large round nucleus with 1~2 nucleoli. Refractile lipid granules are seen in the cytoplasm of a few cell.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