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CULTIVATION OF FUSED CELLS RESULTING FROM TREATMENT OF CELLS WITH HVJ

I. SYNCHRONIZATION OF THE STAGES OF DNA SYNTHESIS OF NUCLEI INVOLVED IN FUSED MULTINUCLEATED CELLS

TADASHI YAMANAKA and YOSHIO OKADA

Department of Preventive Medicine, Research Institute for Microbial Diseases, Osaka University, Osaka

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SUMMARY 1) Multinucleated giant KB cells fused by UV-irradiated HVJ were readily cultivated for several days in Eagle medium containing bovine serum. However, division of these fused cells was rarely seen. On the contrary, mononuclear cells grew actively in the same medium even after treatment with HVJ. It seems that the absence of division of the fused cells is due to polynucleation of the cells and not to treatment with HVJ.

2) DNA synthesis of nuclei in the fused cells was found to be much in mononuclear cells.

3) KB cells were fused randomly by HVJ irrespective of their stage in the generation cycle. Immediately after fusion, G- and S-phase nuclei (corresponding to DNA synthesis) were distributed randomly in the multinucleated cell. When the fused cells were cultured, the stages of DNA synthesis in all the nuclei in a cell became synchronized, even though division of the fused cell was not seen.

4) The time of culture of the cells required for the synchronization increased with increase in the number of nuclei involved in the fused cell.

INTRODUCTION

Fusion of animal cells easily occurs with HVJ (Hemagglutinating Virus of Japan) (OKADA *et al.*, 1957: OKADA and TADOKORO, 1963). The fusion activity of the virus particle is located in the envelope, composed of lipoprotein, and its activity is more stable to UV-irradiation than the infectivity of the virus (OKADA and TADOKORO, 1962). The mechanism of cell fusion has been proposed to be as follows (OKADA *et al.*, 1966: MURAYAMA and OKADA, 1965: OKADA and MURAYAMA, unpublished). I) Virus adsorption onto cells and agglutination of these cells at 4°C. II) Disconnection of the cell surfaces at the sites adsorbing virus particles by the fusion activity of HVJ at 37°C. III) Reconnection of the disconnected sites utilizing energy from nucleotide triphosphate (probably ATP) under con-

ditions in which Ca ions are present. If the disconnected site of one cell is in contact with a similar site of another cell, fusion of these cell membranes occurs at this site. IV) Spherical giant cell formation which requires energy.

The cells fused by UV-irradiated HVJ synthesize as much DNA and RNA as control cells which have not been treated with virus (OKADA and MURAYAMA, 1965: HARRIS and WATKINS, 1965). It could be expected that cellular regulation of multinucleate cells (heterokaryons) shifts from the parental mononuclear cells. In the present report, the DNA synthesis and cell division of heterokaryons were analyzed.

MATERIALS AND METHODS

Virus. The egg adapted Z-strain of HVJ was propagated in the allantoic sacs of 10-day-old chick embryos. Diluted inoculum of the infected allantoic fluid was used. The infected allantoic fluid was harvested and dialyzed against about 500 volmes of saline at 4°C for about 20 hours. The dialyzed fluid was stored in a refrigerator at −20°C. Virus was used for cell fusion after inactivation by UV-irradiation, because virus infection of fused cells would greatly complicate the analytical results. The UV-dose used for irradiation of the virus sample was about 4-fold the dose which completely inactivated the infectivity.

Cells. KB cells were cultured as monolayers in YLH medium containing 20 per cent bovine serum.

Cell fusion.

1. *Fusion on a Petri dish.* A suspension of KB cells was prepared by treatment of the monolayer of KB cells with trypsin. The cells were plated on a Petri dish of 9.1 cm diameter at a concentration of about 10^4 cells/plate. During incubation at 37°C for several hours, these cells became attached and spread over the glass (Fig. 1, A). Then the medium was removed and one ml of UV-irradiated HVJ sample (2,000 HAU/ml) was added to the plate. During incubation for 10 minutes in the cold, virus particles were adsorbed onto the cells (Fig. 1, B). The plate was washed 3 times with cold Eagle medium to remove unadsorbed virus particles, and then a suspension of KB cells was added. When most of the added cells had become attached to the adherent cells, the remaining free cells were removed by washing (Fig. 1, C). Fresh medium was added to the plate and the culture was incubated at 37°C in a CO_2 incubator. Within one hour, the added cells fused with the adherent cells (Fig. 1, D). Fused multincleated cells (formed from the added and adherent cells) and mononuclear cells (unfused adherent cells) were present on the plate. The ratio of fused cells to mononuclear cells depends on the concentrations of added virus and cells. These plates were incubated in Eagle medium containing 20 per cent

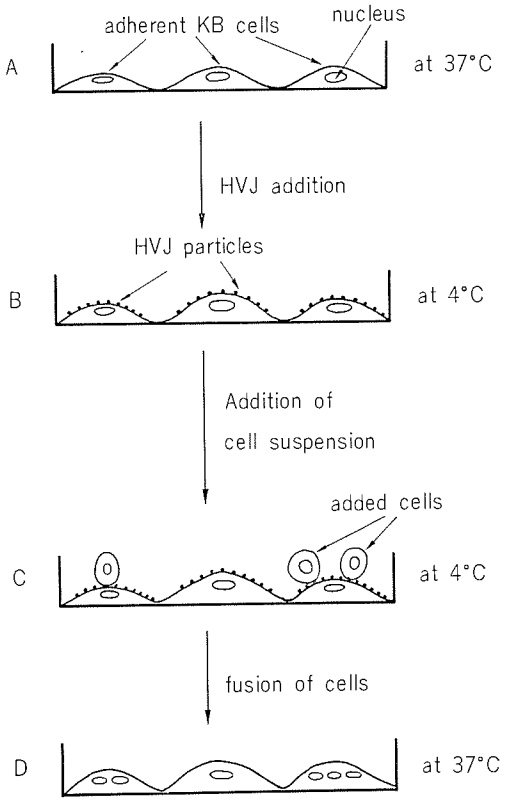


FIGURE 1 A model of a method of fusion on a Petri dish.
A: Adhesion of KB cells on the glass wall at 37°C.
B: Adsorption of HVJ particles onto the adherent cells in the cold.
C: Attachment of added cells onto the adherent cells in the cold.
D: Fusion of the adherent cells with the added cells at 37°C.

bovine serum in a CO_2 incubator at 37°C.

2. *Fusion of cells in suspension.* (OKADA and TDOKORO, 1962).

One ml of suspension of KB cells and an equal volume of 10-fold diluted HVJ sample were mixed in a test tube. The tube was incubated at 4°C for 10 minutes and then at 37°C with shaking for 60 minutes. Then, the cells were washed 3 times and plated in a Petri dish containing coverslips. The cells were cultured in Eagle medium containing 20 per cent bovine serum in a CO_2 incubator.

Autoradiography. The cells cultured on coverslips were incubated with ^{3}H-thymidine dissolved in LE or Eagle medium. Then the coverslips were washed 3 times with PBS, fixed with methanol and treated with 5 per cent PCA at 4°C for 20 minutes. Smear preparations were pre-treated with 25 μg/ml of RNAase at 37°C for 60 minutes before this PCA treatment. Then, preparations were washed three times with distilled water, and dipped in 2-fold diluted Kodak NTB-2 emulsion at 45°C in a dark room. After exposure for 3–6 days at 4°C, the samples were developed and fixed. Finally, they were stained with Giemsa at pH 6.2. For analysis of labelled cells, 500–1,500 cells/coverslip were counted.

RESULTS

Culture of cells after treatment with UV-irradiated HVJ.

In this experiment, fused cells were prepared by method (1). A black plate with small windows was attached to the bottom of a Petri dish. After treatment with UV-irradiated HVJ, cells were plated on the Petri dish and incubated at 37°C in a CO_2 incubator. Then the cells were photographed through each window at intervals of 24 hours. The growth of cells was estimated from the negatives of the photographs. As indicated in Fig. 2, mononuclear cells grew actively during incubation for 7 days, but scarcely multinucleated cells (heterokaryons) divided during the 7 day incubation period. It seems that 1) HVJ which has been UV-irradiated scarcely influences cell division, 2) the loss of the capacity of heterokaryons to divide was due to poly-nucleation.

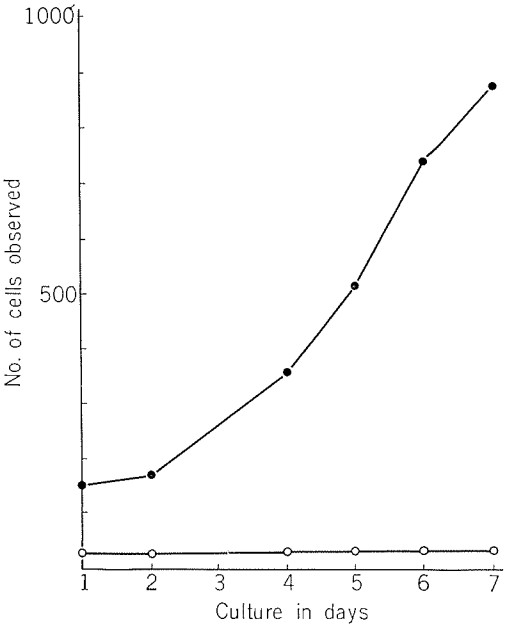


FIGURE 2 Growth curves of mononuclear cells (●) and multinucleated cells (○) after treatment with UV-irradiated HVJ.

Continuous labelling with ^{3}H-thymidine was carried out to test whether the heterokaryons synthesized DNA. At 24 hours after fusion, 0.2 μC/ml of ^{3}H-thymidine was added to the sample which was incubated for a further 72 hours. One series of the photographs of this experiment are shown in Fig. 3. After 2 days-incubation (Fig. 3 a), a tri-nucleated cell and four mononuclear cells were present. After a further 2 days-incubation (Fig. 3 b), the mononuclear cells each divided giving 8 mononuclear cells, but the tri-nucleated cell did not divide. However, the three nuclei in the fused cell synthesized as much DNA as mononuclear cells (Fig. 3 c, d). Many cells were found to be in the same series when autoradiographed at the end of the experiment and the frequency distributions of the nuclei showing, and not showing DNA synthesis were compared with those of similar cells in the control which had not been treated with HVJ. As indicated in Fig. 4, almost all

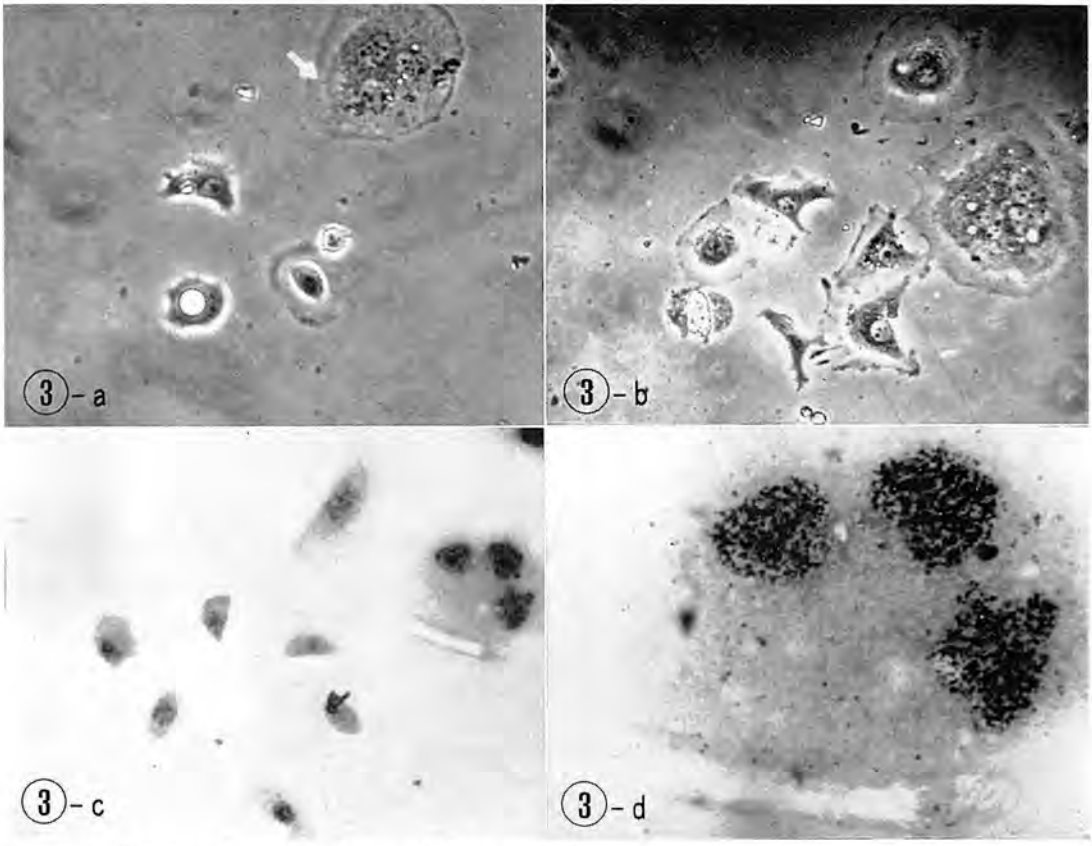


FIGURE 3 Photographs of cell growth and DNA synthesis.
One day after treatment with UV-irradiated HVJ, ^{3}H-thymidine (0.2 μC/ml) was added. The ^{3}H-thymidine was present in the medium during further culture.
3a: Phase contrast micrograph 2 days after HVJ treatment. One trinucleated and four mononuclear cells are observed.
3b: Phase contrast micrograph at the end of the culture. (4 days after HVJ treatment). The tri-nucleated cell in 3a remained. However, four mononuclear cells in 3a divided into 8 cells. A bi-nucleated cell locating in the upper portion was out of place in 3a.
3c: Autoradiograph at the end of the culture.
3d: Highly magnified autoradiograph of the tri-nucleated cell in 3c.

nuclei in the mono-, bi- and tri-nucleated cells in the samples exposed to the virus showed as high activity in DNA synthesis as the control which had not been treated with the virus.

Alteration of the stage of DNA synthesis in the nuclei involved in the fused cells during cultivation.

A) *Distribution of nuclear-members in the S- and G-phases in cells immediately after fusion.*

KB cells cultured as monolayers were pulse-labelled with ^{3}H-thymidine for 60 minutes at 37°C and then washed 3 times with PBS and trypsinized. After removing trypsin, cells were resuspended in medium and fused with HVJ by method (2). The cells were smeared on a slide glass and autoradiographed. The frequency distribution of labelled and non-labelled nuclear-members appearing in the nuclei of the fused cells was calculated and

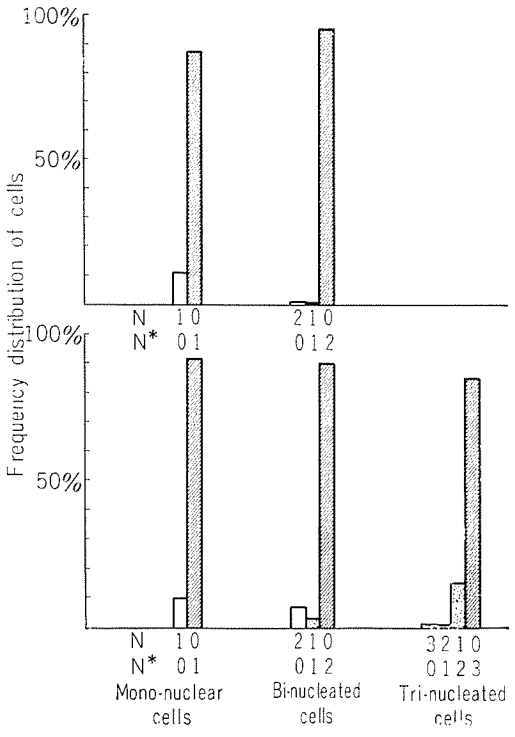


FIGURE 4 Frequency distribution of labelled and non-labelled nuclei in samples treated with ^{3}H-thymidine for 72 hours.
The mean values of per cent labelling of nuclei were 88% in mononuclear cells and 96.4% in bi-nucleated cells in control samples not treated with HVJ; 90.9% in mononuclear cells, 91.7% in bi-nucleated cells and 95.2% in tri-nucleated cells in test samples treated with HVJ.
N: Number of nuclei not labelled,
N*: Number of nuclei labelled.

compared with the theoretical value for cells in the sample fusing together without regard to their generation stage (the cells were in the condition of random growth). The theoretical value was calculated from the following formula (OKADA and MURAYAMA, 1965),

Frequency distribution $= {}_nC_rP^r(1\text{-}P)^{n-r}$

n: total number of nuclei in the fused cell,

r: number of labelled nuclei in the fused cell,

P: ratio of number of labelled cells to total number of cells in the initial cell sample (theoretical value I), or ratio of number of labelled mononuclear cells to total number of mononuclear cells remaining in the sample treated with HVJ (theoretical value II).

As shown in Fig. 5, the observed value coincided with both theoretical values (I and II). This clearly indicates that cells were fused randomly by HVJ irrespective of their stage in the generation cycle. A fused cell containing both S- and G-phase nuclei is shown in Fig. 8 a.

B) *Alteration of the distribution of S- and G-phases of nuclei in fused cells during cultivation.*

As indicated above, multinucleated cells, immediately after fusion, contained a random mixture of S- and G-phase nuclei. These fused cells were cultured in Petri dishes containing coverslips and the coverslips were sampled after appropriate intervals of culture. Then, the coverslip was pulse-labelled with 5 μC/ml of ^{3}H-thymidine for 60 minutes at 37°C. After autoradiography, these preparations were analyzed as in the case of (A). The theoretical value was calculated from the per cent of labelled mononuclear cells appearing in the preparation. In Fig. 6, the frequency distribution of nuclei involved in bi-nucleated cells is compared with the theoretical value. The distribution in the sample pre-labelled before fusion coincided with the theoretical value. However, with increase in the time of culture, the observed value shifted from the theoretical value. The frequency of cells containing nuclei in both S-and G-phases decreased, and the frequency of cells containing two nuclei in the same stage (S or G) increased. It seems that the phases of the nuclei involved in a fused cell became synchronized during cultivation. These tendencies were also observed in tri- and tetra-nucleated cells (Fig. 7). The mean value of the per cent of nuclei labelling was nearly equal in mono-, bi-, tri-, tetra- and penta-nucleated cells. In Figs. 8 b, 8 c and 8 d, huge multinucleated cells containing synchronized nuclei (S- or G-phase only) are shown. On comparing the results indicated in Fig. 6 and Fig. 7, the time of cultivation

FIGURE 5 Frequency distribution of S- and G-phase nuclei per fused cell immediately after fusion.
A: Theoretical value (I),
B: Theoretical value (II),
C: Observed value estimated from the samples treated with HVJ,
D: Observed value estimated from the samples treated with ten-times concentrated HVJ.
N: Number of G-phase nuclei,
N*: Number of S-phase nuclei.

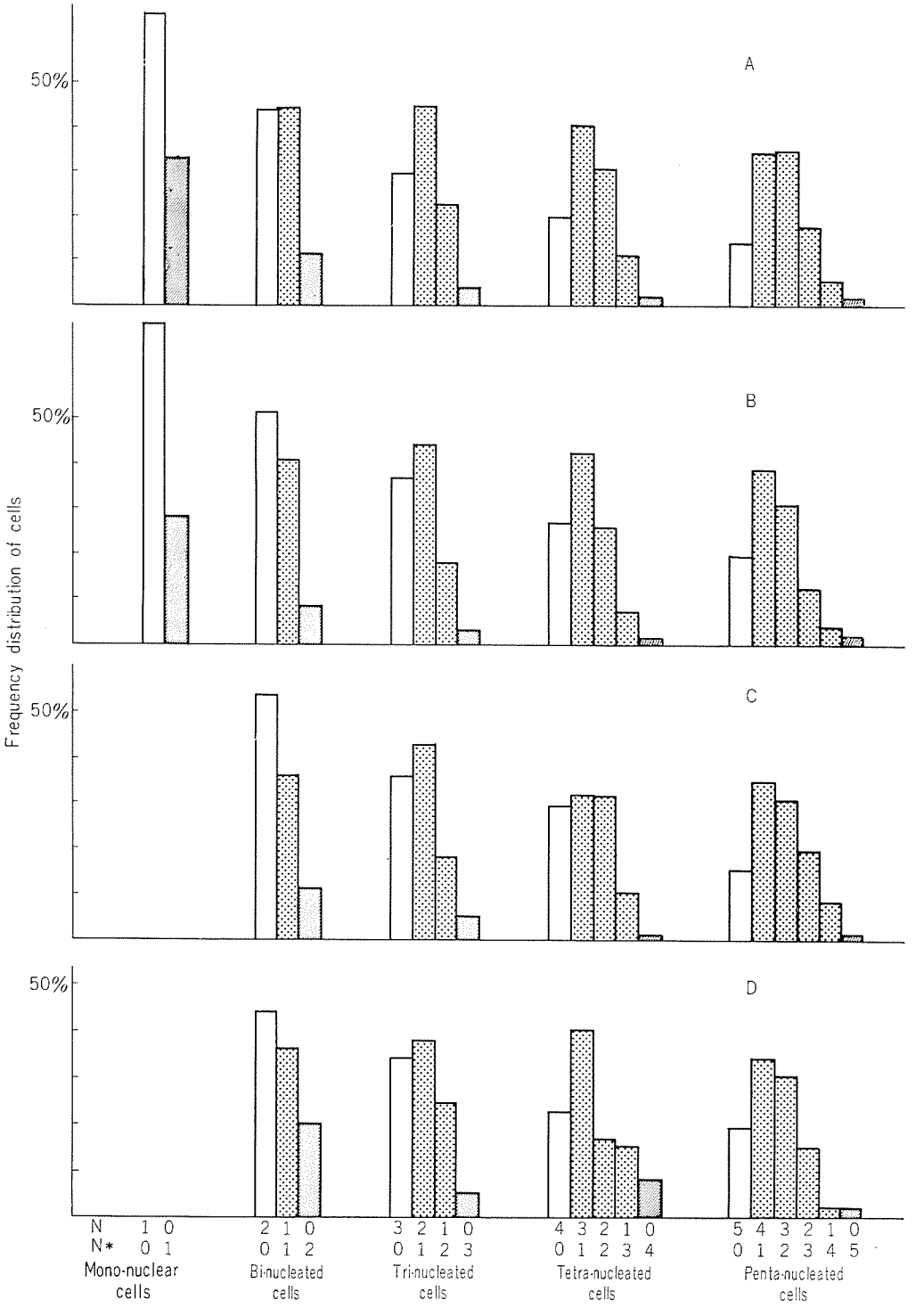

50%
A
50%
B
50%
C
50%
D
Frequency distribution of cells
N 1 0
N* 0 1
Mono-nuclear cells
2 1 0
0 1 2
Bi-nucleated cells
3 2 1 0
0 1 2 3
Tri-nucleated cells
4 3 2 1 0
0 1 2 3 4
Tetra-nucleated cells
5 4 3 2 1 0
0 1 2 3 4 5
Penta-nucleated cells

FIGURE 6 Synchronization of phases of DNA synthesis of nuclei involved in bi-nucleated cells during cultivation.
N : Number of G-phase nuclei,
N* : Number of S-phase nuclei.
a : samples pre-labelled before fusion,
b : samples labelled immediately after fusion (about 1- 1.5 hours after fusion),
c : samples labelled 24 hours after fusion
d : samples labelled after 28 hours,
e : samples labelled after 44 hours,
f : samples labelled after 49 hours.

FIGURE 7 Synchronization of phases of DNA synthesis of nuclei involved in tri- and tetra-nucleated cells during cultivation.
N : Number of G-phase nuclei,
N* : Number of S-phase nuclei.
a : samples pre-labelled before fusion,
b : samples labelled immediately after fusion (about 1- 1.5 hours after fusion),
c : samples labelled 24 hours after fusion,
d : samples labelled after 28 hours,
e : samples labelled after 44 hours,
f : samples labelled after 49 hours.

Fig. 6

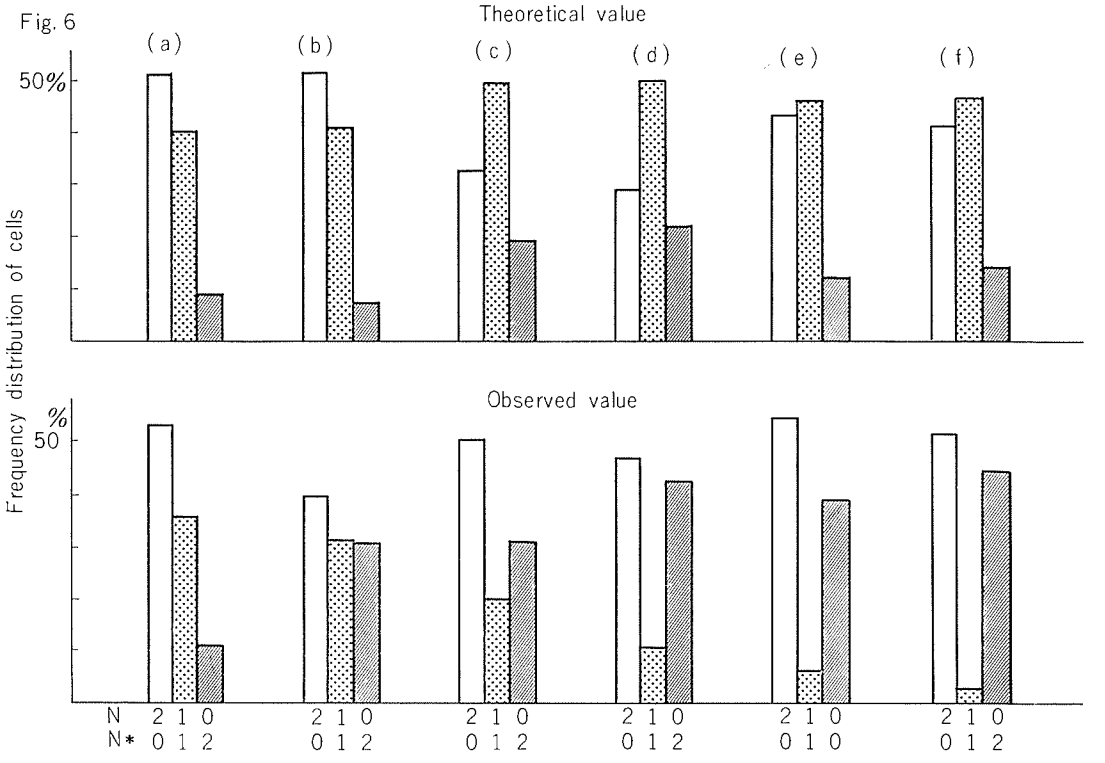
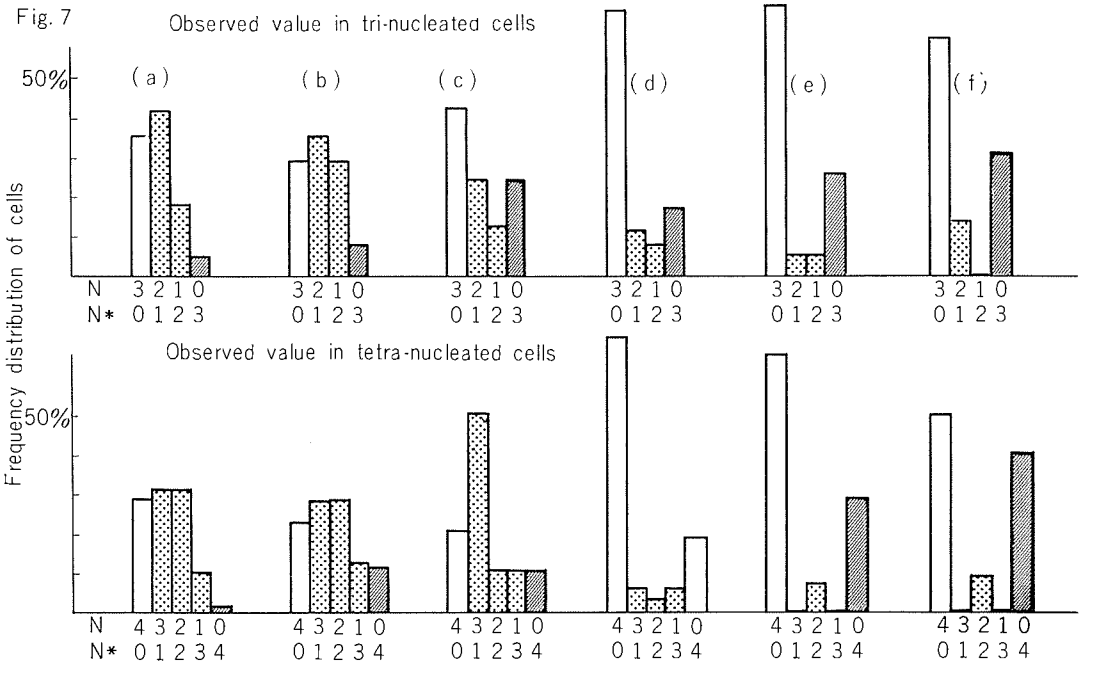

Theoretical value
(a)
(b)
(c)
(d)
(e)
(f)
50%
Frequency distribution of cells
Observed value
%
50
N 2 1 0
N* 0 1 2

Fig. 7


Observed value in tri-nucleated cells
(a)
(b)
(c)
(d)
(e)
(f)
50%
Frequency distribution of cells
N 3 2 1 0
N* 0 1 2 3
Observed value in tetra-nucleated cells
50%
N 4 3 2 1 0
N* 0 1 2 3 4

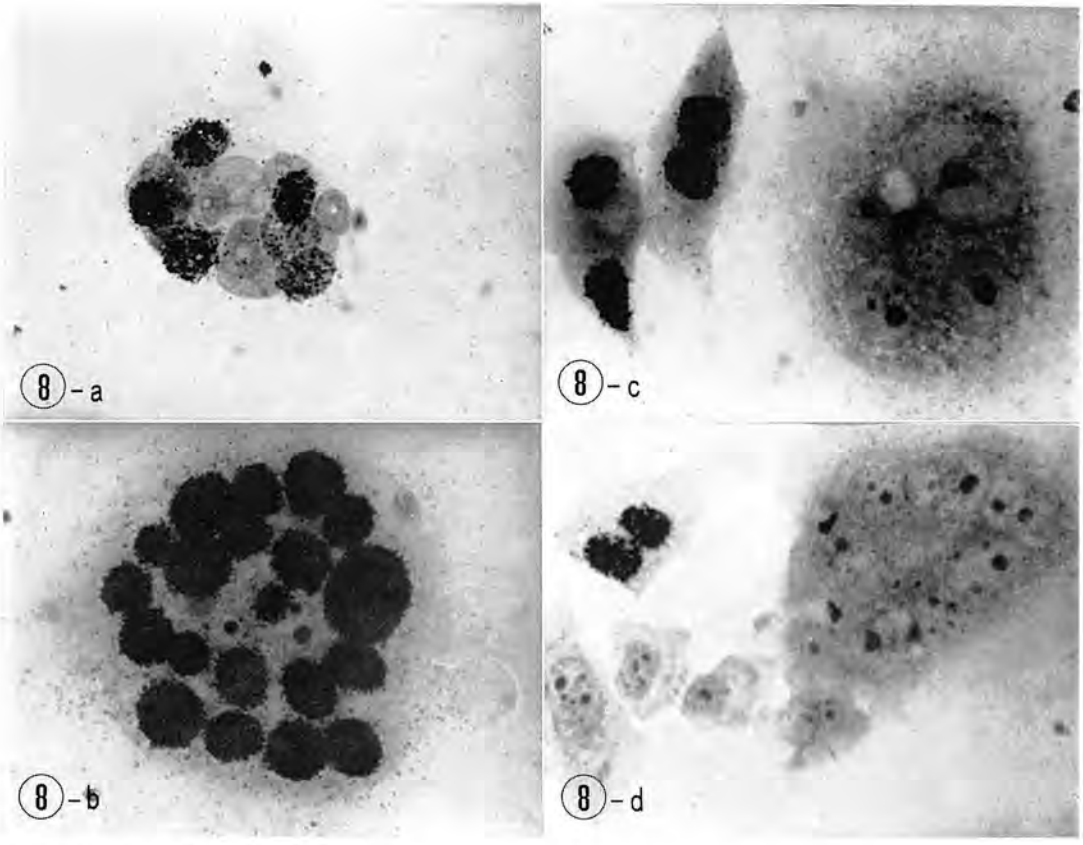


FIGURE 8 Autoradiographs of fused cells.
8a: prelabelled cell; labelled and unlabelled nuclei were mixed in the cell,
8b: A huge multinucleated cell containing S-phase nuclei alone appearing in culture,
8c and 8d: Multinuceated cells containing G-phase nuclei alone or S-phase nuclei alone.

required for the synchronization seems to be longer with the increase in the number of nucclei involved in the fused cell.

C) *Next, it was determined whether S-phase nuclei synchronized in a giant cell moved to G-phase around the similar course as the normal generation cycle in the culture.*

For this analysis, the thymidine phasing method developed by PUCK (1964) and BOOTSMA *et al.* (1964) was adopted. Firstly to determine the pattern of synchronization in the original KB cells, excess thymidine (final concentration 2 mM) was added in monolayer-cultured KB cells which were incubated for 24 hours at 37°C. Then, the medium was replaced by normal medium and the incubation was continued at 37°C. At appropriate intervals, the cells were harvested and pulse-labelled with ^{3}H-thymidine, then S- and G-phase cells and methaphase cells were analyzed. In Fig. 9, 0 time was fixed at the moment when the excess thymidine was removed. In about 4 hours after removing the excess thymidine, the per cent of S-phase cells was found to be maximum, having reached 90 per cent. With the further cultivation, the per cent sharply decreased and after 11 hours, the per cent was minimum (3.5 per cent), then it gradually increased once again. The per cent of S-phase

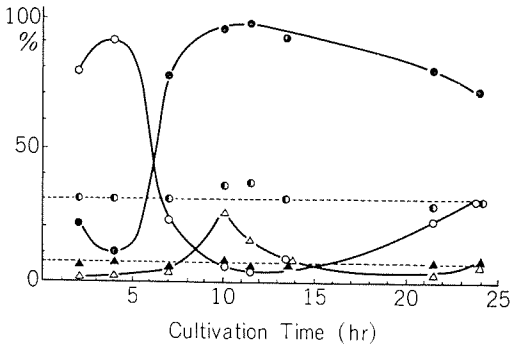


FIGURE 9 Phasing of original KB cells by the addition of excess thymidine.
An excess thymidine (final concentration 2 mM) was added in monolayer-cultured KB cells which were incubated for 24 hours at 37°C. Then the medium was replaced by normal medium (0 time) and the incubation was continued. At appropriate intervals, the cells were harvested and pulse-labelled with ^{3}H-thymidine, then S-phase (○) and G-phase (●) cells and methaphase cells (△) were analyzed. The results were compared with the per cent of S-phase cells (◐) and of metaphase cells (▲) in the control which had not been treated by excess thymidine.

cells in the control which had not been treated with the excess thymidine was constant and it was about 30 per cent. The per cent of mitosis was maximum in 10 hours and it was about 26 per cent. In the control the per cent of mitosis was constantly about 6–7 per cent. These patterns were very similar to the results reported by GALAVAZI *et al.* (1966) in a heteroploid cell line derived from a human kidney. The pattern shown in Fig. 9, was the standard for the following experiments.

Fused cells were prepared by the same method as that described in sections (A) and (B). This sample was cultured in Petri dishes for 16 hours at 37°C, then thymidine was added at the final concentration of 2 mM and the cells were incubated for 24 hours for phasing. After removing the excess thymidine, the cells were harvested and pulse-labelled with ^{3}H-thymidine. In these preparations, mono-, bi-, tri-, tetra- and penta-nucleated cells were selected, and the alteration of the frequency distribution of S-phase nuclei at these cultivation time were determined. As indicated in Fig. 10, the phasing with the excess thymidine in the mononuclear cells was observed same as in the case of the cells not treated with HVJ. In the case of the bi-nucleated cells, the phasing was very similar to that of the mono-nuclear cells, and the synchronism of both nuclei, involved in a bi-nucleated cell, was kept throughout these cultivations. In 4 hours, almost all the cells had a pair of S-phase nuclei and after 11 hours both nuclei moved to G-phase. After 25 hours-cultivation, these G-phase nuclei again move to S-phase. The length of time required for the conversion from S-phase nuclei to G-phase, or from G-phase to S-phase once again was very similar to that of mononuclear cells. The conversion of the phases of DNA synthesis with keeping the synchronism of the phase of nuclei, involved in a giant cell, was also observed in tri-, tetra- and penta-nucleated cells, but the degrees of the frequency of the conversion became low in the cells as the number of nuclei involved in a giant cell increased (Figs. 10 and 11). It seems that the manipulation of the cellular control became difficult with the increasing of the number of the nuclei.

These results clearly indicate that the nuclei in a giant cell were synchronized and able to move around the similar course of the normal generation cycle.

DISCUSSION

A heterokaryon immediately after fusion contained a random mixture of S- and G-phase nuclei but the phases of DNA synthesis in each nucleus became synchronized during culture. It seems that there is some regulatory control in the fused cells. Some kind of material regulating DNA synthesis may flow from one nucleus to other nuclei and control their phase. HARRIS and WHATKINS (1965) analyzed DNA synthesis in heterokaryons resulting from fusion of HeLa cells and Ehrlich ascites tumor cells. They stated that about 80 per cent of the Ehrlich ascites nuclei synthesized DNA, while only about 30 per cent of

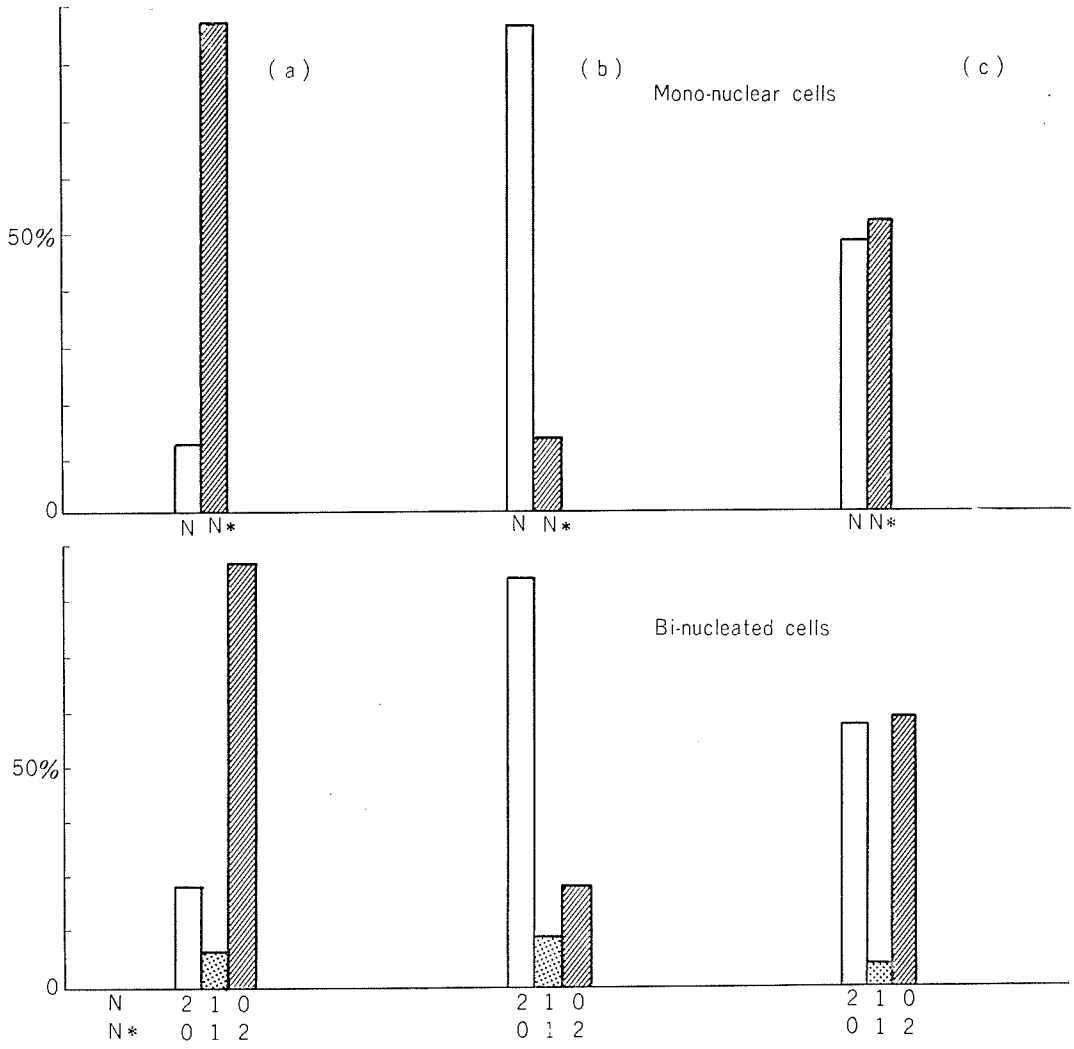
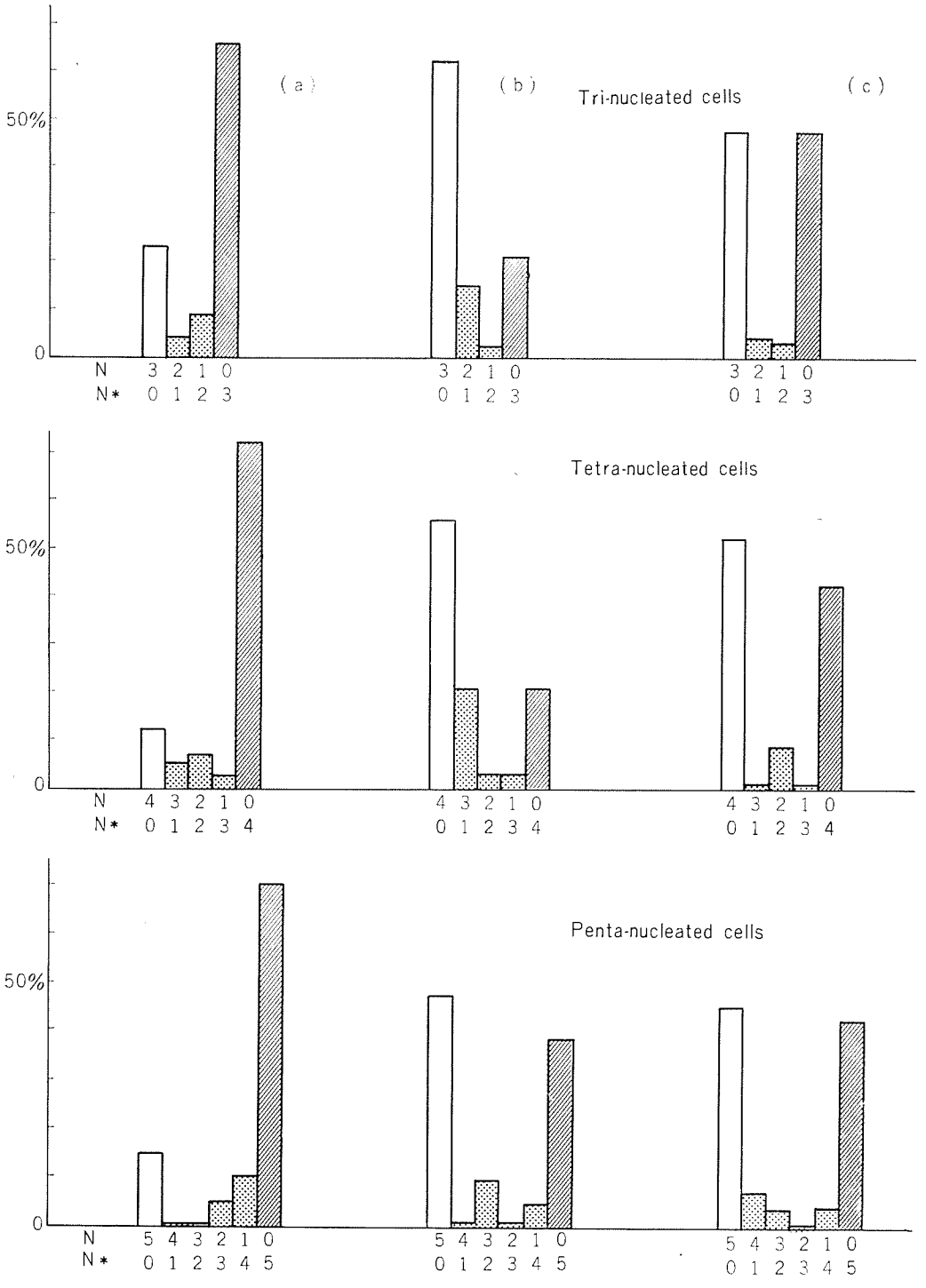


FIGURE 10 Frequency distribution of S- and G-phase nuclei in fused cells which had been treated with excess thymidine.
N: Number of G-phase nuclei,
N*: Number of S-phase nuclei.
a: samples pulse-labelled 4 hours after removing the excess thymidine,
b: samples pulse-labelled after 11 hours,
c: samples pulse-labelled after 25 hours.


(a)
(b)
(c)
Tri-nucleated cells
50%
0
N 3 2 1 0
N* 0 1 2 3
3 2 1 0
0 1 2 3
3 2 1 0
0 1 2 3
Tetra-nucleated cells
50%
0
N 4 3 2 1 0
N* 0 1 2 3 4
4 3 2 1 0
0 1 2 3 4
4 3 2 1 0
0 1 2 3 4
Penta-nucleated cells
50%
0
N 5 4 3 2 1 0
N* 0 1 2 3 4 5
5 4 3 2 1 0
0 1 2 3 4 5
5 4 3 2 1 0
0 1 2 3 4 5

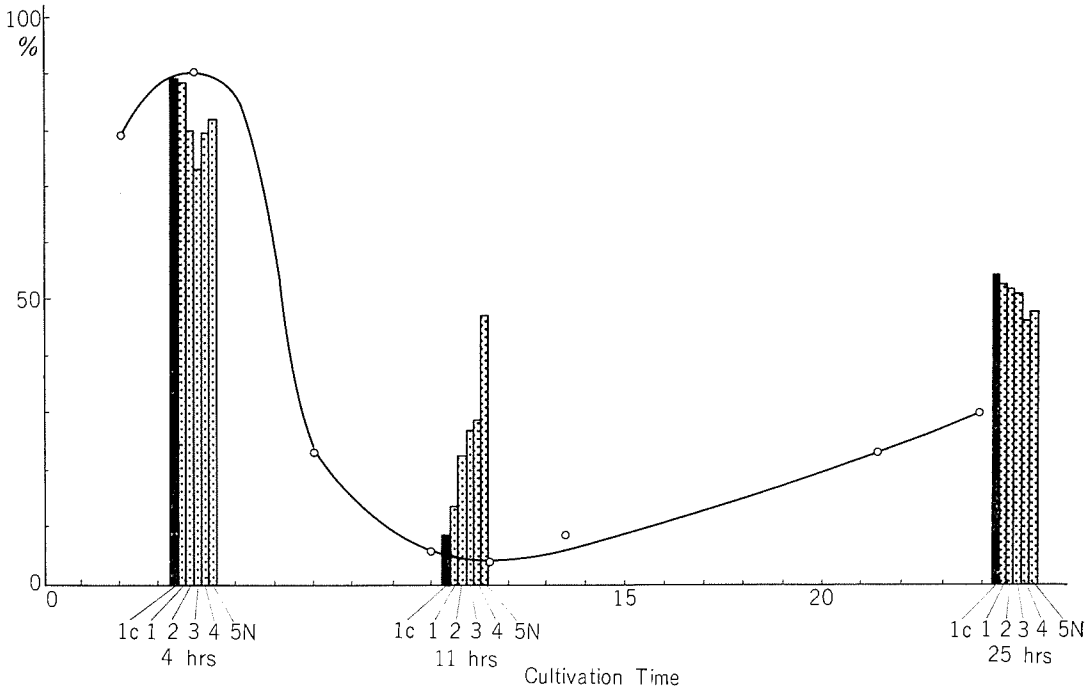


FIGURE 11 Comparison of the patterns of phasing of nuclei after treatment with excess thymidine in the original KB cells and KB cells treated with HVJ. The curve of solid line indicates the per cent of S-phase cells in the original KB cells shown in Fig. 9 (standard curve). The columns show the mean per cent of S-phase nuclei (N*/N+N*) estimated from the experiment shown in Fig. 10.
1c: the mean per cent of S-phase cells in the control sample in this series, not treated with HVJ.
1, 2, 3, 4, 5 N: the mean per cent of S-phase nuclei of mono-, bi-, tri-, tetra- and penta-nucleated cells in the test sample, treated with HVJ.

the HeLa nuclei in the heterokaryons did so, the proportions of Ehrlich ascites nuclei and HeLa nuclei which were synthesizing DNA underwent little change during 7 days culture.

In our present systems, some mononuclear cells remained in samples after treatment with HVJ. The fact that they remained as mononuclear cells was not because their fusion capacity was lower than that of the cells which fused, but because they had no opportunity to fuse. In the present experiment 200 or more virus particles per cell (one HAU is equvalent to 2.4×10^7 virus particles: OKADA and NISHIDA, 1961) were added, so that these mononuclear cells should have been exposed to HVJ just like the fused cells. The fact that the mononuclear cells showed cell division while fused cells did not, indicates that fused cells did not divide because they were polynucleated and not because they were exposed to HVJ. It is not yet clear, why polynucleation results in a lack of capacity of cells to divide. It might be due to the formation of some factors which disturb metabolic control in the fused cell. However, there is a tendency in fused cells on culture to synchronize the DNA synthesis in each nucleus. There may be a tendency for the disturbing factors formed by fusion to be neutralized during culture of fused cells. From the present results, the synchronization of the stages of DNA synthesis seems easiest in bi-nucleated cells. Moreover, evidence for cell division of bi-nucleated cells was obtained. In Fig. 12, one bi-nucleated cell is seen divided

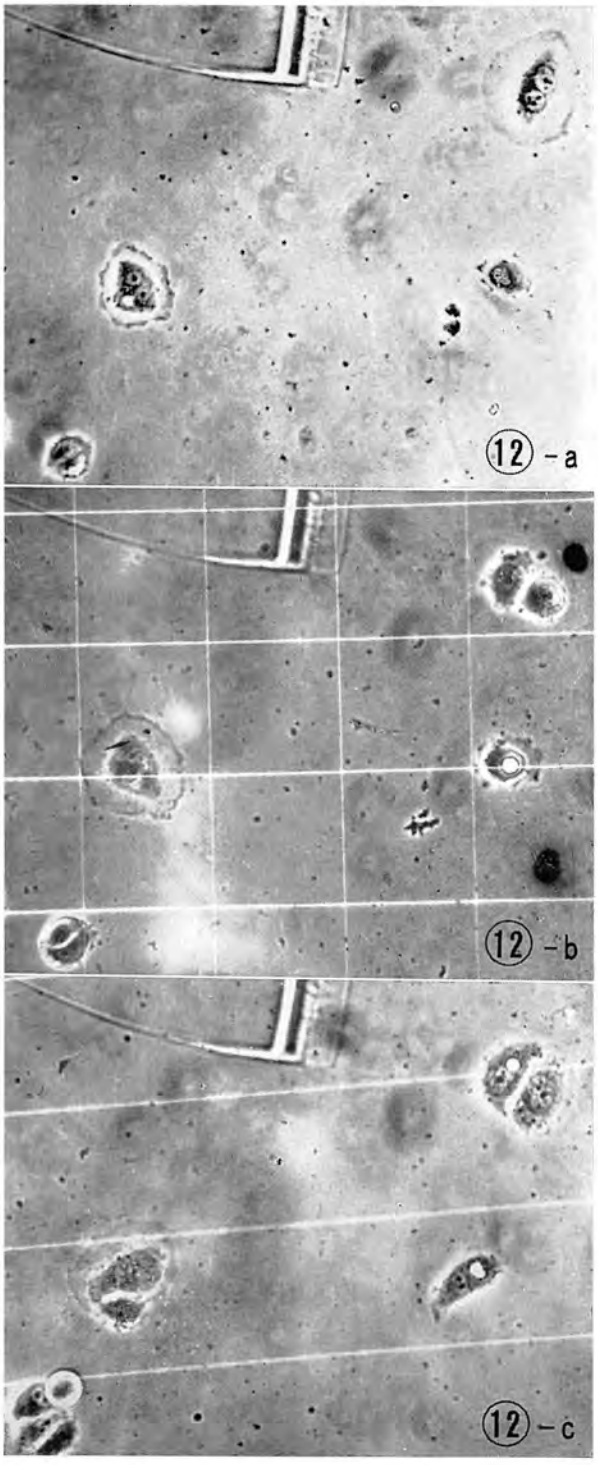


FIGURE 12 Photographs of bi-nucleated cells showing cell division. At the start in the culture (12 a), two bi-nucleated cells were present. After 12 hours in culture (12 b), in bi-nucleated cell (located at the right) divided into two mononuclear cells. Another bi-nucleated cell (located at the left) divided into one bi-nucleated cell and one mononuclear cell after 24 hours culture (12 c).

into two mononuclear cells and another bi-nucleated cell divided into one bi-nuclear and one mononuclear cell. Conversion of fused cells to metaphase was also observed in a few case (without colchicine). (From these observations, it seems that some of the mono-nuclear cells formed from fused cells would have been counted as mono-nuclear cells in the experiment on cell growth shown in Fig. 2). We assume that if a binucleated cell divides into two mononuclear daughter cells, the daughter nuclei have twice as many chromosomes as the parental nucleus.

In 1961, BARSKI *et al.* reported hybridization of animal cells *in vitro* using the method of mixed culture of cells of two strains. Hybrid cell formation of animal cells in vitro would open a new field in cytology. However, the frequency of the hybridization was very low in mixed cultures: *Littlefield* (1966) reported that in a hybridization experiment with two variants of L strain cells the frequency was about 10^{-6}. Fusion of cells of two different strains occurs efficiently with HVJ (OKADA, 1961: OKADA and MURAYAMA, 1965: HARRIS and WATKINS, 1965). Thus, it would be useful for hybridization if the fused cells could be efficiently converted to hybrid cells. One of our aims is to estimate the conditions required for this. As a first analysis, we cultured fused cells derived from homologous cells in which disturbances resulting from cell fusion would be minimal. In a subsequent experiment, fused cells resulting from cells in the same stage will be studied.

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