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MULTINUCLEATED GIANT CELL FORMATION BY FUSION BETWEEN CELLS OF DIFFERENT STRAINS*

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SUMMARY KB cells from man fuse well with HeLa cells and with FL cells of human origin due to HVJ and giant cells composed of two different cells were frequently found. KB cells also fuse well with L cells, Ehrlich ascites tumor cells of mouse origin and PS cells from pig. However, mouse embryo fibroblasts scarcely fuse with Ehrlich ascites tumor cells or KB cells.

These results show that giant cell formation by fusion between cells of two different strains occurs well only among such cells as malignant cells or undifferentiated cells of established tissue culture strains and is not affected by the species from which the cells originate. The results also suggest that the surface of these cells has lost some specificity.

Fused giant cells formed from homologous cells synthesized DNA and RNA in the same manner as normal single cells.

INTRODUCTION

In 1957, OKADA *et al.* reported the appearance of many giant polynuclear tumor cells formed by fusion within an hour after a large dose of HVJ had been inoculated into the abdominal cavity of a mouse implanted with Ehrlich ascites tumor cells. A quantitative experimental method was developed and this phenomenon was analyzed *in vitro* (OKADA, 1958, 1962 a, b; OKADA and TADOKORO, 1962). Then, it was reported that malignant tumor cells and cells of established tissue cultured strains have high cell fusion capacities, while cells of short-term-cultured or mature somatic cells have very low or no cell fusion capacities (OKADA and TADOKORO, 1963).

The reaction steps in the fusion are (I) adsorption of HVJ onto the cell surface and mutual attachment of the participant cells at 4°C, (II) loosening or disconnecting of the virus adsorption sites of the cell surface structure, (III) reconnection of the disconnection sites or fusion of the site on one cell with that on a neighbouring cell surface, and (IV) mixing of the inner components of the participant cells and spherical giant cell formation. Reaction steps (II) to (IV) when carried out at 37°C under aerobic conditions, are complete within 30 minutes.

It is very easy to mix two different kinds of

* The results in this paper were reported at the 17th meeting of the Japan Society for Cellular Chemistry, on November 26, 1964.

cells in any ratio for the cell fusion reaction, since the cells are utilized in the form of suspensions. Then, giant cell formation by fusion between the different cells is considered to be possible when the cells of the two different strains in a mixed cell sample agglutinate together at random after the addition of HVJ at 4°C. Under the microscope it was observed that red blood cells from man, mouse and fowl agglutinate at random with Ehrlich ascites tumor cells or KB cells on addition of HVJ. This observation stimulated us to carry out experiments on giant cell formation between different lines of cells induced by HVJ.

Multinucleated giant cell formation between cells of the different strains of mouse ascites tumor has been described (OKADA, 1961). In the present work, the distribution of demonstrable fusion of cells of different strains to form multinucleated giant cells was examined.

MATERIALS AND METHODS

Virus

The egg-adapted Z strain of HVJ was used. The virus was propagated in the allantoic sacs of 10 day old chick embryos, using a diluted inoculum. The allantoic fluid was centrifuged at $1,400 \times g$ for 20 minutes. Then the supernatant was centrifuged at $34,850 \times g$ for 30 minutes. The sediment was suspended in modified Hanks' solution and was recentrifuged at $1,400 \times g$ for 20 minites to eliminate aggregates.

Hemagglutination (HA) titration

Salk's pattern method, employing 0.5 ml of 0.5 per cent fowl red blood cells, was used with phosphate-buffered saline as the diluent. Under these conditions, one HA unit corresponds to about 2.4×10^7 virus particles (OKADA *et al.*, 1961).

Modified Hanks' solution

Hanks' balanced salt solution, without glucose or bicarbonate, was used throughout the work on cell fusion. The pH was adjusted to 7.6 with tris (hydroxmethyl) aminomethane-HCl buffer (0.01 M).

Ehrlich ascites tumor cells (ETC)

Ehrlich hyper-tetraploid ascites tumor cells were withdrawn from the peritoneal cavity of adult ddO strain mice inoculated 7 days previously with 0.1 ml of ten-fold diluted tumor-bearing ascites fluid. The cells were washed three or four times with modified Hanks' solution to remove contaminating red blood cells and leucocytes and were suspended in the same solution at a concentration of about 1×10^7 cells/ml.

Tissue culture cells

KB cells, HeLa-S3 cells and FL cells from man, L cells from mouse and PS cells from pig were grown in YLH medium containing 20 per cent bovine serum at 37°C. For mouse embryo fibroblasts, 18 day old embryos were trypsinized and cultured in YLH medium containing 10 per cent bovine serum for one week at 37°C, as reported previously (OKADA and TADOKORO, 1963). To distinguish one of the cell lines in experiments on giant cell formation between cells of two different strains, monolayer-cultured cells of some lines were heavily labeled with ^{3}H-thymidine by incubation in bovine serum-YLH medium containing 1 to 5 μC/ml of ^{3}H-thymidine for 72 hours at 37°C. These monolayer-cultured cells were washed three times with phosphate-buffered saline and treated with 0.25 per cent of trypsin solution in PBS at 37°C for the minimum time require for exfoliation of the cells from the glass wall. The cells collected from the bottles were filtered through a platinum grid of 80 mesh, washed three or four times with Hanks' solution and then filtered through a platinum grid of 150 mesh. These cells were suspended in the Hanks' solution at a concentration of about 1×10^7 cells/ml.

Determination of the degree of giant cell formation between cells of different strains

One ml of a cell sample containing ^{3}H-thymidine labeled cells of one strain, non-labeled cells of another strain and 0.5 ml of HVJ sample were mixed in a test tube. After keeping the tube at 4°C for 10 minutes to allow cell agglutination, it was incubated in a water bath at 37°C for 60 minutes with shaking. During the incubation the cells in the clumps fused together or became dispersed in the medium; no cell clumps remained at the end of the incubation.

One part of the sample was used for determination of the cell fusion index using the following fomula (OKADA and TADOKORO, 1962):

$$\text{Fusion Index} = \frac{\text{cell number in non-virus control tube}}{\text{cell number in test tube}} - 1.0$$

And another part of the sample was smeared on a slide glass, dried and fixed with methanol. Then,

it was treated with 5 per cent PCA (perchloric acid) for 16 hours at 10°C to elute the RNA. DNA was scarcely liberated by this treatment. In some case RNAase treatment was used instead of PCA treatment. When the RNA was not eluted, the β-rays from ^3H were absorbed with RNA. The treated sample was dipped in autoradiographic emulsion (Kodak NTB-2 or Konishi-Roku NR-MI), exposed for 7 days in a dark cold room and then developed and stained with giemsa. Five hundreds to one thousand giant cells with between two and seven nuclei were counted on each plate and the numbers of labeled and non-labeled nuclei in each giant cell were determined. The frequency distribution of labeled nucleus-members appearing in the nuclei of the giant cells was calculated and compared with the theoretical values for (i) cells of the two strains fusing independently, and (ii) cells of the two strains fusing together as readily as homologous cells. The theoretical value was calculated from the following formula,

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

n: total number of nuclei in the giant cell,
r: number of labeled nuclei in the giant cell,
P: ratio of number of labeled cells to total number of cells in the initial cell sample.

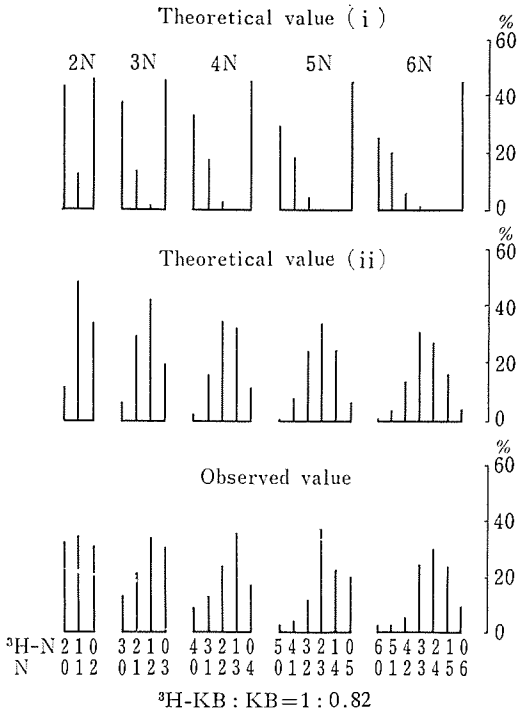


FIGURE 1 Distribution of giant cells with labeled and non-labeled nuclei on fusion of KB cells induced by HVJ. The starting cell sample containing KB cells labeled with ^3H-thymidine (88.6 per cent of the cells had been labeled) and non-labeled KB cells in the ratio of 1 : 0.82. This Fig. shows the data observed when 320 HAU/ml of HVJ was added. Theoretical value (i) indicates the case, when these cells fuse independently; theoretical value (ii) indicates the case when these cells fuse together.

RESULTS

Multinucleated giant cell formation between cells of human origin

As a control, labeled KB cells were mixed with non-labeled KB cells and fusion was induced by HVJ. Fig. 1 shows that the ob-

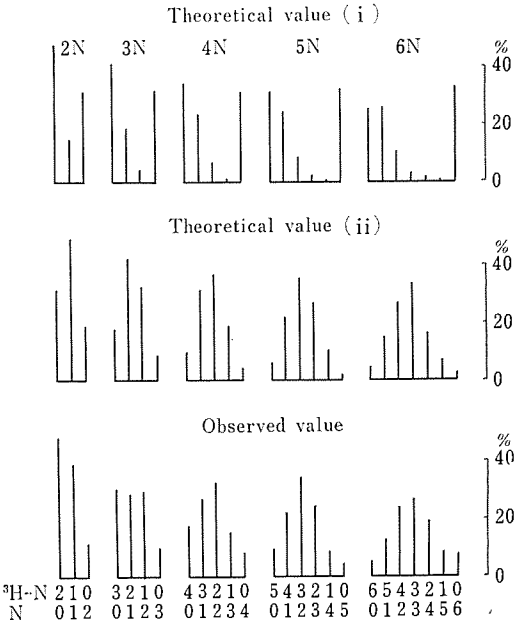


FIGURE 2 Distribution of giant cells with labeled and non-labeled nuclei on fusion of KB and HeLa cells induced by HVJ. The ratio of KB cells to HeLa cells in the starting sample was 2.1 : 1, and 85.6 per cent of the KB cells had been labeled with ^3H-thymidine. This Fig. shows the results observed 320 HAU/ml of HVJ were used.

served frequency distribution of labeled nucleus-members in giant cells agreed well with the theoretical value for (ii) that these cells fused together and was very different from the theoretical value for (i) that these cells did not fuse together. This shows that the method is useful for analysis of giant cell formation between cells of different strains.

In the next experiment, labeled KB cells were mixed with non-labeled HeLa cells and fusion was induced by HVJ. As shown in Fig. 2, KB cells fused as readily with HeLa cells as with homologous cells. The values found for giant cells with two and three nuclei varied from the theoretical values for these cells fusing together, because the initial sample was contaminated with giant cells with two and three nuclei. KB cells also fused well with FL cells.

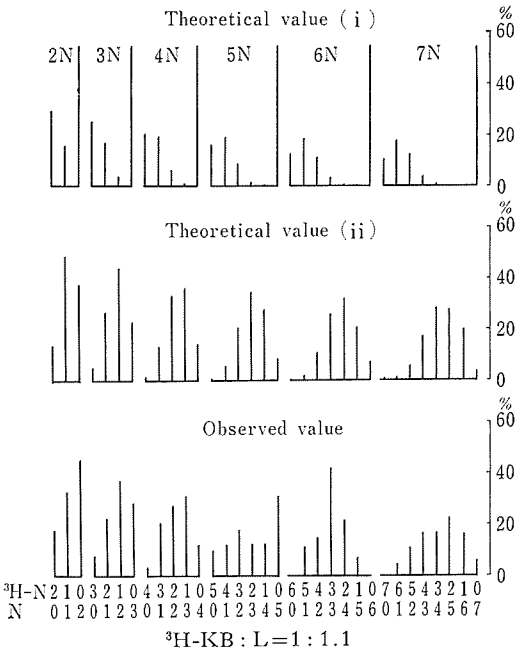


FIGURE 3 Distribution of giant cells with labeled and non-labeled nuclei on fusion of KB and L cells induced by 320 HAU/ml of HVJ. The ratio of KB cells to L cells in the starting sample was 1 : 1.1, and 80.8 per cent of the KB cells had been labeled with ^{3}H-thymidine.

Multinucleated giant cell formation between cells from different animals

^{3}H-labeled KB cells and non-labeled L cells were mixed and fusion was induced by HVJ. The distribution of labeled nucleus-members in the giant cells agreed well with the theoretical value for (ii) that these cells fused together, as indicated in Fig. 3. KB cells also fused well with PS cells, as shown in Fig. 4.

These results show that the cells of the established culture strains have a kind of " homologous " surface structure. It was thought that their loss of specificity was due to long-term-culture *in vitro*. This suggested the further test of whether the cells of the tissue culture lines could fuse with mouse ascites tumor cells which has been serially passaged *in vivo*. ^{3}H-labeled KB cells were mixed with non-labeled Ehrlich ascites tumor cells (ETC) and fusion

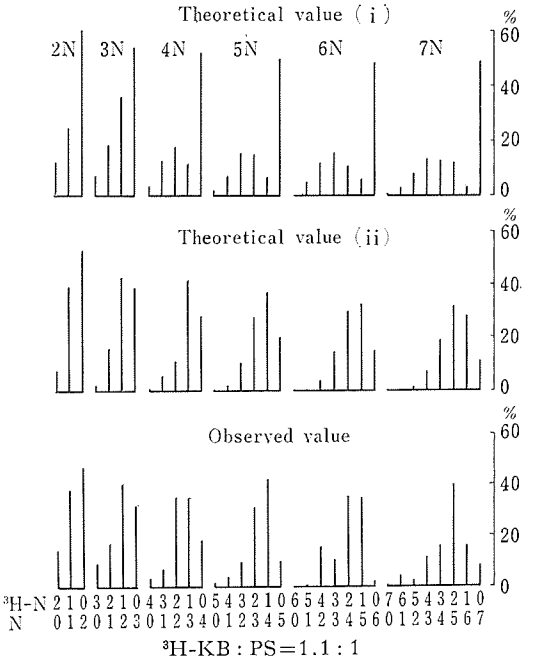


FIGURE 4 Distribution of giant cells with labeled and non-labeled nuclei on fusion of KB and PS cells induced by 640 HAU/ml of HVJ. The ratio of KB cells to PS cells in the starting sample was 1.1 : 1, and 51.0 per cent of the KB cells had been labeled with ^{3}H-thymidine.

was induced by HVJ. In this case, KB cells also fused with ETC. However, when a low titer of HVJ (e.g. 400 HAU/ml) was used, labeled nuclei were predominant in all giant cells. On increasing the HVJ titer, fusion between these cells, as well as between tissue culture line cells, occurred more readily, as shown in Fig. 5. This phenomenon could be attributed to the fact that ETC requires more HVJ than KB cells for cell fusion (OKADA and TADOKORO, 1963). In Fig. 6, the effect of the input virus titer on the fusion indices of ^{3}H-labeled KB cells, non-labeled ETC and a mixture of these cells is shown. This mixed cell sample and the sample of giant cells induced by 1,600 HAU/ml of HVJ from this mixture were analyzed and the results are indicated in Fig. 5.

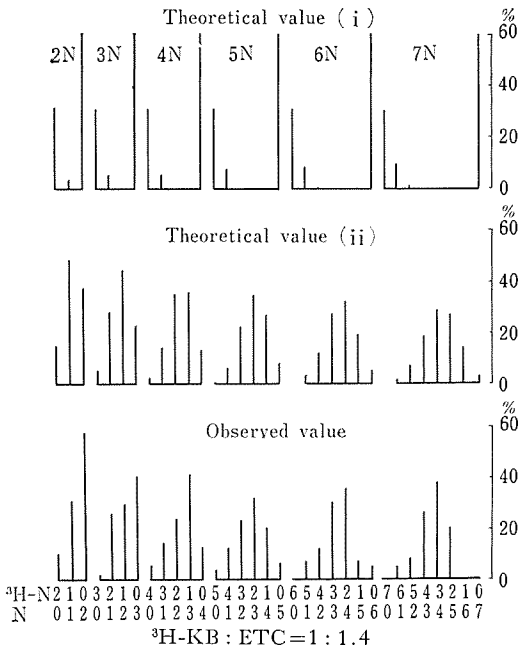


FIGURE 5 Distribution of giant cells with labeled and non-labeled nuclei on fusion of KB cells and Ehrlich ascites tumor cells induced by HVJ. The ratio of KB cells to Ehrlich ascites tumor cells in the starting sample was 1 : 1.4 and 96.0 per cent of KB cells had been labeled with ^{3}H-thymidine. This Fig. shows the results observed when 1,600 HAU/ml of HVJ was added.

Fusion between cells with a high and a very low cell fusion capacities

Mouse embryo fibroblasts (MEF) were derived from 18-day-old embryos and cultured on monolayers for one week. During this period the MEF were labeled with ^{3}H-thymidine. About 74 per cent of the cells were heavily labeled. The MEF were mixed with non-labeled Ehrlich ascites tumor cells (ETC) and fusion was induced by HVJ. The results observed are shown in Fig. 7. MEF and ETC, both of mouse origin, scarcely fused together, while most of the giant cells were composed of ETC only and almost all the MEF remained as single cells, although a few

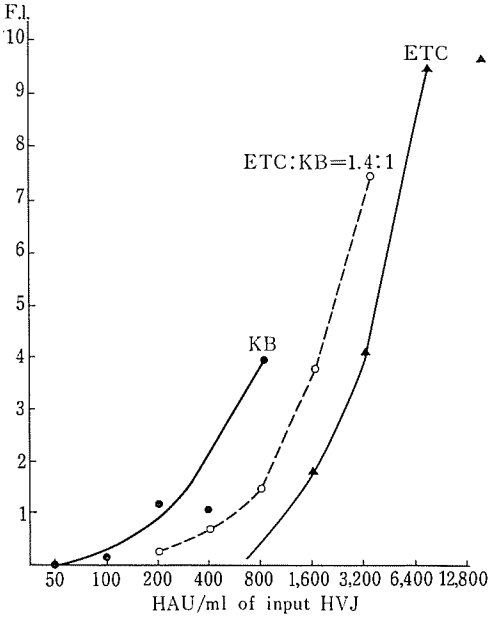


FIGURE 6 Comparison of fusion capacities of ^{3}H-labeled KB cells, non-labeled Ehrlich ascites tumor cells and a mixture of the two. The cell samples used were the same as for Fig. 5.

giant cells containing many ETC-nuclei and one or two MEF-nuclei were observed in fusion. When HVJ was added to a mixture of MEF and ETC at 4°C, much cell agglutina-

tion occurred and no free cells remained, and the MEF clumped together with ETC. However, during the cell fusion reaction at 37°C almost all the MEF were excluded from the cell fusion process. MEF did not fuse with KB cells either, as indicated in Fig. 8.

These results support the conclusion that MEF are a type of cell with a very low cell fusion capacity (OKADA and TADOKORO, 1963).

Metabolic patterns of fused giant cells

As indicated above, the formation of giant cells from cells of different kinds of animals is readily induced by HVJ, when cells having high cell fusion capacities were used. It is of interest to compare the metabolic patterns of these giant cells with patterns of giant cells formed from homologous cells.

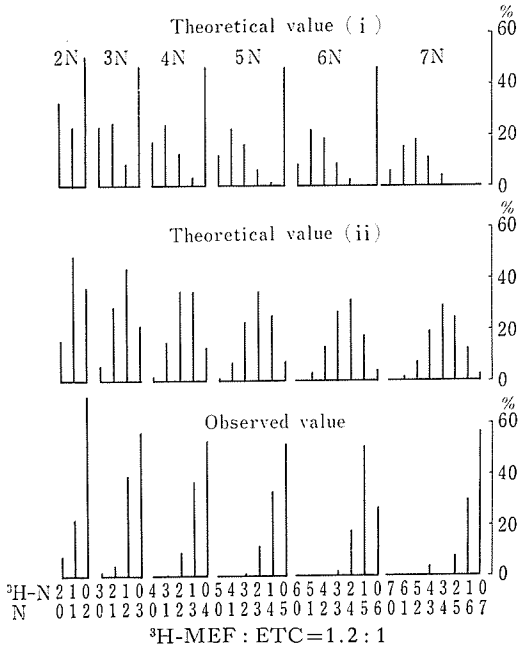


FIGURE 7 Distribution of giant cells with labeled and non-labeled nuclei on fusion in a mixture of mouse embryo fibroblasts and Ehrlich ascites tumor cells. The ratio of MEF to ETC was 1.2 : 1, and 73.2 per cent of MEF had been labeled with ^{3}H-thymidine. This Fig. shows the results obtained when 2,560 HAU/ml of HVJ was used.

However, there are certain difficulties in carring out such experiments. Some HVJ particles adsorbed onto the cells would enter the cells during the cell fusion reaction at 37°C, and the viral RNA would be uncoated in the cytoplasm and affect the control mechanism of the cells. To prevent this affect of viral RNA, the active substance of HVJ causing cell fusion should be separated from the viral RNA, but this has not yet been achieved. Therefore, the infectivity of the HVJ used for the cell fusion reaction in this experiment, was completely destroyed by UV irradiation so that the biological activities of the viral RNA were minimized. KB cell fusion was induced by this HVJ sample. The fused cells were washed with medium containing anti-HVJ

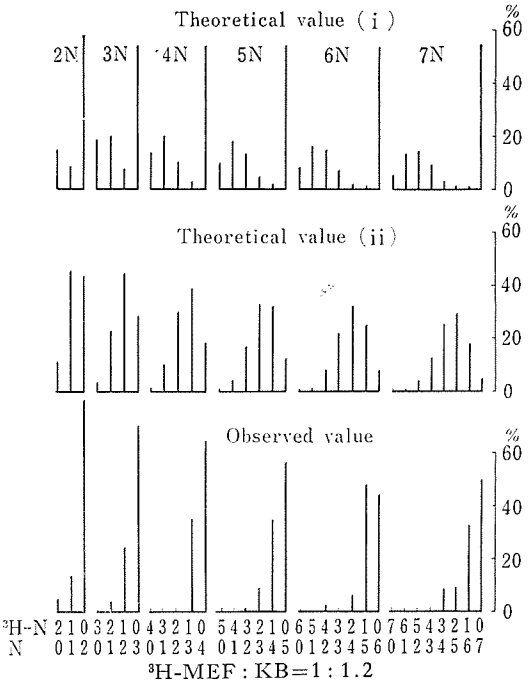


FIGURE 8 Distribution of giant cells with labeled and non-labeled nuclei on fusion of a mixture of mouse embryo fibroblasts and KB cells. The ratio of MEF to KB cells was 1 : 1.2, and 73.2 per cent of MEF had been labeled with ^{3}H-thymidine. This Fig. shows the results obtained when 1,280 HAU/ml of HVJ was used.

serum and then resuspended in YLH medium containing 1 μC/ml of ^{3}H-thymidine or ^{3}H-uridine and incubated for 60 minutes in a water bath at 37°C with shaking. After washing with Hanks' solution, the sample was smeared on a slide, dried and fixed with methanol. When ^{3}H-thymidine was used, the sample was treated with 5 per cent PCA for 16 hours at 10°C; when ^{3}H-uridine was used, the sample was treated with 5 per cent PCA for 10 minutes at 4°C. Then, autoradiography was carried out. As a control, KB cells alone (without HVJ) were treated as above. The number of grains in each nucleus in the giant cells was counted and compared with that of the control sample. As indicated in Table 1, the synthesis of DNA in giant cells was not affected by fusion. The synthesis of RNA in the nucleus was also not affected (Fig. 14). This shows that the membrane structure of the giant cells was not damaged by fusion and culture of the fused cells may be possible.

The metabolic patterns of the giant cells formed from cells from different animals should be compared with those of giant cells formed from homologous cells.

TABLE 1 *Comparison of the grain numbers in the nuclei of giant cells formed from KB cells with those in the nuclei of control cells*

Grain No. per nucleus	0~	20~	100~	total nuclei No. observed
Nuclei number in control sample (without HVJ)	277 (78.1%)	8 (2.3%)	70 (19.7%)	355 (100%)
Nuclei number in test sample (fused cell)	370 (75.6%)	18 (3.7%)	102 (20.7%)	490 (100%)

KB cells were fused by UV-irradiated HVJ, washed with anti-HVJ serum and then resuspended in YLH medium containing 1 μC/ml of ^{3}H-thymidine and incubated for 60 minutes at 37°C with shaking. The smear sample was autoradiographed after treatment with PCA. As a control KB cells alone (without HVJ) were treated as above.

DISCUSSION

From the above results, it is concluded that giant cell formation by fusion between different kinds of cells occurs well between cell strains with high cell fusion capacities, and this kind of cell fusion was not affected by the species of animals from which the cells originated. As reported by Coombs *et al.* (1961), the cells of established tissue culture strains maintain the species specificity of the original animals and their specificity is detectable immunologically in the cell surface. Nevertheless, our results indicate that the cell membrane of cells with high cell fusion capacities can connect in a way which maintains functional structure with the membranes of cells from different animals as well as with those of homologous cells. It seems that the membrane structure of these cells loses some kind of specificity. Cells with high cell fusion capacities may be malignant cells or long-term-cultured undifferentiated cells, while short-term-cultured or mature somatic cells have a very low or no cell fusion capacity (OKADA and TADOKORO, 1963).

Recently, the authors observed that in the MEF-HVJ system, reaction steps (I), (II) and (III) of fusion can be demonstrated; HVJ particles were adsorbed onto MEF and the MEF agglutinated together at 4°C, during incubation at 37°C the cell surface was disconnected by HVJ and then reconnected. Nevertheless, the cells were scarcely induced to fuse by HVJ. It seems that the cell surface disconnected by HVJ only reconnects at its own disconnection sites. In the case of cells with high cell fusion capacities, the reconnection occurs at disconnection sites on neighbouring cell as well as at its own disconnection

sites. Hence, it seems that there is a kind of specificity of cell surface structure in cells showing a very low, or no cell fusion capacity, but that this specificity disappears in malignant cells or undifferentiated cells of established tissue culture strains and a new homologous structure appears which is not affected by the species of animal from which the cells originate.

As indicated in this report, synthesis of DNA or RNA of giant cells from KB cells were demonstrable as well as those of the original ones. MAENO and MATSUMOTO (1964) reported that HeLa cells infected with HVJ were capable of mitosis as in the case of non-infected cells, while permitting the synthesis of the virus antigen; the HeLa cells became persistently infected with HVJ. From these results, it is considered that long-term-culturing of fused giant cells would be possible. MAENO and MATSUMOTO also indicated that the HeLa cells infected with HVJ aquired immunity against re-infection of HVJ, while these cells showed higher susceptibility to infection and synthesis of another kinds of animal viruses than that of normal HeLa cells. It shows that conditions of the HeLa cells are influenced by the viral RNA. In this paper, UV irradiated HVJ was used but it does not clear whether biological activities of HVJ-RNA against the host cells were completely inactivated by this treatment. Analyses of the metabolic patterns and the control mechanisms of giant cells formed by fusion between cells of different animals should become a new experimental field in biological science. However, if this field is to develop greatly, the substance with cell fusion activity should at least be separated from viral RNA.

ACKNOWLEDGEMENTS

The authors wish to express their thanks to Dr. K. FUKAI for his kind guidance and encouragement. Thanks are also expressed to Dr. J. TADOKORO for his advice and for supplying tissue culture cells.

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OKADA, Y. and MURAYAMA, F. unpublished data.

ADDENDUM

Recently, H. HARRIS and J. F. WATKINS reported data on this subject in a paper entitled "Hybrid cells derived from mouse and man: Artifical heterokaryons of mammalian cells from different species" in *Nature*, **205**, 640–646, 1965.

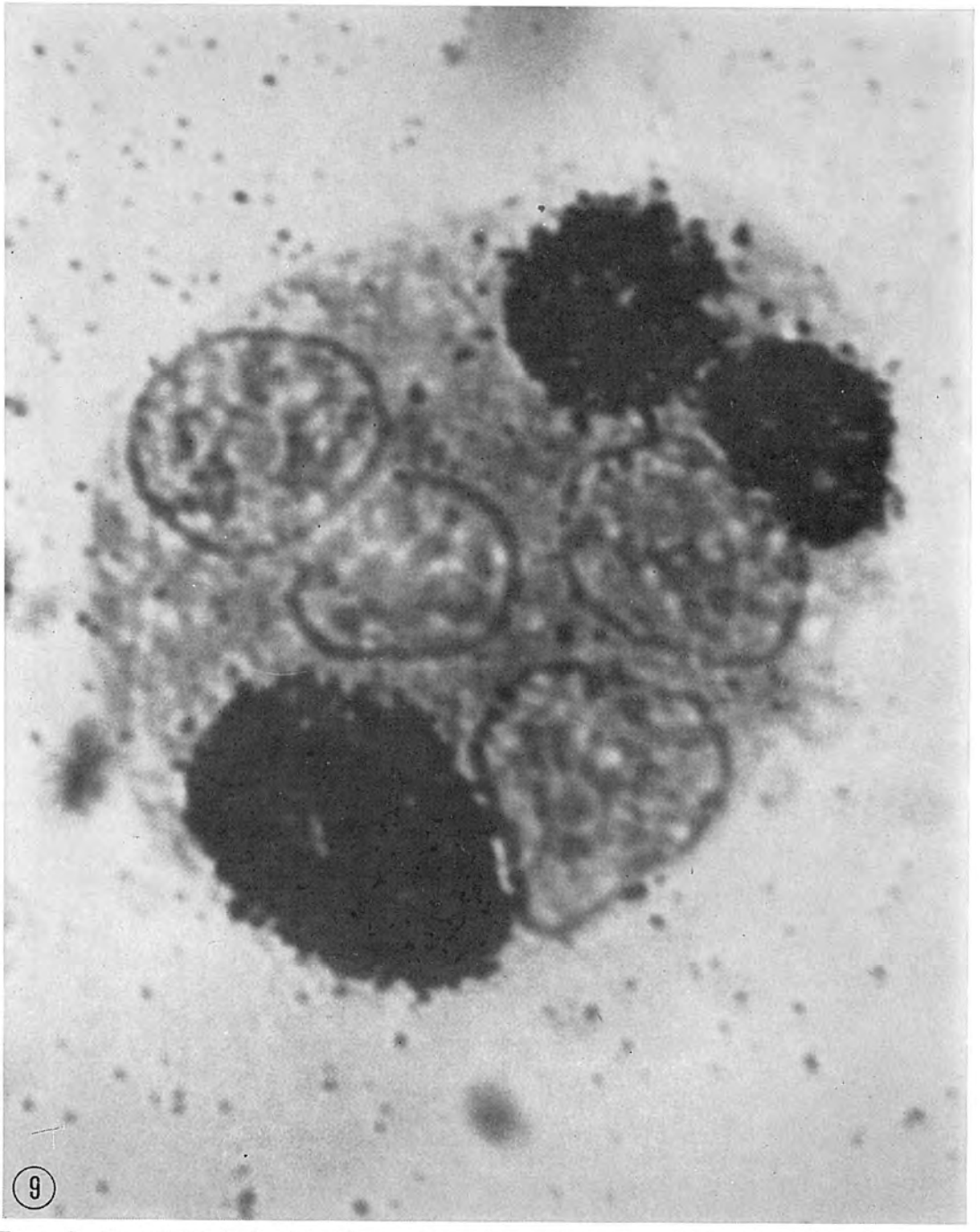
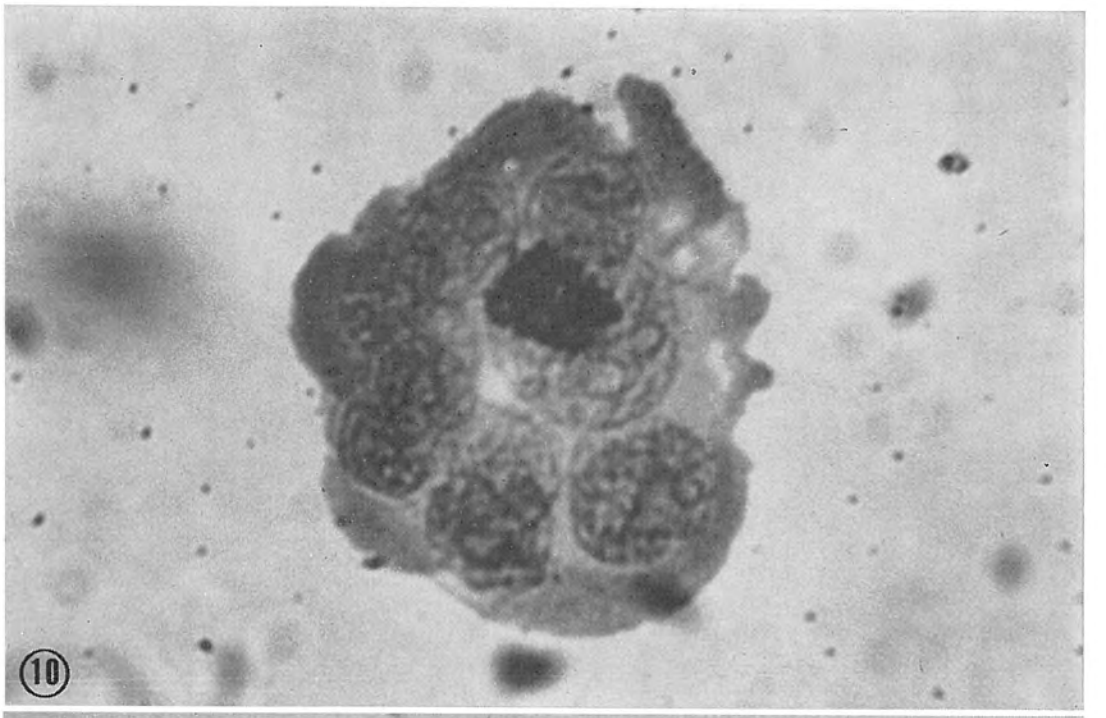


FIGURE 9 Autoradiograph of a giant cell appearing in a fused cell sample containing labeled KB cells and non-labeled KB cells. Three labeled nuclei and four non-labeld niclei are seen.

FIGURE 10 Autoradiograph of a giant cell appearing in a fused cell sample containing labeled mouse embryo fibroblasts and non-labeled Ehrlich ascites tumor cells. One labeled nuclei and eleven non-labeled nuclei are seen

FIGURE 11 Autoradiograph of a giant cell appearing in a fused cell sample containing labeled mouse embryo fibroblasts and non-labeled KB cells. One labeled nuclei and nine non-labeled nuclei are seen.


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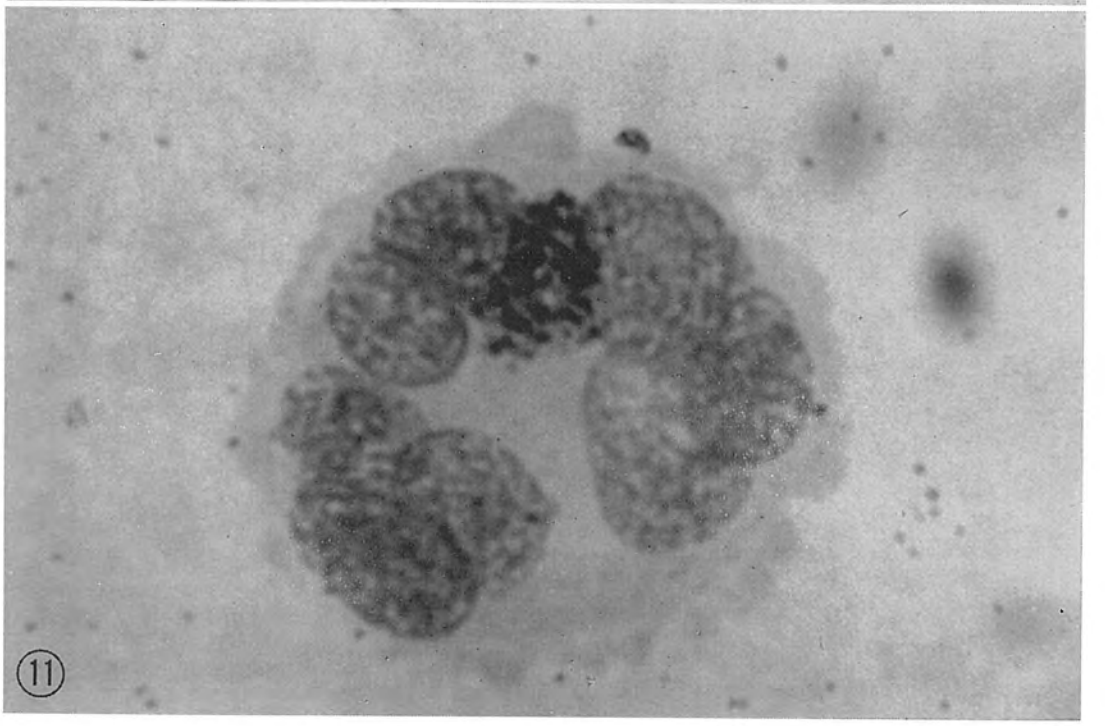
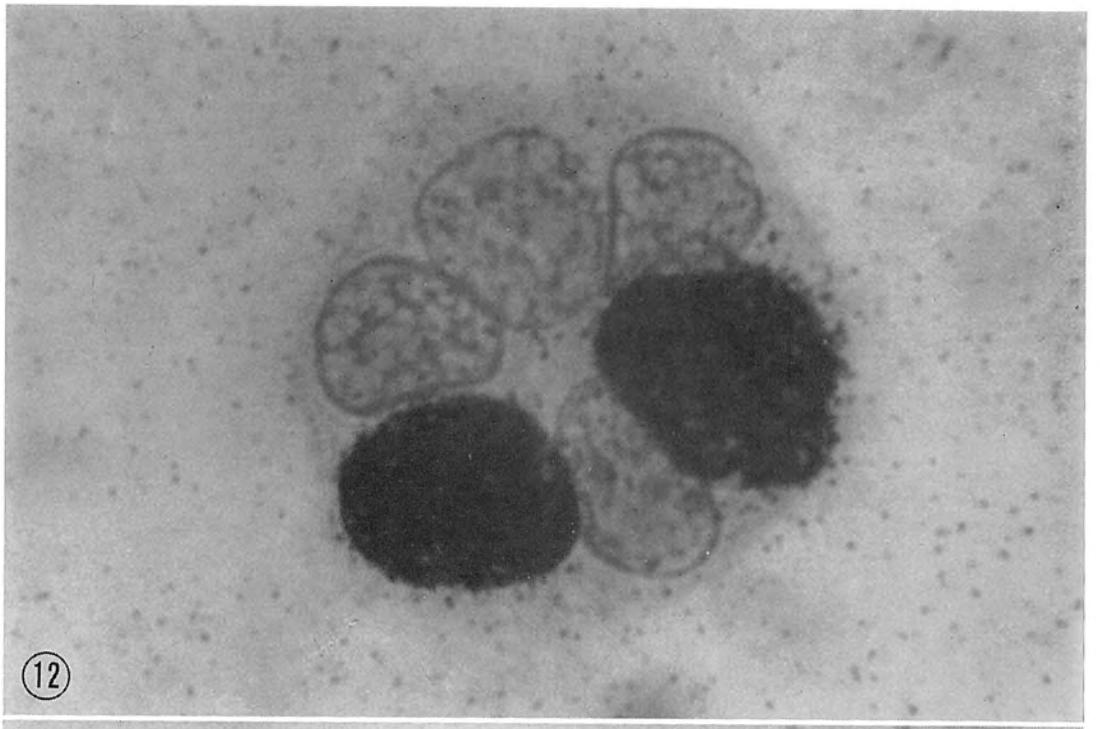
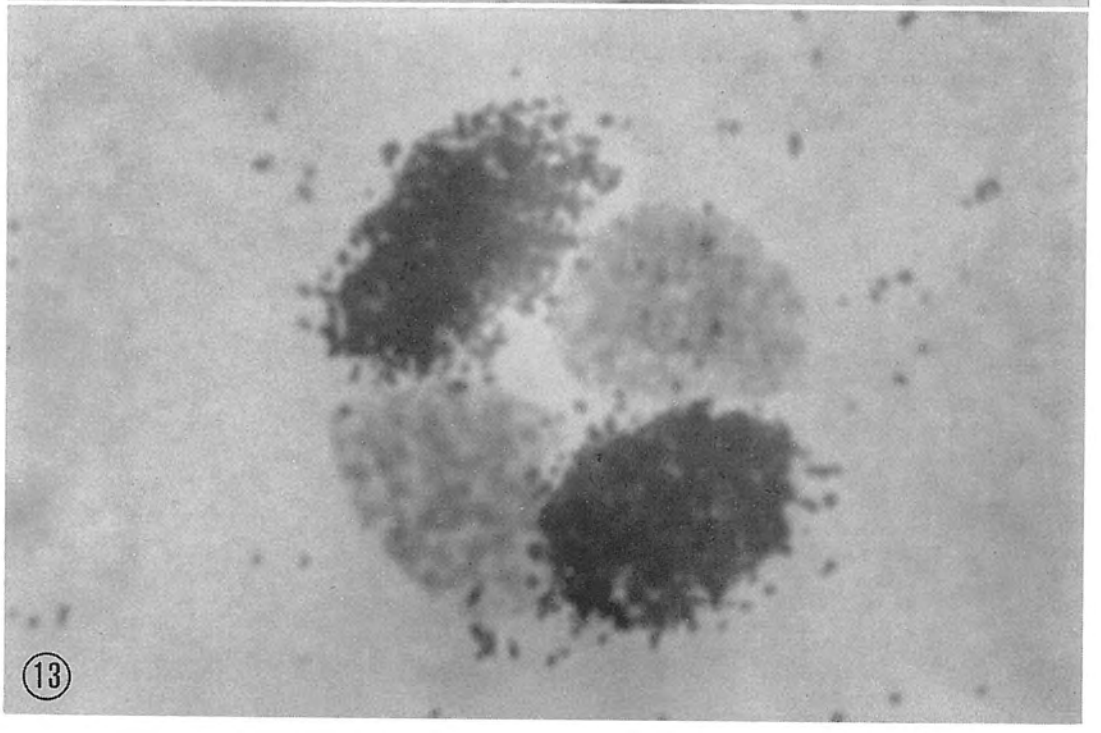

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FIGURE 12 Autoradiograph of a giant cell appearing in a fused cell sample containing labeled KB cells and non-labeled PS cells. Two labeled nuclei and four non-labeled nuclei are seen.

FIGURE 13 Autoradiograph of a giant cell appearing in a fused cell sample containing labeled KB cells and non-labeled Ehrlich ascites tumor cells. Two labeled nuclei and two non-labeled nuclei are seen.



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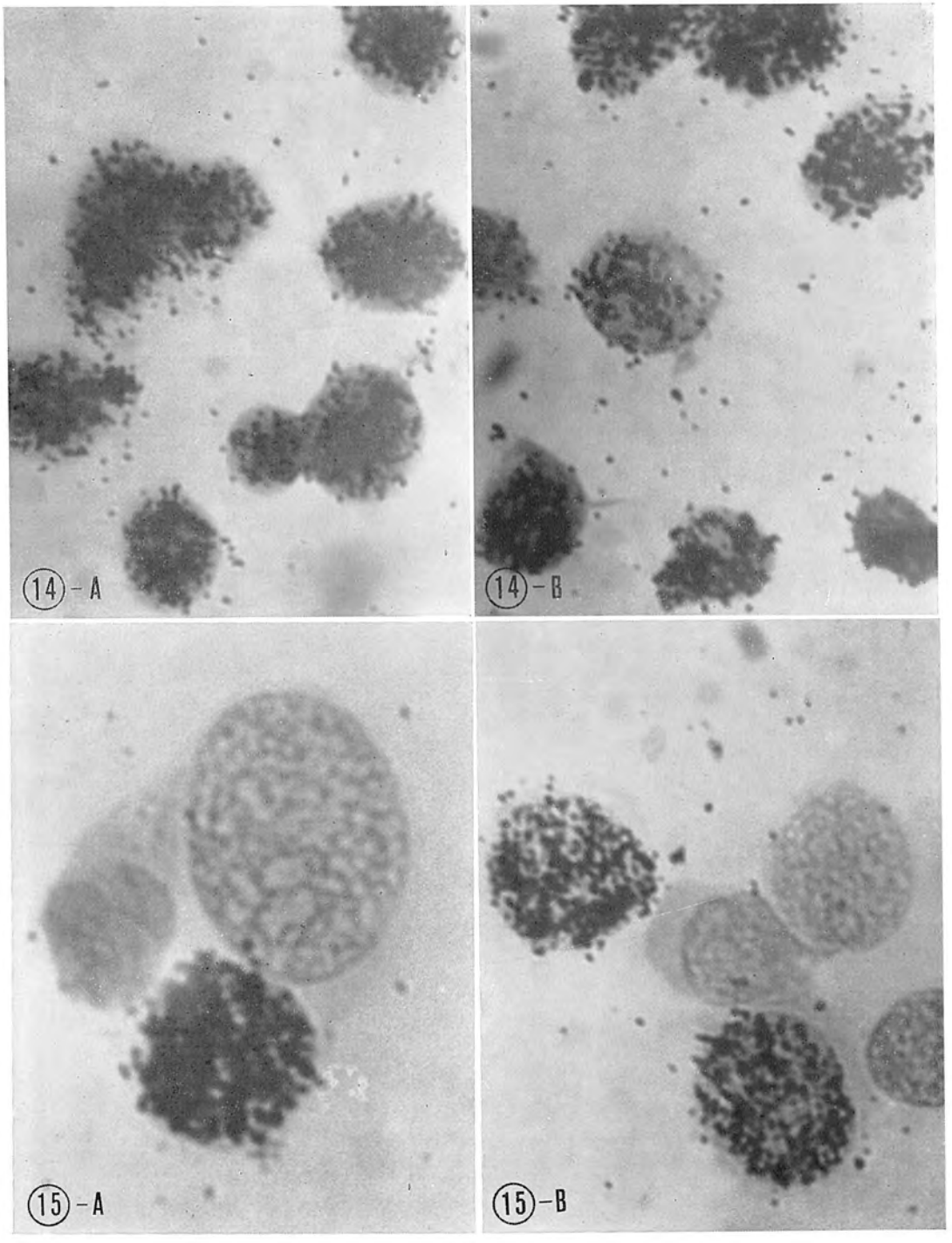
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FIGURE 14 Autoradiographs showing RNA synthesis of nuclei of giant cells from KB cells (A) and of normal KB cells (B). KB cells were fused by UV-irradiated HVJ, washed with anti-HVJ serum and fused giant cells alone were prepared from this sample. The giant cells were resuspended in YLH medium containing 1 μC/ml of ^{3}H-uridine and incubated for 60 minutes at 37°C with shaking. After washing, the sample was homogenized by a teflon homogenizer and free nuclei were prepared and autoradiographed, due to free from absorption of β-rays from ^{3}H with substances of cytoplasm. As a control KB cells alone (without HVJ) were treated as above.

FIGURE 15 Autoradiographs showing DNA synthesis of a giant cell formed from three KB cells (A), and normal KB cells (B), corresponding to Table 1.

A : In this giant cell, one nucleus is in a stage synthesyzing DNA (S-phase) and two nuclei are in G-phase.

B : Two cells are in S-phase and three cells are in G-phase.


14-A
14-B
15-A
15-B