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Cytopathic Changes Induced by Herpes Simplex Virus* **

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SUMMARY

Cytopathic changes induced by three substrains of the Miyama strain of herpes simplex virus are described. Morphological changes induced in cells are controlled genetically by these strains. In FL cells infected with strain —GCf, agglutination and fusiform development were observed as the characteristic changes induced by this variant. With strains —GCf and —GCr, FL cells were infected and the number of cells in replicate cultures were counted at various times after virus inoculation. Proliferative changes were not induced by infection with either strain. L cells infected with a high virus titer of strain +GC showed a very early appearance of giant cells. The formation of giant cells on Earle's L cells was dependent on the concentration of input virus. +GC virus partially inactivated with ultraviolet light also induced remarkable effects on L cells and under some experimental conditions giant cells not accompanied by intranuclear inclusion bodies appeared. These indicated that giant cells were formed by the mass effect of the virus suspension of strain +GC on the cells and not on intracellular virus growth.

From the above data it is deduced that these different types of transformation of cells by different strains are mainly due to the primary interaction of some agents in the virus particles of these strains on the cells.

INTRODUCTION

The isolation of strains of herpes virus which cause different cytopathic effects on a variety of host cells was described by McNair-Scott *et al* in 1959. Similar results were presented by Hoggan *et al.* (1959) and by us (Nii and Kamahora, 1961a). A biological comparison between the strains has been reported in detail by McNair-Scott *et al.* (1961). Essentially the same phenomenon has recently been reported by Falke (1961), who isolated two variants, fR and rR, from a strain of herpes B virus.

In the previus report we described three substrains of the Miyama strain of herpes simplex virus, which were distinguishable from each other by their effects

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on FL cells; one produces giant cells, another causes rounding of infected cells and the third causes cells to become fusiform. These strains were designated as strains +GC, —GCr and —GCf respectively (Nii, 1961b).

This report describes further investigations on these substrains. The difference in the cytopathic changes caused by each, and the conditions necessary for giant cell formation by herpes virus on monolayer cultures were studied.

MATERIALS AND METHODS

1. *Virus*

The Miyama strain of herpes simplex virus was used throughout this series of experiments. It had been isolated from the vesicular fluid of a patient with *herpes labialis* and three substrains were obtained (Nii *et al.*, 1961a, Nii, 1961b). Each was serially passaged on FL cells. The supernatant fluid of the infected culture of each substrain reached a titer of about 10^8 $TCID_{50}$ or more per ml. As viral sample, these tissue culture fluids were used after centrifugation at 2,500 rpm for 10 minutes.

2. *Tissue culture cells*

FL cells and Earle's L cells were used. The former was from the National Institute of Health, Japan, and the latter line, from the Institute for Infectious Diseases, Tokyo University. Each was subcultured in this Institute using 200 ml and 50 ml prescription bottles.

3. *Culture media*

The L cell strain of mouse fibroblasts was grown in a medium consisting of 95 parts of Hanks' balanced salt solution containing 0.1 per cent Yeast Extract and 0.5 per cent lactalbumin hydrolyzate and 5 parts of bovine serum. The growth medium for FL cells was composed of 85 parts of Earle's saline containing 0.5 per cent lactalbumin hydrolyzate (L-E solution) and 15 parts of bovine serum.

4. *Preparation of virus for UV (ultraviolet) inactivation*

FL cell monolayers containing about 10^7 cells were inoculated with 1-2 ml of virus suspension. After a one hour adsorption period at 37°C, the inoculum was removed. Then the monolayers were washed three times with 5 ml of L-E solution not containing phenol red. Eight ml of fresh L-E solution without phenol red were introduced onto each monolayer. When the cytopathic changes were maximal, the infected cells were detached from the glass wall of the culture bottle by spraying the wall vigorously with the culture fluid by pipette and cell suspensions were made. They were freeze-thawed rapidly six times using a dry ice-acetone mixture and warm water. Disrupted cell suspensions were centrifuged at 4,500 g for 20 minutes using a refrigerated centrifuge at 0°C. The supernatant fluid was used for ultraviolet inactivation of the virus.

5. *Ultraviolet (UV) inactivation*

A 15W germicidal lamp fitted with a reflector (Toshiba Electrics) was employed for inactivation. Two ml of the virus sample in a petri dish (87 mm I. D.) were irradiated at a distance of 21 cm with continuous shaking.

6. *Infectivity titrations*

Infectivity of FL cells was titrated by the 50 per cent endpoint method by tube titration ($TCID_{50}$) as described in the preceding report (Nii *et al.*, 1961a).

7. *Cell counting*

The nuclei were counted in a haemocytometer. The method for preparations of cell nuclei was in principle similar to that reported by Katsuta (1955).

8. *Morphological observations*

The method described in the preceding report (Nii *et al.*, 1961a) was used.

RESULTS

1. *Cytopathic effects on FL cell monolayers induced by strain —GCf*

The method of isolation of strain —GCf from the parent virus population of the Miyama strain has already been reported (Nii, 1961b). In this article a detailed study on the cytopathic changes in FL cells infected with this variant will be described.

Replicate monolayer cultures in 50 ml bottles were prepared. Each bottle contained 3.5×10^6 cells (Fig. 1). These were infected with 2 ml of virus suspension of a titer of 2×10^8 $TCID_{50}$. The cultures were incubated at 37°C for 2 hours with occasional shaking. After the adsorption period, the inoculum was removed. Then monolayers were washed 5 times with 10 ml of Hanks' BSS and 5 ml of fresh medium were introduced onto each monolayer. The bottles were incubated again at 37°C. Two bottles were taken out at intervals to photograph the infected FL cells and to titrate viral infectivity.

During the course of infection, considerable cytopathic changes in the FL monolayers were observed. These were arbitrarily classified into three or four stages.

Stage I: At earlier stages of infection (8 hours) the cytoplasm of the cells is spread out on the glass and the cells looked flattened. Borders between neighbouring cells in the sheet were apparently lost, although syncytia were not formed (Fig. 2). These earlier changes induced by strain —GCf were compared with those resulting from infection with a high viral dose of strains +GC and —GCr. Cellular responses to strain +GC resembled those induced by strain —GCf only in the earliest stage of infection, when syncytia had not yet been formed in the sheet. Such cohesive changes of infected cells, however, were not apparent in experiments using strain —GCr. Fig. 3 shows FL cells 6 hours after infection with a high titer of strain —GCr. Almost all the cells looked rounded and separate without signs of cohesion.

Stage II: Twelve hours after infection the cells became slightly rounded and the border of each cell appeared to be clear, but the cytopathic changes at this stage were not specific for strain —GCf. (Fig. 4)

Stage III: After about 20 hours many cells looked fusiform and some rounded cells had agglutinated with one another although giant cells had not formed. As infection proceeded, these fusiform cells became more elongated. The appearance of such fusiform shape cells is one of the most conspicuous changes induced by strain —GCf. (Fig. 5)

Stage IV: After about 70 hours rounded cells which were pyknotic increased

in number and they were present together with long fusiform cells. (Fig. 6) These shrunken round cells seemed to become detached from the glass wall and dispersed in the liquid phase of the culture. Even in the latest stage of infection the conspicuous pattern of cellular morphology, *i.e.* the fusiform shape of the infected cells, remained in the culture.

These experiments indicated that the characteristic changes produced on FL monolayers by strain —GCf were agglutination and fusiform development of infected cells. These changes have consistently been observed on serial passage of this variant on FL cells ever since its isolation. For a comparative study of the cellular responses to three substrains of the Miyama strain of herpes simplex virus, typical changes produced by the other two strains, *i.e.* strains +GC and —GCf are shown in Figs. 7 and 8.

Strain —GCf as well as the other two strains grow rapidly on FL cells. In the above experiment the titer of the culture fluid reached 2×10^8 $TCID_{50}$ per ml 40 hours after infection.

In preparations stained with haematoxylin and eosin, intranuclear inclusion bodies with an oval form were observed in the fusiform cells and sometimes they were very long along the long axis of the cells. (Fig. 9) The shape of the inclusion bodies may be more or less influenced by the cellular morphology.

2. *Response of FL cell to strains —GCf and —GCr*

Gray *et al.* differentiated three types of cellular responses to a newly isolated herpes simplex virus *in vitro*. Proliferation of cells exposed to virus is an interesting problem.

Experiments were performed to study the FL cell response to substrains of the Miyama strain and to see whether there was a correlation between the types of cellular responses reported by Gray *et al.*, *e.g.* proliferation or non proliferation and the cytopathic effects revealed by strain —GCf or —GCr.

Replicate monolayer cultures were prepared in 50 ml bottles. Each contained 4.9×10^6 cells. These were infected with 2 ml of virus suspension of strain —GCf or —GCr of variable viral titers. The cultures were incubated at 37°C for 2 hours. After the adsorption period, the inoculum was removed and 5 ml of fresh medium was introduced onto each monolayer.

Cultures were reincubated at 37°C. At intervals after infection cultures were removed and the number of nuclei remaining attached to the wall of the bottle was counted. It was noticed that nuclei changed in appearance as infection proceeded and in the later stages they became pyknotic. Dead cells with pyknotic nuclei may have been counted, especially in later stages, but cellular viability was not strictly considered in the experiment.

The results are shown in Fig. 10. Neither substrains —GCr or —GCf caused cellular proliferation. Higher concentrations of virus in the inoculum did no

cause proliferation of FL cells.

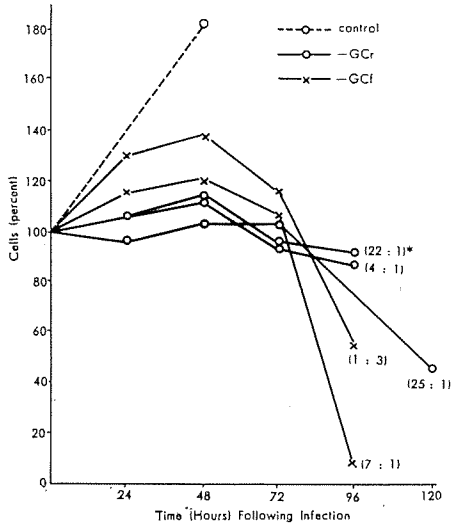

Fig. 10 FL Cell Response to Infection with the Miyama Strain of Herpes Simlex Virus

* Input virus : number of cell

Control cells increased in number rapidly by a factor of 1.8 in 48 hours. From this experiment it was shown that strain −GCf induced earlier destructive effects on FL cells than did strain −GCr and this phenomenon was constantly observed in other experiments.

3. *Cytopathic changes induced by strain +GC*

Syncytium formation of FL cells, that is a characteristic change induced by strain +GC, has been constantly observed since isolation of this strain from the parent virus population. (Fig. 7) The response of Earle's L cells to the Miyama strain of herpes simplex virus was presented in an earlier report and it was shown that the response of these cells to the Miyama strain differed a little from that of FL cells (Nii *et al.*, 1961a).

Experiments were repeated on L cells using strain +GC. A syncytium was not always formed by cells infected with the virus and it was indicated that a genetic marker revealed by strain +GC on FL cells could not be expressed well on L cells. From the data on the interactions of strain +GC and L cells, the following facts were recognized. When a high titer of strain +GC was inoculated onto an L cell monolayer, many giant cells were formed, although as large a syncytium as that seen in FL monolayer cultures was rarely observed and in the later stages of cytopathic changes, giant cells were not so clearly seen so that the cellular response was more like the cytopathic effects to type −GCr. In cells

infected with a low infective titer of virus, the first cytopathic change often appeared as formation of single giant cells surrounded by apparently normal cells and as infection proceeded the number of rounded cells with a single nucleus increased and the —GCr type of cytopathic effect appeared.

A series of experiments, as described below, were performed to see what regulates the formation of giant cells in L cell cultures.

L cells were prepared on 10×50 mm coverslips in Leiton tubes. Each contained about 5×10^5 cells. The cell sheets formed about 24 hours after planting were used, as the cytopathic changes in these cells infected with the Miyama strain of herpes virus were influenced more or less by the age of monolayer and the cells of older cultures showed milder responses to the virus.

a) *Time of appearance of giant cells after infection*

The purpose of this experiment was to find the relationship between the time of appearance of giant cells and the stage of intracellular virus growth. The latter was indicated by the development of intranuclear inclusion bodies.

Each monolayer on a coverslip in a tube was infected with 1 ml of virus suspension containing about 10^8 $TCID_{50}$ and incubated at 37°C. After a 2 hours adsorption period the medium of the inoculum was renewed. At appropriate intervals after infection samples were withdrawn and fixed.

As early as 2 hours after virus inoculation, that is, at the end of the adsorption period, a dramatic appearance of giant cells was seen. (Figs. 11, 12) At this stage of infection no nuclei in the sheet showed any sign of formation of eosinophilic masses. (Fig. 13)

To analyze this phenomenon more precisely and quantitatively, the number of giant cells and of nuclei contained in each of them were counted under the microscope. The results of two separate experiments are shown in Table 1. The percent of nuclei which participate in the formation of giant cells and the average number of nuclei in them are recognized as indices of the grade effected by viral material in the cells. Giant cell formation on L and FL cells is thought from the following facts to be caused by fusion of infected cells.

a) The number of cells decreases progressively after infection

b) Amitotic nuclear division is observed in too low frequency to cause formation of the multinucleated cells induced by the virus.

As early as 1 hour after infection, agglutination and fusion of two or three neighbouring cells was recognized and as shown in Table 1 about 60 per cent of the nuclei were present in giant cells at this time. The formation of giant cells seemed to begin as soon as the cells were infected. Thus the phenomenon seemed to be due to the primary interactions between virus material and cells regardless of intracellular viral growth. An eosinophilic mass was observed in all the nuclei 4 hours after infection in exp. 2. Formation of giant cells was complete about 6 - 8 hours after infection with viral doses as high as those used in the experiments.

Table 1. Appearance of Giant Cells on L Cell Monolayers after Infection with Strain +GC

	number of nuclei	1	2	3	4	5	6	7	8	9	10	11～20	21～30	31～40	41～100	100〈	per cent of total nuclei in giant cells*	average number of nuclei in all cells	average number of nuclei in all giant cells
number of cells having the number of nuclei indicated in the line above	1 hour after inoculation	100	16	13	5	4	2	1	0	1	0	1	0	0	0	0	60	1.7	3.5
	2 hours 〃	100	32	17	15	10	5	5	7	5	4	23	5	5	1	0	92	5.2	8.4
	7 hours 〃	100	16	12	8	2	2	4	0	1	2	12	8	4	3	1	92	6.7	14.4
	8.5 hours 〃	122	15	10	7	11	0	1	3	3	1	5	4	3	3	0	84	4.0	9.5

Exp. 1

	number of nuclei	1	2	3	4	5	6	7	8	9	10	11～20	21～30	31～40	41～100	100〈	per cent of total nuclei in giant cells	average number of nuclei in all cells	average number of nuclei in all giant cells
number of cells having the number of nuclei indicated in the line above	uninfected	117	1	0	0	0	0	0	0	0	0	0	0	0	0	0	2	1.0	2.0
	2 hours after inoculation	105	27	12	6	8	6	2	2	2	1	1	0	0	0	0	71	2.1	3.9
	4 hours 〃	102	25	17	6	3	8	2	5	2	3	14	13	0	2	0	90	5.0	9.0
	6 hours 〃	105	17	11	3	5	4	2	3	4	0	11	7	3	1	0	87	4.6	9.9
	8 hours 〃	100	18	7	5	3	3	1	4	2	2	6	4	3	6	0	90	6.1	14.1
	13 hours 〃	102	27	11	7	2	4	0	4	3	3	17	5	4	2	1	91	5.7	11.1

Exp. 2

* The term "giant cell" is used to describe a cell having two or more nuclei.

Table 2. Relationship between the Concentration of Input Virus and the Formation of Giant Cells in the Infection of L cells with Strain +GC of the Miyama Strain

Exp. 1a* Six and a half hours after infection

number of nuclei / number of cells having number of nuclei indicated in the line above in cells infected with each viral dilution	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	percent of total nuclei in giant cells	average number of nuclei in all cells	average number of nuclei in all giant cells	other morphological changes in the cells
1 : 3^0**	1112	227	107	41	28	14	12	8	6	4	1	1	1	2	1	57	1.7	3.3	all nuclei have eosinophilic masses
1 : 3^1	1029	117	53	23	11	2	3	0	0	0	0	0	0	0	0	36	1.3	2.7	〃
1 : 3^2	1038	75	19	6	2	1	1	0	0	0	0	0	0	0	0	20	1.1	2.4	〃
1 : 3^3	1023	23	1	0	0	0	0	0	0	0	0	0	0	0	0	5	1.0	2.0	〃
1 : 3^4	1041	8	2	1	0	0	0	0	0	0	0	0	0	0	0	2	1.0	2.4	no change

Exp. 1b* Twenty hours after infection

number of nuclei / number of cells having number of nuclei indicated in the line above in cells infected with each viral dilution	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	percent of total nuclei in giant cells	average number of nuclei in all cells	average number of nuclei in all giant cells	other morphological changes in the cells
1 : 3^0	1009	158	48	21	6	10	4	3	2	0	0	2	0	0	0	42	1.4	2.9	all nuclei have inclusions, all cells become rounded pyknosls (++)
1 : 3^1	1006	113	32	13	7	0	1	0	1	1	0	1	0	0	0	31	1.2	2.6	〃
1 : 3^2	996	44	8	6	1	2	1	0	0	0	0	0	0	0	0	14	1.1	2.6	〃
1 : 3^3	1012	55	10	1	2	0	0	0	0	0	0	0	0	0	0	13	1.1	2.3	all nuclei have inclusions, some cells become rounded pyknosls (+)
1 : 3^5	1239	4	2	0	0	0	0	0	0	0	0	0	0	0	0	1	1.0	2.3	no change

Exp. 1c* Fortyeight hours after infection

number of nuclei		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	percent of total nuclei in giant cells	average number of nuclei in all cells	average number of nuclei in all giant cells	other morphological changes of the cells
number of cells having corresponded number of nuclei	$1:3^4$																15	1.1	2.3	all nuclei have inclusions, some cells become rounded
	$1:3^5$																			focal degeneration in a monolayer

Exp. 1d* One hundred and twenty hours after infection

In the monolayer infected with a viral dilution of $1:3^6$, focal degenerations were observed.

Exp. 2 Six and a half hours after infection

number of nuclei		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	15–20	percent of total nuclei in giant cells	average number of nuclei in all cells	average number of nuclei in all giant cells	other morphological changes of the cells
number of cells having number of nuclei indicated in the line above in cells infected with each viral dilution	$1:3^0$	508	166	76	33	14	13	11	8	7	8	4	1	1	0	6	4	73	2.2	3.8	All nuclei have inclusions
	$1:3^1$	513	63	21	7	6	3	2	2	0	0	0	0	0	0	0	0	37	1.3	2.7	〃
	$1:3^2$	518	21	1	0	0	0	0	0	0	0	0	0	0	0	0	0	8	1.0	2.0	Some nuclei have inclusions
	$1:3^3$	572	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	〃	〃	no change
	$1:3^4$	530	6	0	0	0	0	0	0	0	0	0	0	0	0	0	6	2	〃	〃	〃
	$1:3^5$	512	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	〃	〃	〃

* In Exp. 1 infected preparations were obtained at four different intervals after infection, i.e. 6.5, 20, 48 and 120 hours after inoculation.

** Virus suspensions having a titer of approximately 10^8 $TCID_{50}$/ml were serially diluted three fold.

Desruption of cells began after 10 hours and became pronounced 13 hours after infection.

b) *Relations between concentration of input virus and giant cell formation*

Viral material derived from FL monolayers infected with strain +GC was serially diluted threefold. One ml aliquots of each dilution were each introduced onto monolayer cultures. The preparations were incubated for 2 hours and then the inoculum was replaced by fresh culture medium. At various times after infection samples were taken out and tested as described above. The results are shown in Table 2.

The cytopathic changes 6 and a half hours after virus inoculation were related to the concentration of input virus. More dilute viral material induced milder effects on cells. (Figs. 14, 15) The cells, which were inoculated with viral dilutions of $1:3^0$, $1:3^1$, and $1:3^2$ in exp. 1 and of $1:3^0$ and $1:3^1$ in exp. 2, had intranuclear eosinophilic masses in all their nuclei. (Fig. 16) The inclusions formed were recognized to be due to the first cycle of virus growth, although there were slight differences in their development in preparations infected by different dilutions of virus. It is interesting to see that a higher concentration of viral material is required to form giant cells than to infect all the L cells in a sheet. The data described above, however, do not mean that the results were due to differences in the stage at which intracellular virus growth occurred caused by different viral concentrations. This is shown by the following facts.

In exp. 2 parallel samples were removed 20 and 45 hours after infection and the cytopathic changes studied. In the preparations which were infected with a lower concentration of virus, infection proceeded further and virus grew until finally all cells contained intranuclear inclusion bodies, but at this stage there was not much formation of giant cells.

It is worthy of note that L cells produce large syncytia only when a high infective titer of virus derived from the culture fluid of infected FL cultures is used for inoculation but rarely as the result of viral growth itself.

c) *Effect of virus material which has been partially inactivated by ultraviolet irradiation*

Experiments were performed to see whether the dissociation between the formation of giant cells and intracellular viral growth could be seen in the interaction between L cells and the Miyama strain.

The effect of irradiation of strain +GC of the Miyama strain is shown in Fig. 17. Two ml viral samples containing about 10^8 $TCID_{50}$ per ml were irradiated for 1, 2 and 3 minutes as described earlier in this report. One and a half ml of active and inactivated virus samples were introduced into replicate cultures, which were then incubated at 37°C. After an adsorption period the inoculum was removed and fresh medium added.

Eight and a half hours after infection the cultures were removed and fixed.

The preparation infected with the active viral sample showed the severest cytopathic changes in the cells. Intranuclear eosinophilic masses could be seen in all the nuclei. (Figs. 18 and 19) In cultures inoculated with partially inactivated virus, giant cells were also produced. Giant cell formation was almost completed within about 8 hours and thereafter the number did not increase. This result was the same as with active virus. Eosinophilic masses, however, were not found in preparations inoculated with samples inactivated with UV for 1 minute. (Figs. 20 and 21)

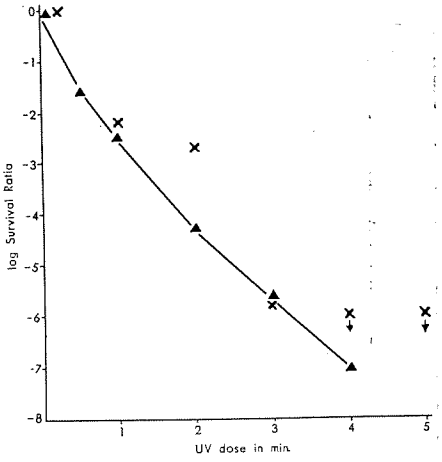

Fig. 17 Inactivation of Herpes Simplex Virus (+GC Miyama Strain) by UV-irradiation
A 15W germicidal lamp fitted with a reflector was employed for inactivation at a distance of 21 cm.

The degree of these cytopathic effects is shown in Table 3.
A similar marked change was also found using viral material inactivated by 3 minutes treatment (Figs. 22 and 23) and in this case the giant cells usually degenerated and did not form intranuclear inclusion bodies.

As shown in Fig. 17 a viral suspension with a titer of 10^8 $TCID_{50}$ per ml was inactivated to a level of 10^2 - 10^3 $TCID_{50}$ by 3 minutes exposure. Active virus of the Miyama strain with this titer had little effect if any within 3 or 4 days on L cells, because L cells are less susceptible to this strain than FL cells (Nii *et al.*, 1961a). The effect of virus suspensions of such a low infective titer on L cells is indicated in Table 2, and they cause focal degeneration of L cell monolayers. (Fig. 24)

In the above experiment a very low infective titer if any, (less than 10 $TCID_{50}$) was detected from the culture fluid of the L cell monolayer on the 2nd or 3rd day after inoculation with virus which had been irradiated for 3 minutes.

A high concentration of UV inactivated virus of strain —GCr did not induce

Table 3. Effect of Ultraviolet Inactivated Virus of Strain +GC on L Cell Monolayers

number of nuclei		1	2	3	4	5	6	7	8	9	10	11~20	21~30	31~40	41~100	100<	percent of total nuclei in giant cells	average number of nuclei in all cells	average number of nuclei in all giant cells	other morphological changes of the cells
number of cells having corresponded number of nuclei	virus sample inactivated for 0 min	106	14	11	3	6	1	4	6	4	1	10	7	3	6	1	91	6.8	14.7	oll nuclei have inclusions
	〃 for 1 min	100	7	7	10	5	3	1	0	0	2	13	1	0	0	0	79	3.2	7.7	inclusions are not found
	〃 for 2 min	109	22	10	5	4	7	4	1	3	0	3	1	0	0	0	72	2.3	4.6	〃
	〃 for 3 min	110	14	7	4	4	4	0	1	2	0	11	0	0	0	0	72	2.5	6.1	〃
	uninoculated culture	122	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1.0	—	〃

Preparations were obtained eight and a half hours after inoculation.

giant cells in L cell monolayers.

From these data it seems that giant cells are formed by the massive effect of virus suspensions of strain +GC on cells regardless of intracellular virus growth.

DISCUSSION

Two types of variants of herpes simplex virus had already been isolated by some workers: one produces multinucleated giant cells in monolayer cultures and the other rounding of infected cells (McNair-Scott *et al.*, 1959; Hoggan *et al.*, 1959; Nii *et al.*, 1961). On passage of a strain of herpes simplex virus (Miyama strain) *in vitro* a variant unlike either type was obtained (Nii, 1961b).

In this paper further studies on the morphological changes in FL cells induced by the —GCf Miyama strain are presented. The chacteristic cytopathic effects produced by this variant are agglutination and fusiform development of infected FL cells. These changes are distinguishable from those caused by other substrains, i.e. strain +GC or strain —GCr. Although the extent of cytopathic changes in monolayer cultures after virus inoculation varied with the titer of the input virus and the morphology of the infected cells varied during infection, fusiform cells always appeared in the sheet when the cytopathic effects were maximal.

The microscopic plaques which were produced in a monolayer culture infected with a low titer of herpes simplex virus may be useful to distinguish this strain from others which each cause their own conspicuous morphological changes. The plaques produced by strain —GCf, however, sometimes resembled those induced by strain +GC. Therefore to distinguish the substrains of the Miyama strain clearly it is best to study monolayer cultures infected with a high viral titer of each substrain.

Besides the morphological characteristics induced by strain —GCf, another character was studied. Changes of the number of FL cells infected with various titers of strain —GCf were followed after virus inoculation and it was found that this variant caused earlier disruption of FL cells than strain —GCr.

Strain —GCf as well as the other two strains were neutralized with a specific rabbit antiserum against strain HF.

It is interesting that the three substrains, *i.e.* +GC, —GCr and —GCf, were originally derived from the same material isolated from a patient although the origin of each substrain is unknown. Strains +GC and —GCr were isolated after consecutive passages of the parent virus population on HeLa and FL cell cultures and further passages of the latter on L cells and FL cell cultures resulted in the isolation of —GCf strain. Recent studies showed that the parent virus population, from which strain —GCf was isolated, contained all three types of viruses, *i.e.* +GC, —GCr and —GCf and that type —GCf was predominant, (unpublished data). However, it is uncertain whether any viral clone obtained

from the parent virus population of the Miyama strain induces the same type of cellular response as those of one of the three substrains. There might be other types of cellular responses which are slightly different from the types already known.

The question therefore arises as to what controls the change in cellular morphology after infection with herpes simplex virus and various possibility may be considered. First, the genetic material in the virus particle which seems to be specific for each substrain, may play a role in determining cellular changes which occur immediately after infection. Second, each substrain of herpes virus may have its own metabolic pattern of virus reproduction in infected cells thus causing different degenerative changes. Third, the variant which induces multinucleated giant cell formation may induce formation of "a giant cell producing agent" in association with virus particles which causes giant cells formation regardless of intracellular virus growth. Fourth, such a variant may produce "a toxin-like agent" in infected cells which is separable from virus particles as in adeno virus.

The data presented in this paper support the third possibility. Changes in L cells infected with strain +GC vary with the concentration of viral sample in the inoculum and large syncytia are formed only when infected with a higher infective titer of virus. Inoculation with a low titer results in formation of few giant cells even when infection has proceeded and cytopathic effects have become maximal. This suggested that giant cell formation is due to the primary effect of input virus.

UV inactivated +GC virus caused formation of giant cells but not of intranuclear inclusion bodies and this phenomenon indicates that giant cell formation is not directly related to intracellular viral growth. Although satisfactory data have not been obtained in FL cells with UV inactivated virus, this conclusion is supported by the following observations on them. Microscopic plaques of FL cells infected with strain +GC, showed that some cells with apparently normal nuclei participated in syncytial formation and mitotic cells in metaphase which did not seem to play a role of viral growth were sometimes also engulfed in a syncytium. Giant cell formation as the result of the primary interaction between virus particles and cells was reported in detail by Okada *et al.* (1958).

It is said that nucleic acids of virus particles play an important role in initiating virus reproduction. UV light destroyed the infectivity of virus particles, while their ability to produce giant cells was unaffected. Therefore the agent inducing giant cell formation may be present in a different portion of the virus particle from that causing infectivity, *e.g.* in the protein coat of virus. It is interesting to see that the reproduction of such an agent which is separable in its function from the infectivity of the virus particle is genetically controlled.

Genetical relationships between strains +GC, −GCr and −GCf have not been clarified and studies must be made on whether −GCf type virus is a hybrid type.

Finally we would like to discuss some roles of host cells in producing giant cells after infection. Syncytia containing several hundred nuclei are consistently produced on infected FL cells regardless of the infective titer of input +GC virus, while giant cells containing about ten to a hundred nuclei appear in L cells only when a high titer of virus was inoculated onto them.

This variation in response of L cells was first attributed to the lower susceptibility of L cells to the Miyama strain of herpes virus. Quantitative analysis of the cytopathic changes induced by +GC virus showed that too low viral yields were obtained from infected L cell cultures to form syncytia. The earlier disruption of L cells than of FL cells after infection is regarded as another factor in inducing formation of smaller giant cells in L cells.

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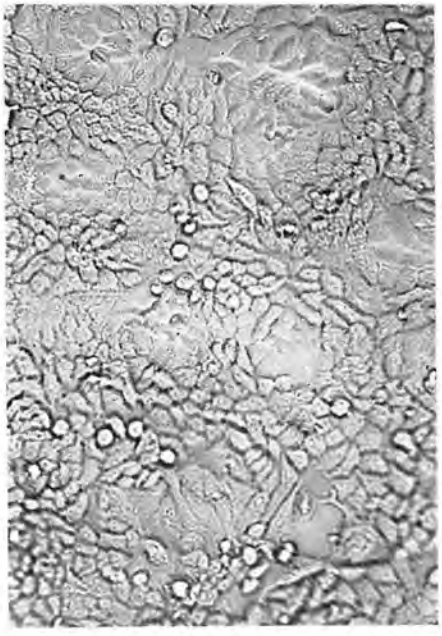
EXPLANATION OF PHOTOGRAPHS

Fig. 1. Uninfected FL cells (× 120)
Fig. 2. FL cells infected with a high viral titer of strain−GCf. Eight hours after inoculation (×120)
Fig. 3. FL cells infected with a high viral titer of strain−GCr. Six hours after inoculation (×120)
Fig. 4. FL cells infected with a high viral titer of strain −GCf. Twelve hours after inoculation (× 120)
Fig. 5. FL cells infected with a high viral titer of strain −GCf. Twenty hours after inoculation (× 120)
Fig. 6. FL cells infected with a high viral titer of strain −GCf. Seventy hours after inoculation (× 120)
Fig. 7. Cytopathic changes induced by strain +GC of herpes simplex virus (× 120)
Fig. 8. Cytopathic changes induced by strain −GCr of herpes simplex virus (× 120)
Fig. 9. FL cells infected with strain −GCf
Intranuclear inclusion bodies of an oval form are shown.
Fixed with Bouin's solution and stained with haematoxylin and eosin. (×320)
Fig. 11. Uninfected L cells (× 160)
Fig. 12. Giant cell formation on an L cell monolayer 2 hours after inoculation with strain +GC (× 160)
Fig. 13. Giant cell formation on an L cell monolayer 2 hours after inoculation with strain +GC
Intranuclear inclusion bodies are not shown in these giant cells. (× 320)
Fig. 14. L cell monolayer which was inoculated with viral dilutions of 1 : 3 as shown in exp. 1 in Table 2 (× 160)
Fig. 15. L cell monolayer which was inoculated with viral dilutions of 1 : 3^2 as shown in exp. 1 in Table 2 (× 160)
Fig. 16. L cell monolayer which was inoculated with viral dilutions of 1 : 3^2 as shown in exp. 1 in Table 2
All cells have intranuclear eosinophilic masses in their nuclei. (× 640)
Fig. 18. L cells infected with unirradiated +GC virus
Eight and a half hours after inoculation (× 160)
Fig. 19. L cells infected with unirradiated +GC virus
All nuclei show intranuclear changes. Eight and a half hours after inoculation. (×640)
Fig. 20. Syncytial formation of L cells on inoculation with +GC virus, inactivated by UV for 1 min.
Eight and a half hours after inoculation (× 160)
Fig. 21. Syncytial of L cells on inoculation with +GC virus, inactivated by UV for 1 min.
Eight and a half hours after inoculation. No nuclei show intranuclear inclusion bodies at this stage. (× 640)
Fig. 22. Giant cell formation of L cells on inoculation with +GC virus, inactivated by UV for 3 min.
Eight and a half hours after inoculation (× 160)
Fig. 23. Giant cell formation in an L cell monolayer on inoculation with +GC virus inactivated by UV for 3 min.
Eight and a half hours after inoculation. Eosinophilic masses are not seen in their nuclei. (× 640)
Fig. 24. A focal degeneration in an L cell monolayer caused by a low infective titer of the Miyama strain of herpes virus (× 160)



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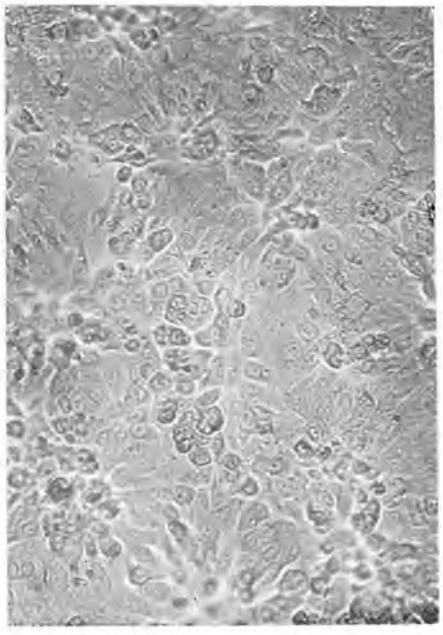

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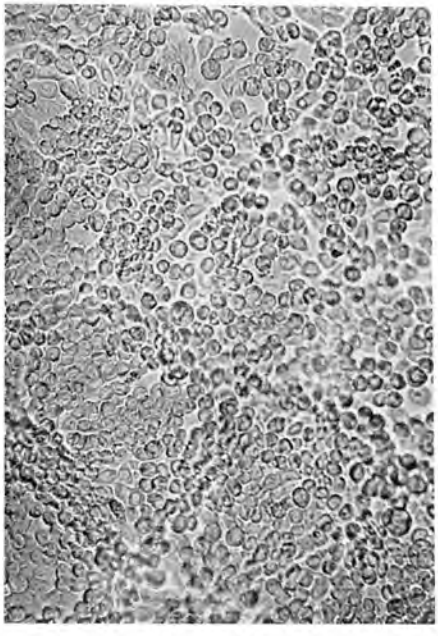

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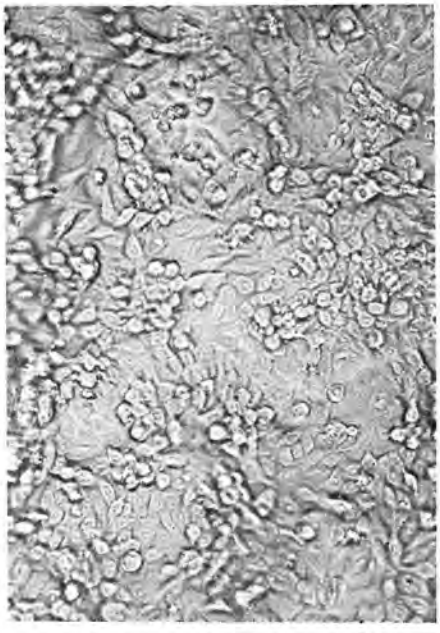

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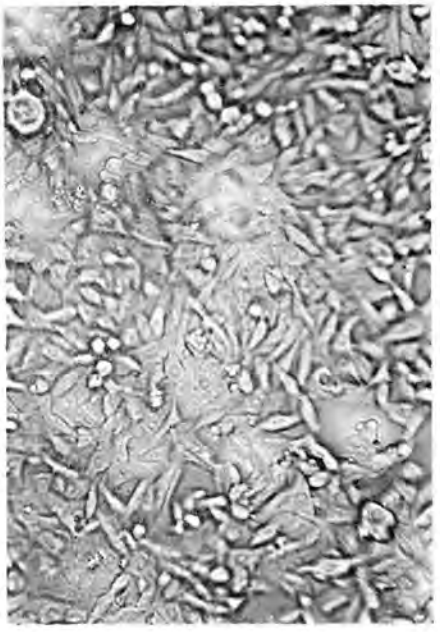

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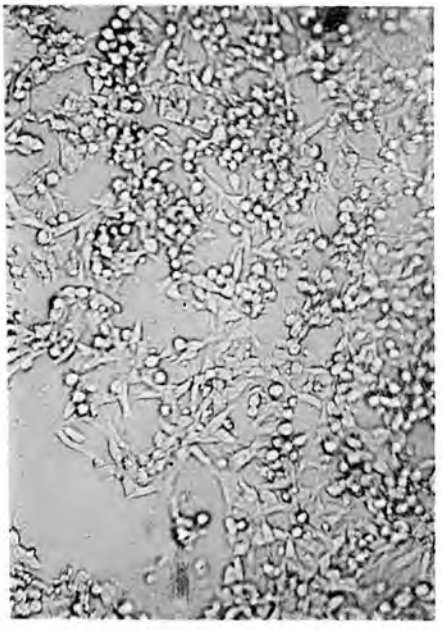

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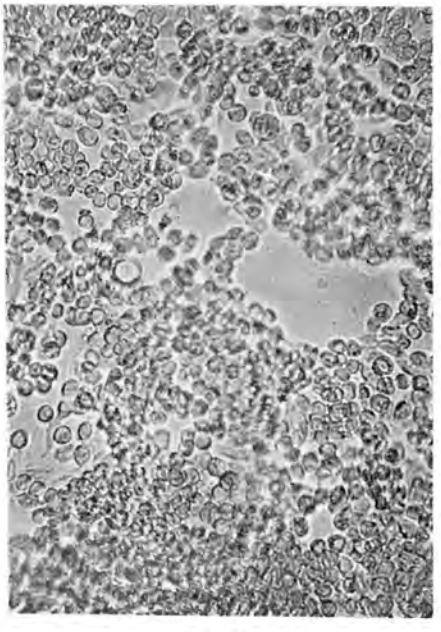
Fig. 7.



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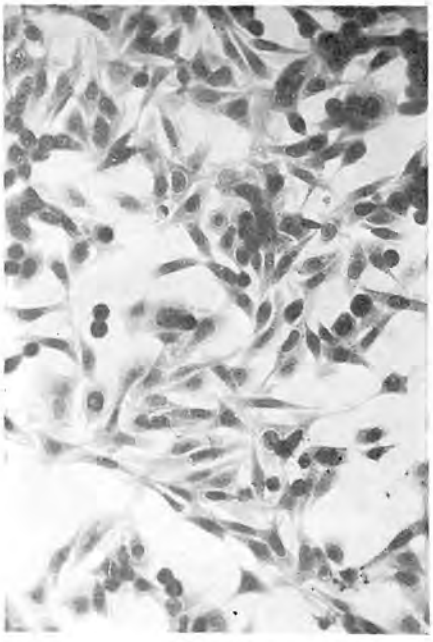

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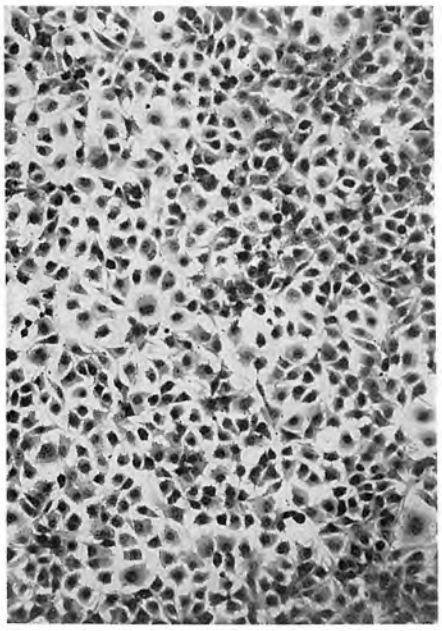

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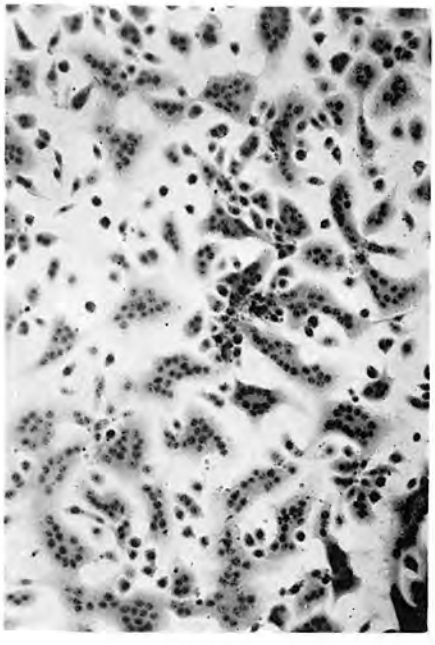

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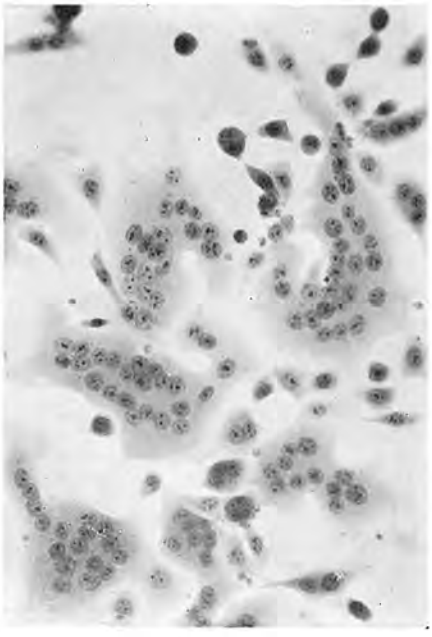

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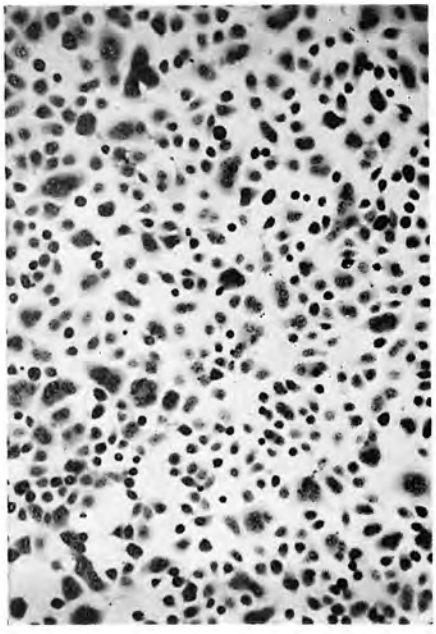

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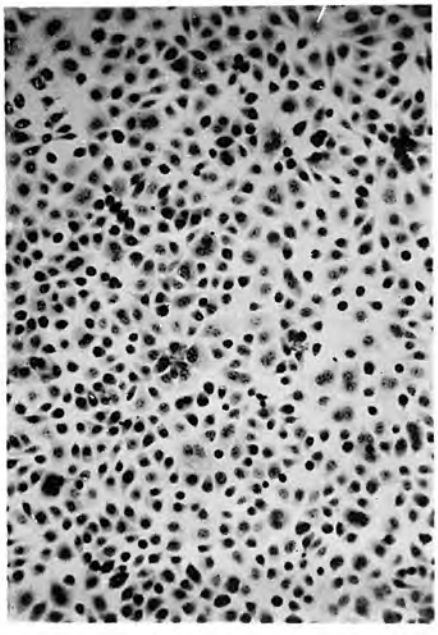

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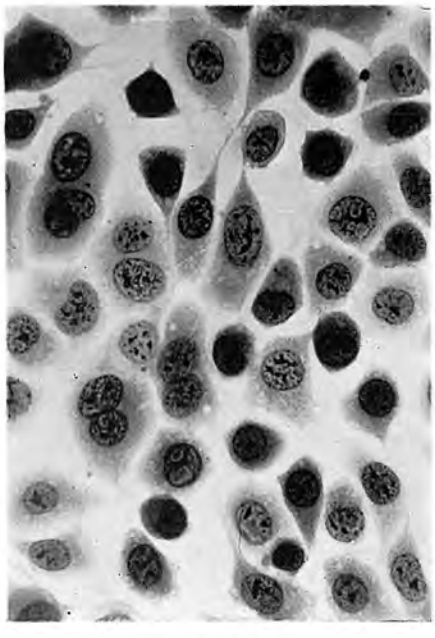

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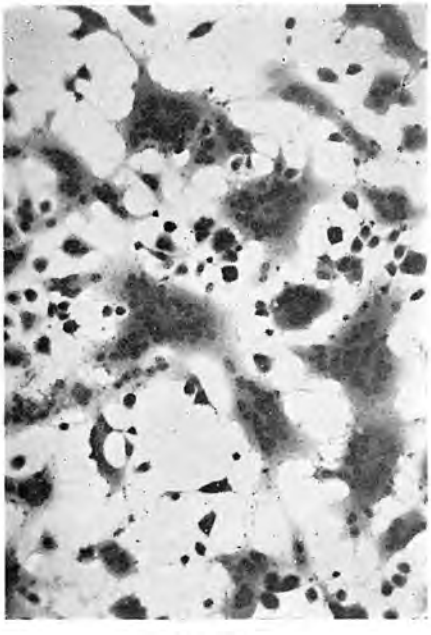

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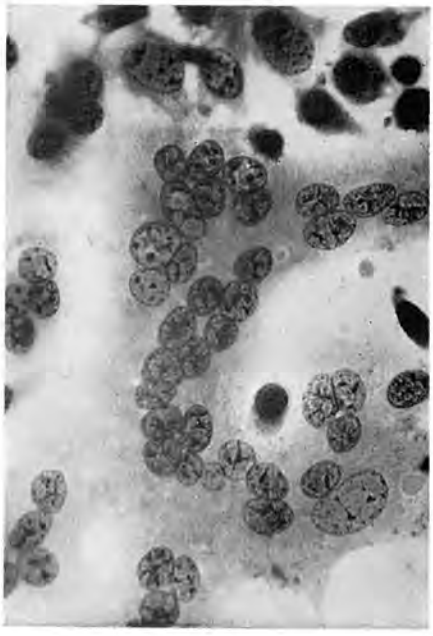

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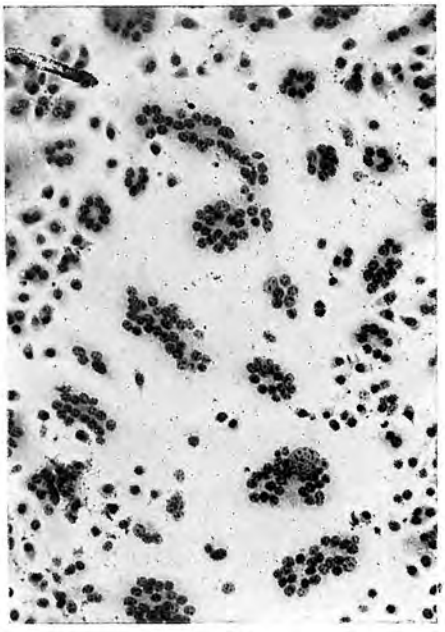

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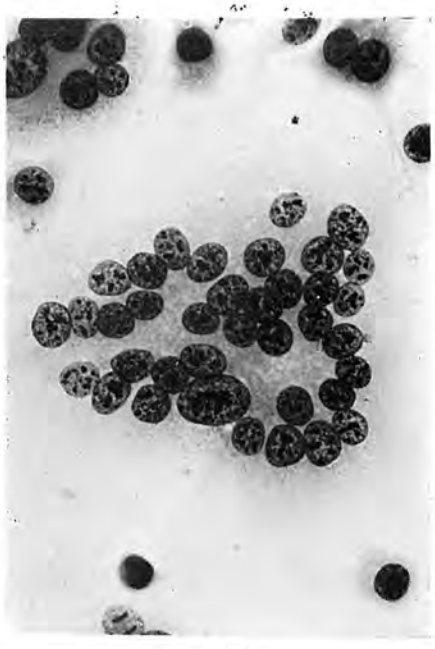

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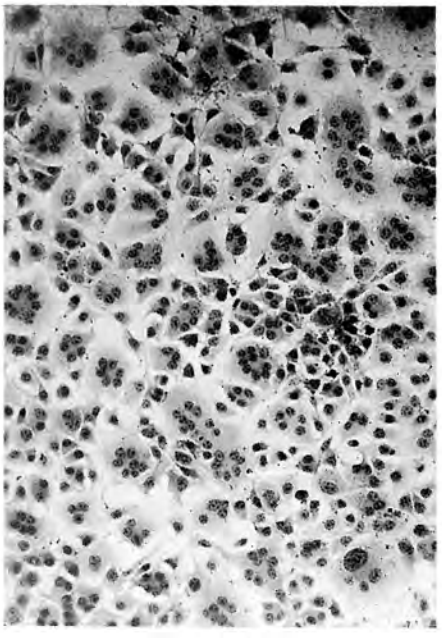

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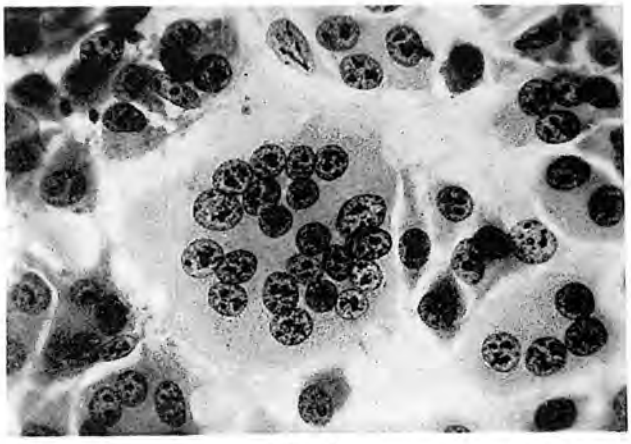

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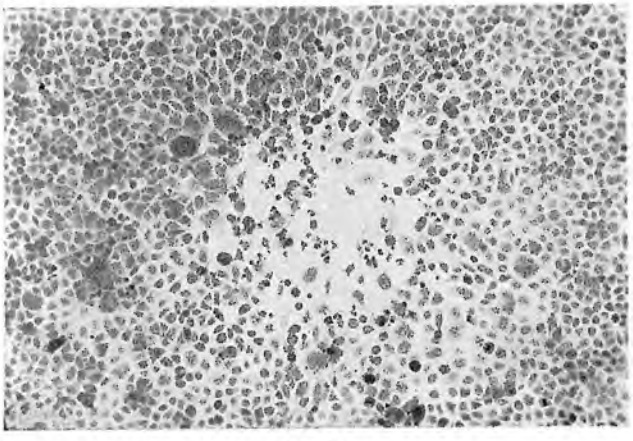

Fig. 24.