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Location of Herpetic Viral Antigen in Interphase Cells

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SUMMARY

Studies were made on the appearance of herpetic viral antigen in monolayer cells *in vitro*. The $^{+}$GC and $^{-}$GCr variants of the Miyama strain and Yamagishi strain virus were used. A high input multiplicity of virus was inoculated onto FL or L cell monolayers. Approximately four hours after virus inoculation fluorescent material appeared at the nuclear membrane and in the perinuclear regions of the cytoplasm. In the early stages of infection the fluorescent granules were very fine. In the nucleus, however, definite fluorescence could not been detected. As infection proceeded, the antigen spread extensively in the cytoplasm.

In experiments with the $^{-}$GCr type of virus, the infected cells became round and in later stages of infection it was difficult to decide the exact site of the antigen in cells, but the nuclei in the syncytium produced by $^{+}$GC virus did not seem to have fluorescent material even in advanced stages of infection.

Syncytial formation induced by a high dose of $^{+}$GC virus was distinct as early as one hour after virus inoculation which was before the antigen began to appear in the cells.

The correlation between the time of appearance of the antigen and the formation of intranuclear inclusion bodies was also studied. The viral antigen was detected earlier than the formation of typical intranuclear inclusion bodies.

INTRODUCTION

Electron microscopic observations on ultrathin sections of herpes infected tissues, made by Morgan *et al.* (1959), revealed the developmental features of virus particles in the nucleus and in the cytoplasm. In earlier reports we described the characteristic pattern of incorporation of H^3-thymidine into the nuclei of herpes-infected FL cells seen by autoradiography (Nii *et al.*, 1960; Nii *et al.*, 1961a). DNA synthesis for herpes virus is supposed to occur in the nucleus. In connection with these findings it is of interest to find where in the cell virus antigen is made.

Herpetic virus antigen in infected cells has been located by several investigators using the fluorescent antibody technique. However their results, do not agree well, as shown in Table 1. Lebrun (1956) observed that virus antigen accumulated in the nucleus in the earlier stages of infection and later it moved from the nucleus to the cytoplasm through the nuclear membrane and spread out in the cytoplasm.

Table 1. Location of Viral Antigen in Cells Infected with Herpes Simpiex Virus

Authors	Date of publication	Experimental host cells or specimens	Site of fluorescence in infected cell
Lebrun	1956	HeLa	First in the nucleus and later in the cytoplasm accompanied by loss of nuclear fluorescence
O'Dea et al.	1957	human amnion cells suckling-mouse kidney cells	cell membrane
Ross et al.	1958	HeLa	Both in the nucleus and in the cytoplasm
Biegeleisen	1959	conjunctival smears from patients	Both in the nucleus and in the cytoplasm
Kaufman	1960	conjunctival smears from rabbits	Both in the nucleus and in the cytoplasm
Vozza et al.	1961	conjunctival smears from rabbits	Both in the nucleus and in the cytoplasm
Roizman	1961	HEp-2 cells	First at the nuclear membrane and then in the cytoplasm

He considered these findings corroborated the cytochemical studies made by Crouse *et al.* (1950). On the contrary Roizman (1961) reported that fluorescent antibody staining of syncytia containing interphase nuclei revealed the early appearance of virus antigen at the nuclear membrane and subsequent accumulation of the antigen in the cytoplasm.

Studies were made to clarify the discrepancy between these results and to study the process of formation of virus particles in infected cells. The possible correlation between the time of formation of syncytia induced by a giant cell forming variant and the appearance of virus antigen was also studied.

MATERIALS AND METHODS

1. *Virus*

Two variants of the Miyama strain of herpes simplex virus, *i. e.* $^{+}$GC and $^{-}$GCr, were used (Nii *et al.*, 1961b). The Yamagishi strain, which was kindly supplied by Dr. Mutai, was also used. The supernatant fluid of infected FL cells was used directly as virus material. Both variants of the Miyama strain produced approximately 10^8 $TCID_{50}$ per ml, while the Yamagishi strain virus formed approximately 10^7 $TCID_{50}$ per ml. Usually the culture fluids were used after centrifugation at 2,500 rpm for 10 minutes. In experiments on the correlation between syncytium formation and the appearance of virus antigen, the culture fluids were centrifuged at $4,500 \times g$ for 20 minutes at 0°C after the above low speed centrifugation.

2. *Cells*

FL cells and Earle's L cells were obtained. Cell monolayers were prepared on coverslips in Leiton tubes. Each coverslip had about 5×10^5 cells on it.

3. *Infection*

One ml of virus inoculum was introduced into each Leiton tube. A one hour adsorption period was chosen. The culture was washed five times with 2 ml aliquots of YLH solution and fresh maintenance medium was then introduced onto it.

4. *Preparation and fixation of cultures for fluorescent antibody staining*

The infected cultures were taken out at appropriate intervals after infection and washed twice with Hanks' solution and once with PBS. The cultures were fixed in one of the following methods.

1) Usually the cultures were air dried and fixed with acetone at room temperature for 10 minutes.

2) Wet culture preparations were immersed directly in acetone at −60°C and kept in it for 40 minutes.

3) After procedure 2), the preparations were kept overnight in acetone at −20°C.

4) Wet culture preparations were immersed directly in acetone at −20°C and kept in it overnight at this temperature.

The appearance of the fluorescent material in these preparations was essentially the same after any of these procedures.

5. *Antiserum*

Three kinds of antisera derived from different adult rabbits were used. Rabbits were infected by intracorneal inoculation or corneal incision and they suffered from keratoconjunctivitis.

1) One conjugated antibody was kindly supplied by Dr. Kawamura of the Institute for Infectious Diseases, Tokyo. This rabbit antiserum had been made as follows: A rabbit was infected with the supernatant fluid of HeLa cells infected with the Yamagishi strain of herpes simplex. Before conjugation with fluorescein isothiocyanate, the titer of this antiserum was × 3600 by the neutralization test and × 256 by the complement fixation test. Conjugated antisera had a titer of × 16 by the complement fixation test. This conjugated antibody was used for staining infected L cells after adsorption with acetone dried mouse liver powder. This was because Earle's L cells derived from mouse fibroblasts and HeLa cells derived from Human cervical carcinomas, are known to be immunologically unrelated (Brand *et al.*, 1962).

2) Two other antisera were made as follows: Rabbits were inoculated by intracornael inoculation with the supernatant fluid of porcine kidney cells infected with the Miyama strain of herpes simplex, and consequently suffered from keratoconjunctivitis. These antisera neutralized more than 10^7 $TCID_{50}$ of virus before conjugation. The two antisera were not mixed and each antiserum was conjugated separately. After conjugation these antisera were absorbed with acetone dried human liver powder and the conjugated antibody was used for staining infected FL cells.

6. *Preparation of fluorescein conjugated antibody*

Essentially the same method as that reported by Tateno *et al.* (1962) was used. The antiserum was fractionated by 33 per cent saturation with ammonium sulphate. The resulting precipitate was separated by regrigerated centrifugation and redissolved in the original volume of saline. This procedure was repeated three times. To remove ammonium ions from the final solution a Sephadex G 50 column was used.

This rabbit globulin was conjugated with fluorescein isothiocyanate (Baltimore Biological Laboratories) essentially by the method of Marshall *et al.* (1958). 0.01 mg of fluorescein isothiocyanate per 1 mg of protein was added to the globulin solution, which had been adjusted to pH 9.0-9.5 with 0.5 M carbonate buffer and the mixture was stirred at 4°C for about four hours by a magnetic stirrer. To remove unconjugated dye from the solution a Sephadex G 50 Column was used. The effluent was passed through a column of DEAE-cellulose and the globulin was fractionated further. All the above procedures were carried out at 4°C.

The virus antigen was specifically stained with fluorescent antibody since: 1) labeled antibody did not stain uninfected cultures and 2) the specific fluorescence was blocked by preincubation of the coverslip preparations with nonconjugated rabbit antiserum, but not with nonconjugated normal rabbit serum.

7. *Cytological observations*

Cultures were washed with Hanks' solution, fixed with Bouin's solution and stained with haematoxylin and eosin.

RESULTS

1. *The -GCr Miyama strain and L Cells*

Definite spots of antigen were detectable about four hours after virus inoculation. The first appearance of fluorescent material was seen at or near the nuclear membrane, but virus antigen could not be seen in the nucleus (Figs. 1 and 2). As infection proceeded the antigen spread in the cytoplasm and looked finely granular as reported by Roizman (1961). (Fig. 3) Sometimes spots of fluorescence laced the nuclear membrane (Fig. 4). By the fifth or sixth hour of infection the fluorescent area was often situated in the perinuclear portion surrounding one side of nucleus. From about this time the infected cells became round. About seven hours after infection the whole cytoplasm was filled with fluorescent materal and then each infected cell appeared as a round fluorescent mass, although the nuclear area showed less fluorescence. At this stage of infection the exact localization of the virus antigen was difficult. A few flat cells sometimes showed cytoplasmic fluorescent masses (Fig. 5). From eight or nine hours after infection detachment of the round cells from the coverslip began to occur.

2. *The +GC Miyama strain and L cells*

As early as one hour after virus inoculation fusion of two or three neighbouring cells is seen here and there on the monolayer (Figs. 6, 7). Syncytial formation became more remarkable at the second hour of infection, when no fluorescent material could detected at all (Figs. 8, 9). The first definite appearance of fine granules of antigen was recognizable after three hours and clearly seen by four hours (Fig. 10), while syncytial formation was pronounced by the end of three hours (Fig. 11). This early appearance of syncytial formation in L cell monolayers confirmed our earlier work (Nii *et al.*, 1961c). The fluorescent material appeared as fine granules visible at the nuclear membrane and in perinuclear portions in the syncytia. No fluorescence was detectable in the nuclei. Five or six hours after infection more extensive accumulation of virus antigen was observed in the cytoplasm (Figs. 12, 13). Fine spots of fluorescence were seen in the projections from the syncytia and in the bridges between neighbouring cells. In cells which were not in the syncytia, the fluorescent material had the same appearance as in experiments with the -GCr variant.

3. *The -GCr Miyama strain and FL cells*

The results were essentially the same as with the same virus strain and L cells. Antigen was definitely recognizable approximately four hours after infection. At this time fine granules or spots of fluorescent material were dispersed in a semicircle lacing the nuclear membrane or inlayed adjacent to it around the nucleus (Figs. 14, 15). As the destructive effect of the virus on this cell line is slower than that of L cells, cells with fluorescence could be seen clearly for a longer period and from about eight hours after infection intense accumulation of viral antigen was seen.

4. *The Yamagishi strain and L cells*

The results were essentially the same with this system as with the -GCr Miyama strain and L cells. The fluorescent mass in the perinuclear portion was very clearly visible four hours after infection, as in other systems (Figs. 16, 17). This suggested that there might be some correlation between the site of virus antigen and cytoplasmic organelles such as Golgi apparatus. This was also noticed in the preparations six hours after infection (Fig. 18), but the fluorescent mass was not so clear at the eight hour, because the viral antigen had spread into all the cytoplasm (Fig. 19).

5. *Correlation of the time of appearance of the fluorescent material and of formation of intranuclear inclusion bodies*

In the above experiments, parallel cultures were prepared for sequential morphlogical studies on the infected cells. In all the series the fluorescence was detectable before typical intranuclear incluson bodies could be seen in the infected cells. When the virus antigen was first detectable, disintegration of the nucleolus and disarrangement of the chromatin network were observed and eosinophilic material giving the appearance of gold dust was often seen in the nuclei. These morphological changes in the nuclei seemed to correspond to the stage of the nucleus with a "finely granular appearance", which was described by Scott *et al.* (1953). A distinct eosinophilic mass appeared about one hour after the first appearance of any definite fluorescence, and typical intranuclear inclusions did not appear till later.

DISCUSSION

Previously, data on the location of the viral antigen of herpes simplex in the cell have been in discrepancy. There are many possible reasons why the results of various workers have not been in agreement.

In our experiments on the development of fluorescence in cells within one cycle of infection, the definite existence of the antigen was not recognizable in the

nucleus, but the first fluorescent material was found lining the nuclear membrane and in the perinuclear region of the cytoplasm. As infection proceeded, the fluorescent material spread diffusely through the cytoplasm. Therefore it seems likely that the viral antigen is formed at or near the nuclear membrane in the cytoplasm and later spreads to the peripheral parts of the cytoplasm. At least two problems made the determination of the site of viral antigen difficult. One was cell rounding after infection with -GCr virus and the other was the interpretation of the nature of the fluorescent material in the nuclear area, which made determination of the true site of antigen formation in cells difficult.

In experiments with the -GCr variant, the infected cells readily became round. In advanced stages of infection extensive accumulation of fluorescent material was recognizable in the cells and they looked like round fluorescent masses. In these cells the exact localization of viral antigen was impossible, although the nuclear area generally showed less fluorescence. This may partly explain why different data were obtained by different workers. However in experiments using the + GC variant the absence of nuclear fluorescence was clearly apparent even in the later stages of infection, because the virus formed syncytia in which the distinct contours of the nuclei were comparatively well recognizable throughout the course of infection.

Another problem, as shown in Figs. 20 and 21, was that in a few cells it was not easy to decide whether the fluorescent material lay adjacent to the nuclear membrane or in the nucleus. However even in these cells the finely granular fluorescence was more intense around the nuclear membrane and was also considered to lie adjacent to and covering the nuclear area.

As described above, nuclear membrane seems to play an important role in the formation of herpetic viral antigen. Electronmicroscopic studies by Morgan *et al.* (1959) revealed that the envelope of the virus particle was formed at the nuclear membrane and therefore it is interesting to speculate on whether the envelope of virus particles may have a relation with a biological characters of the virus such as its antigenicity.

Our present data show that not all parts of the nuclear membrane begin to produce virus antigen at the same time after infection. Possibly only some parts participate in the formation of the antigen at least in the earlier stages of infection, because at the time of infection the fluorescence usually appeared in a semicircle lining the nuclear memrane and the fluorescent area was usually apparent in the perinuclear portion surrounding only one side of the nucleus. The latter area may correspond to the site of the Golgi apparatus and it is very intriguing to wonder if the passage of herpes virus particles from the nucleus to the cytoplasm is related to such cellular organelles.

Discussions must be made on the absence of nuclear fluorescence in our data. Various fixation procedures were tested. Parallel cultures were fixed with ace-

tone at room temperature for 10 minutes, at −60°C for 40 minutes and at −20°C overnight. However, after these different fixation procedures results were the same.

Variation in the host cells, virus strains and antisera were also tested but the results were essentially the same. The antiserum may play a very important role in the present experimental system. Balducci *et al.* (1956) reported that herpetic antigens prepared from suckling mouse brain or the chorioallantoic membrane contained sedimentable ("Virus particles") and non sedimentable ("soluble") antigens and that human sera possessing antibody for the first component often lacked the antibody for the second component. This might be a cause of failure to detect nuclear fluorescence and this possibility is not completely excluded. In our experiments three kinds of rabbit antisera derived from different rabbits were used and the same results were obtained and, by the test, at least one conjugated antiserum was proved to have sufficient complement fixing antibody.

Recent work by Slotnick *et al.* (1963) on the location of varicella virus in tissue culture cells showed that varicella antigen, first detectable by immunofluorescence in the cytoplasm at the border of the nucleus, gradually accumulated in the perinuclear region and finally became concentrated in the cytoplasm. This is in good accordance with our results. Although the two viruses are immunologically unrelated, they produce the same intranuclear inclusions in infected cells.

The appearance of eosinophilic masses in the nuclei is recognized as a characteristic morphological change after infection with the viruses of the "NITA" group (Andrews *et al.*, 1961) and the data of Slotnick *et al.* (1963) and our work showed that these masses have no relation with virus antigen. Correlation of the time sequence of the appearance of intranuclear inclusion bodies and that of fluorescent material was also studied and it was found that the appearance of virus antigen was recognized earlier than the formation of typical intranuclear inclusion bodies.

We reported earlier that after infection with a high virus input L cells produced one log unit or less infective viruses than did FL cells. However, using the fluorescent antibody technique, no definite difference could have been found between FL and L cell preparations.

Cytopathic changes in L cell monolayers induced by the +GC variant of the Miyama strain are dependent on the input virus multiplicity in the first cycle of virus growth and a high dose of virus partially inactivated with ultraviolet light also induced syncytia with no intranuclear inclusions in them (Nii *et al.*, 1961c). In these experiments also syncytia formed progressively as soon as a high multiplicity of virus was inoculated onto the monolayers and this is in contrast to the existence of a latent period before the appearance of fluorescent material in the syncytia. Therefore this data also suggests that syncytial formation is produced as the result of a primary interaction between L cells and +GC virus.

The characteristic pattern of incorporation of H^3-thymidine into the nuclei

of herpes virus-infected cells revealed by autoradiography (Nii *et al.*, 1962) as well as the presence of intranuclear crystals composed of viral particles showed by electronmicroscope (Morgan *et al.*, 1959) indicates that one important process in the formation of the virus particle undoubtedly occurs in the nucleus.

Wildy *et al.* (1960) reported that herpes virus is composed of three main substructures, *i.e.* an envelope, a capsid and a core. The relations between these structures and the biological activity of the virus, such as its infectivity and antigenecity, and the mechanism by which virus particles are endowed with the latter characters are problems to be elucidated in the future.

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EXPLANATION OF FIGURES

Fig. 1. An L cell monolayer inoculated with the −GCr variant of the Miyama strain. One hour after virus inoculation. Fluorescent material cannot be seen. × 1000

Fig. 2. L cells infected with the −GCr Miyama strain. Four hours after virus inoculation. Fluorescence is found at or near the nuclear membrane × 1000

Fig. 3. L cells infected with the −GCr Miyama strain. Five hours after virus inoculation. The fluorescent material looks finely granular. × 1000

Fig. 4. L cells infected with the −GCr Miyama strain. Five hours after virus inoculation. In the middle and lower part of the plate there is a cell, in which the spots of fluorescence seem to lace the nuclear membrane. × 1000

Fig. 5. L cells infected with the −GCr Miyama strain of herpes simplex. Seven hours after infection. In the middle and left part of the plate, a flat cell is shown. In the cytoplasm many cytoplasmic fluorescent masses are shown. Other infected cells in the plate look round and the exact location of the site of viral antigen is uncertain.

Fig. 6. Normal non-infected L cell monolayer. × 360

Fig. 7. L cell monolayer inoculated with the +GC Miyama strain. One hour after virus inoculation. Syncytial formation is shown. × 360

Fig. 8. L cells infected with the +GC Miyama strain of herpes simplex virus. Two hours after virus inoculation. Syncytial formation is shown. × 360

Fig. 9. L cells infected with the +GC Miyama strain of herpes simplex. One and a half hour after infection. No definite fluorescence is seen. × 1000

Fig. 10. L cell monolayer inoculated with the +GC Miyama strain. Three hours after virus inoculation. The early appearance of fluorescence in the cells is apparent. Syncytial formation is also shown. × 1000

Fig. 11. L cell monolayer inoculated with the +GC Miyama strain. Three hours after virus inoculation. Large syncytia are shown. × 360

Fig. 12. L cell monolayer inoculated with the +GC Miyama strain. Five hours after virus inoculation. Fine granular fluorescent material is seen in the cytoplasm of the syncytium but not in the nuclei. × 1000

Fig. 13. L cell monolayer inoculated with the +GC Miyama strain. Six hours after virus inoculation. Intense accumulation of virus antigen in the cytoplasm is seen. × 1000

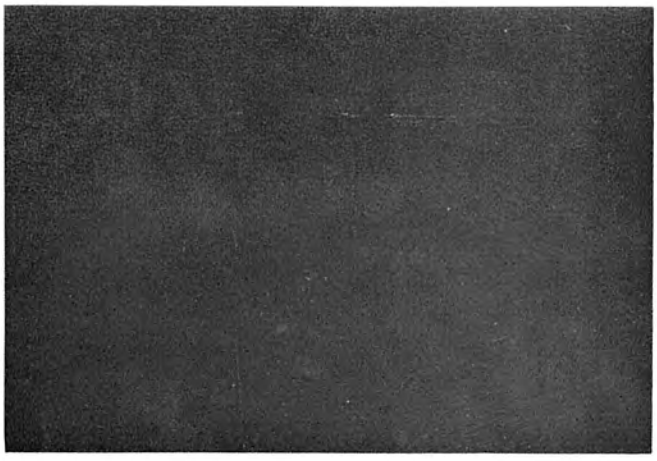
Figs. 14, 15. FL cells inoculated with the −GCr Miyama strain. Four hours after infection. Fluorescence at the nuclear membrane and in the perinuclear regions of the cytoplasm is shown. In Fig. 14, a round fluorescent cell is shown in the upper left of the plate. Due to accumulation of virus antigen in the whole cytoplasm and cell rounding, the true site of virus antigen is uncertain, although there is less fluorescence in the nuclear area. × 1440

Fig. 16. L cells inoculated with the Yamagishi strain. One and a half hour after virus inoculation. No definite fluorescence is shown. × 1800

Figs. 17, 18. L cells infected with the Yamagishi strain. Four hours after virus inoculation. The fluorescent mass in the prinuclear region is very distinct. × 1800

Fig. 19. L cells infected with the Yamagishi strain of herpes simplex virus. Eight hours after virus inoculation. Intense fluorescence is seen in the whole cytoplasm of infected cells. × 1800

Figs. 20, 21. L cells infected with the Yamagishi strain of herpes simplex virus. Four hours after virus inoculation. In Fig. 20, a giant cell is seen in the upper center of the plate. Fine granular fluorescence is seen in the perinuclear portion on the nuclear area, although there is less in the latter area. In Fig. 21, a giant cell is seen in the center of the plate. Fine granular fluorescence is seen at the nuclear membrane, in the perinuclear portions of the cytoplasm and covering the nuclear area.



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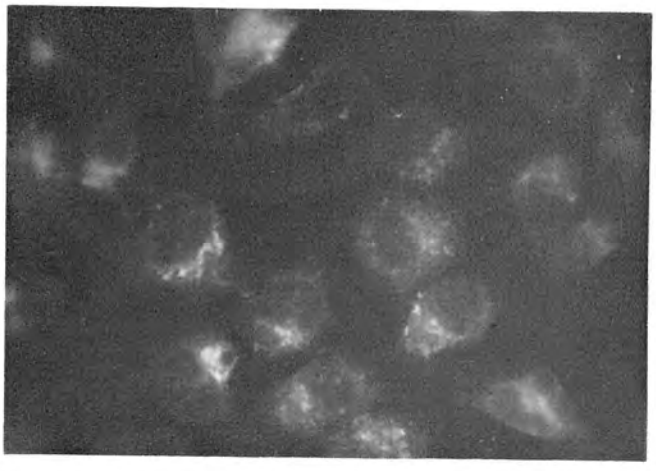

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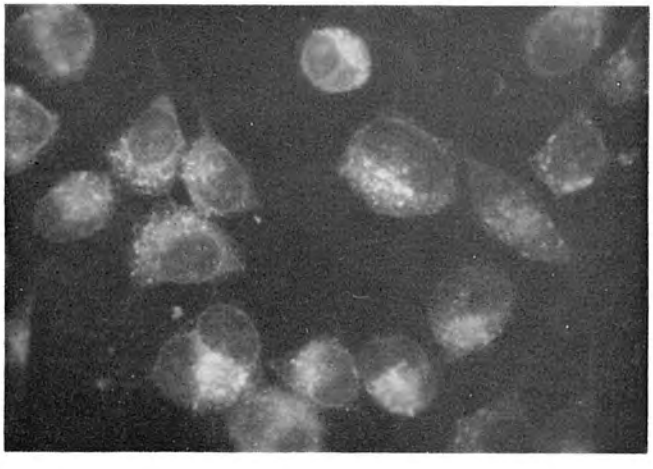

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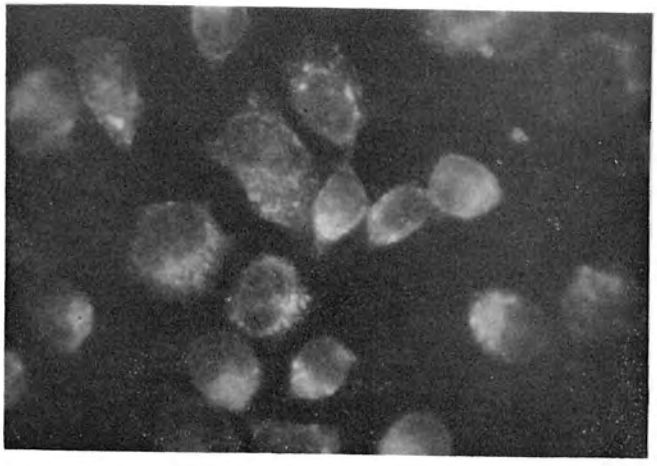

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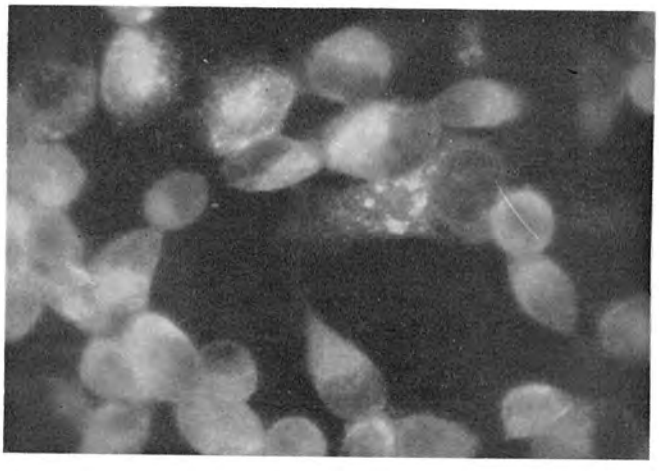

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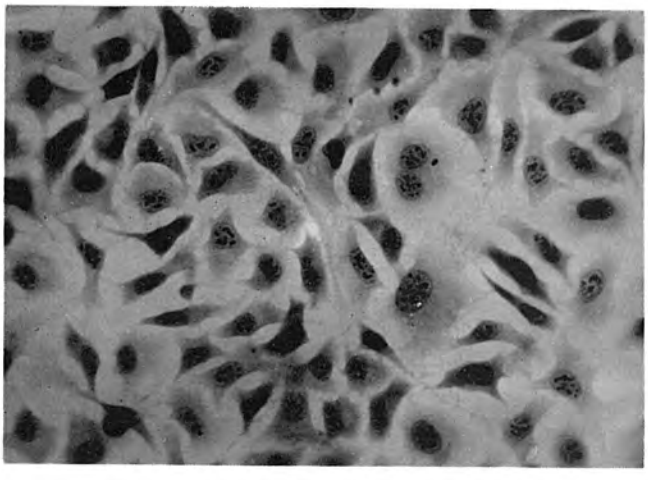

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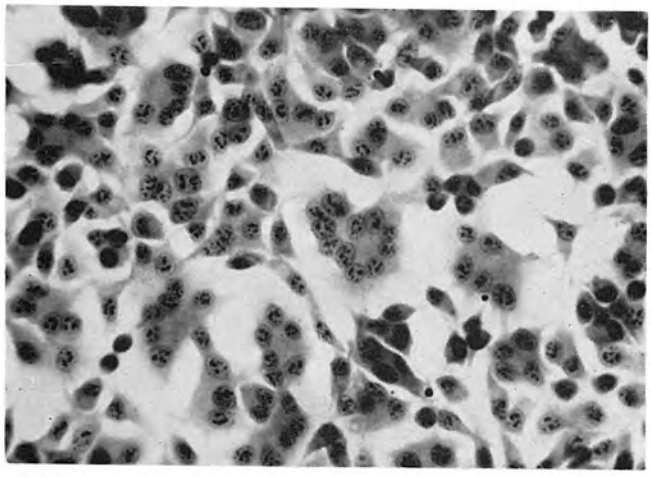

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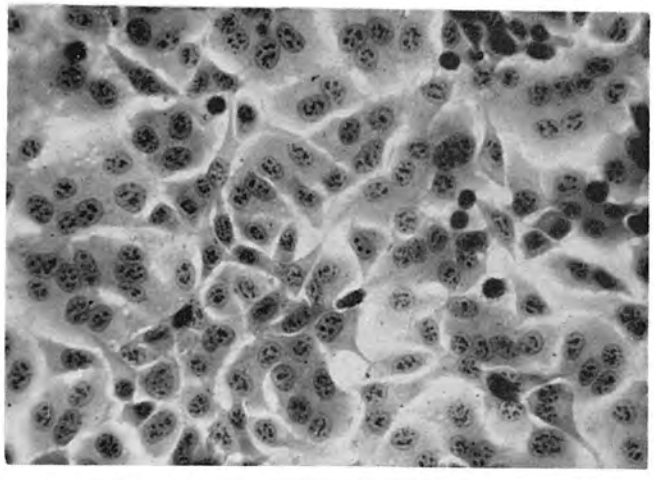

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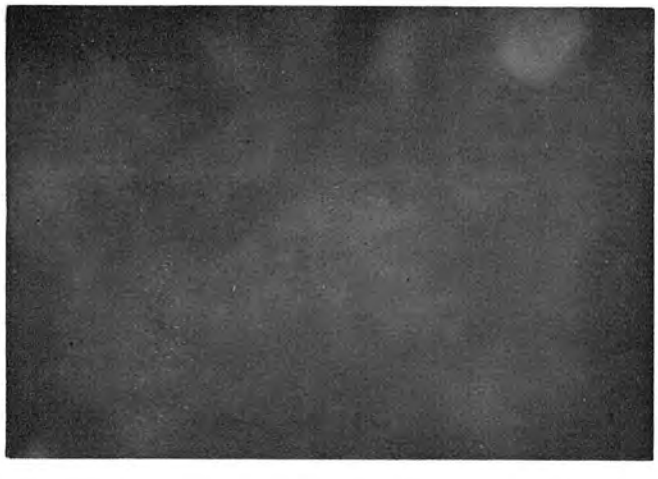

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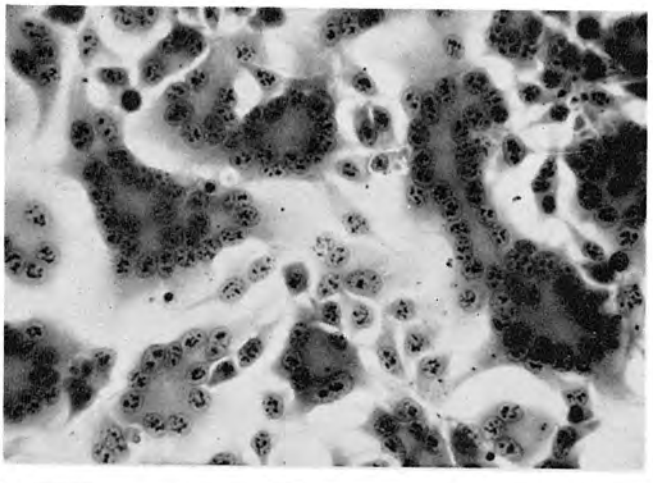

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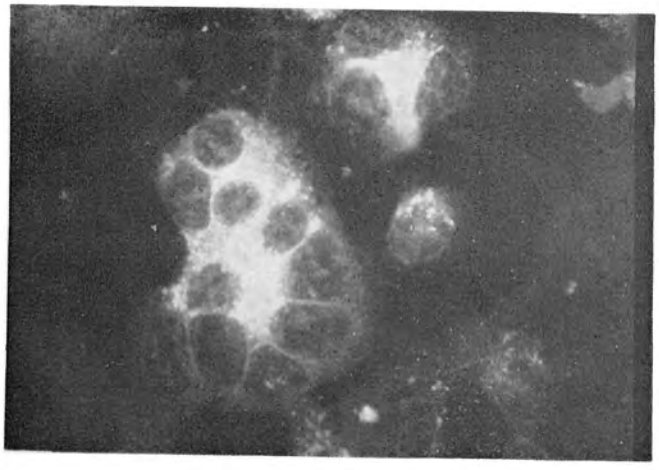

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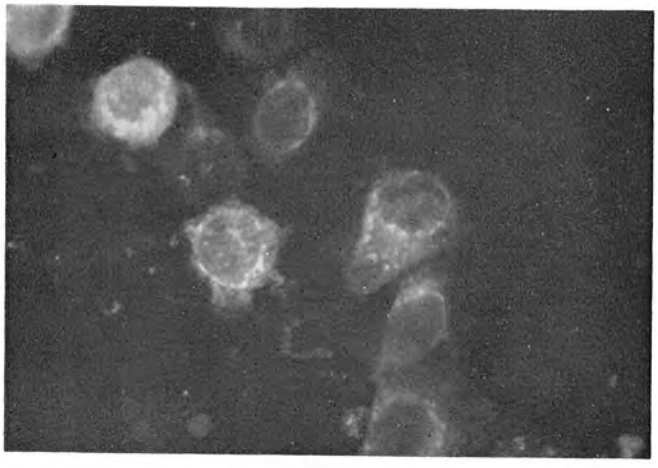

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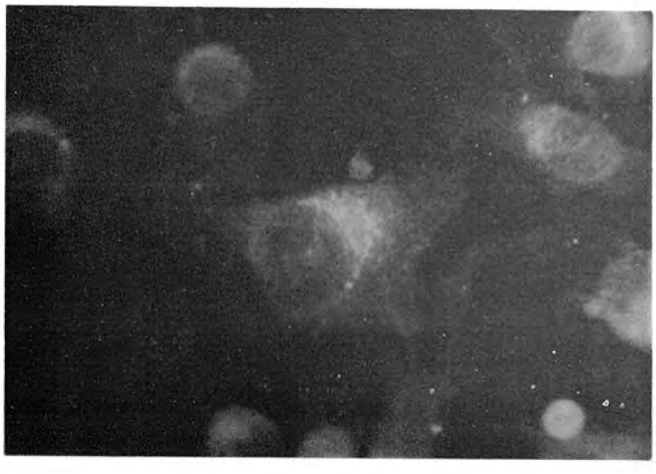

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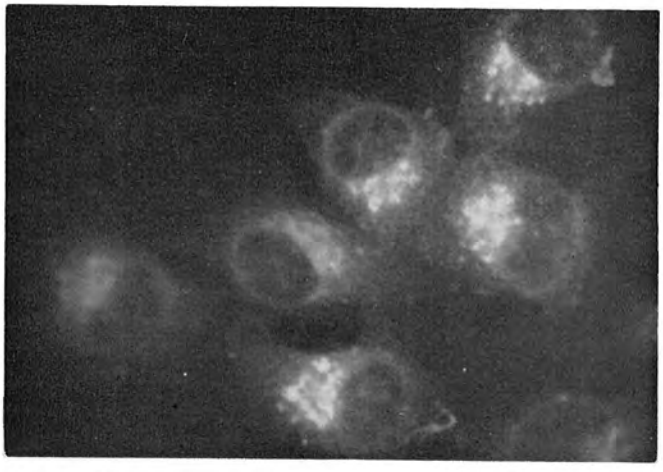

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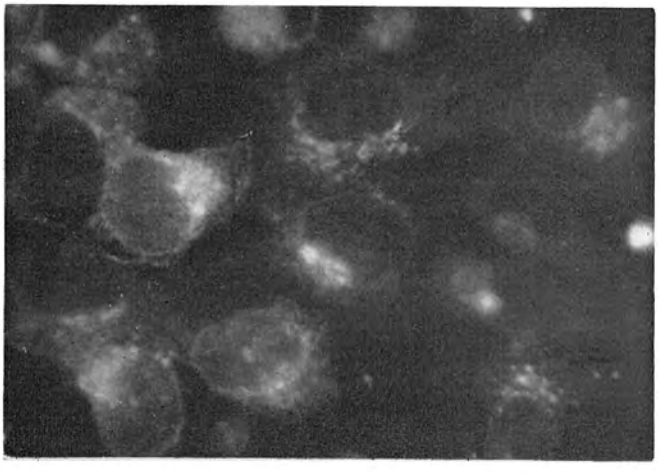

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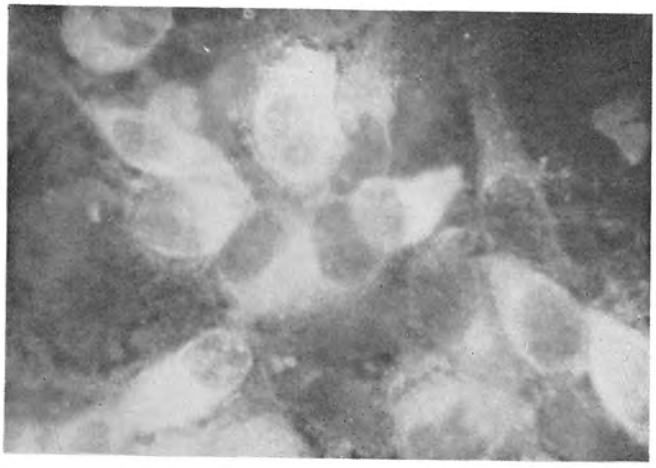

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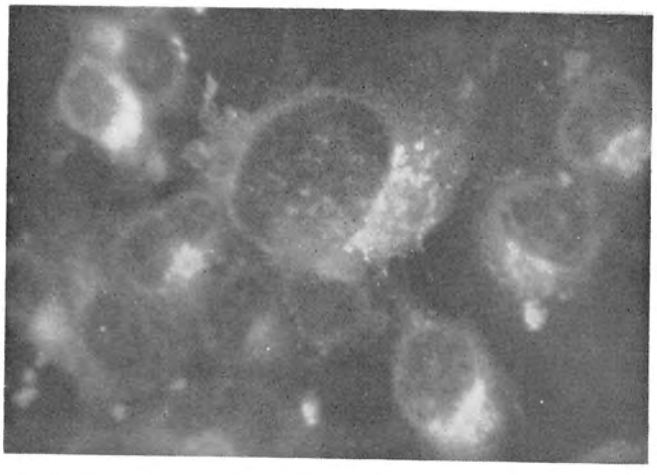

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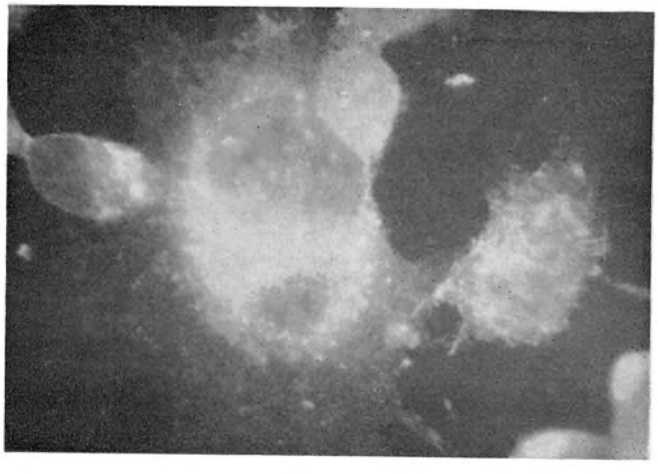

21