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Aspects of influenza C virus replication

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SUMMARY

Influenza C virus antigen could be detected by immunofluorescence in chick amniotic epithelium 4 hr. after inoculation with undiluted amniotic fluid. Antigen could be detected in the nucleus of rhesus monkey kidney tissue cultures 10 hr. after infection. Only cytoplasmic fluorescence was observed 17–21 hr. after infection.

The pattern of replication was similar to that reported for group I myxoviruses, indicating that the virus is correctly classified as an influenza virus.

INTRODUCTION

Taylor (1949) isolated a strain of influenza virus 1233, which appeared to be antigenically unique. Francis, Quilligan & Minuse (1950) isolated a JJ strain which proved to be identical with 1233, and they suggested that these strains should be designated influenza C virus. Quilligan, Minuse & Francis (1951) and, later, Minuse, Quilligan & Francis (1954) reported in detail on the properties of influenza C virus, which differ from those of A and B viruses to a greater extent than the properties of A and B viruses differ from each other.

We investigated the replication of influenza C virus in rhesus monkey kidney cells and in the amnion of chick embryos by immunofluorescent methods to discover whether the pattern resembles that reported for influenza A and B (Watson & Coons, 1954, and many subsequent workers) or that reported for myxovirus group 2 and paramyxoviruses (Wheelock & Tamm, 1959; Traver, Northrop & Walker, 1960).

MATERIALS AND METHODS

Viruses

The following two strains were used: influenza C/Paris/1/67 and influenza A 2/Hong Kong/1/68.

Conjugate

SWAR, swine anti-rabbit globulins, obtained from Nordic Diagnostics, Fraburg Ltd, was used at a 1/20 dilution.

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Antisera

The following were used.

(1) A/4/69. Influenza C antiserum produced by inoculations of infected amniotic fluid into rabbits. It contained antibodies to both V and S antigens. Haemagglutination-inhibition (HAI) titre 1/512; complement fixation (CF) titre 1/64.

(2) A/82/68. Influenza C antiserum produced by inoculations into rabbits of infected rhesus monkey kidney cell culture. HAI titre 1/1280.

(3) Anti-V serum. Influenza C anti-V serum was prepared in guinea-pigs by the method of Lief & Henle (1956, 1959). HAI titre 1/256; CF titre 1/64.

(4) Human convalescent serum. Influenza A/Hong Kong antiserum. CF titre 1/2048.

All antisera were treated with periodate according to the method of Nelson & Lewis (1958), then filtered through a modified Hemmings membrane filter. They were used at dilutions of 1/8 to 1/10.

Tissue cultures

The tissue cultures were maintained in equal parts of Eagle's MEM and 199 solutions containing penicillin (200 units/ml.) and neomycin (50 units/ml.).

Immunofluorescence technique

Primary monkey kidney cell sheets were prepared on cover-slips. The approximate number of cells per cover-slip was $3{\cdot}4 \times 10^5$. One drop of clarified amniotic fluid, containing about 2·5–5 egg infective particles of influenza C per cell, was placed on the cell sheet and left to adsorb at 30° C. for 1 hr. The inocula were then washed off with maintenance medium and the cover-slips incubated at 37° C. in a CO_2 incubator for various times. The cell sheets were fixed in acetone at −20° C. for 30 sec. and, after washing in phosphate buffered saline (PBS), antiserum was added and they were incubated at 37° C. for 30 min. in a moist atmosphere. After thorough washing with PBS, the preparations were counter-stained with conjugate for 30 min. at 37° C. in a damp atmosphere, then stained with Evans Blue, 1/2000 in water (w/v), and finally washed, dried and mounted. A series of preparations were also made with influenza A/Hong Kong virus.

Controls

In every test, one cover-slip was tested for haemadsorption to confirm that the cells were infected. The criteria of specificity of staining were: (*a*) immunological staining of infected but not of uninfected cells, (*b*) no staining when normal serum was substituted for antiserum, (*c*) no staining with influenza C antiserum of cells infected with Hong Kong virus.

Preparation of amniotic sections

Ten-day embryonated eggs were inoculated amniotically with 0·2 ml. of a 1/100 dilution of influenza C/Paris, having an HA titre of 1/64, and incubated at 37° C. for the required number of hours before harvest of the amnion. Uninfected

control amnions treated with SWAR or normal rabbit serum were also examined at every stage.

After washing in PBS the amnion was fixed by immersion in acetone at $-20°$ C. for 30 sec. After a further wash in PBS it was frozen and sections cut on a Slee microtome were collected on cover-slips. Thereafter the immunofluorescent staining procedure already described was followed.

RESULTS

Development of influenza C/Paris in amniotic membrane

A series of sections of infected amnion were examined at intervals of 0–45 hr. after inoculation. The sections were stained with A/82/68 rabbit antiserum and counter-stained with swine anti-rabbit gamma globulins. The sections showed virus development to be negligible at 3 hr., well established at 4 hr., and then progressively increasing until 45 hr., when the entire membrane was stained (Plate 1, figs. 1, 2). Controls throughout the series showed no fluorescence.

Development of influenza C/Paris in rhesus monkey kidney cells

A/4/69 rabbit antiserum (containing both S and V antibodies) and swine anti-rabbit gamma globulins produced no specific fluorescent staining 2–8 hr. after infection. Occasionally very fine, brightly fluorescing individual granules were observed on the surface of single cells, probably representing unadsorbed virus particles (Plate 2, fig. 1). No haemadsorption was detected in the tissue culture at this stage. At 10 hr., strong granular nuclear, nucleolar, and cytoplasmic fluorescence was observed. At no time during the experiments was a cell observed with only nuclear fluorescence (Plate 2, figs. 2, 3). A perinuclear halo was seen in some cells (Plate 2, fig. 3). At this stage the inoculated cell sheets showed slight haemadsorption. By 17 hr. the fluorescence was mainly cytoplasmic. Traces of remnant S antigen were seen in some nuclei giving them a stippled appearance (Plate 3, fig. 1). Perinuclear halos were still present in some cells, and haemadsorption had slightly increased.

At 21 hr. no nuclear fluorescence was seen and many more cells exhibited cytoplasmic fluorescence (Plate 3, fig. 2). Fluorescing multinucleate cells (Plate 4, fig. 1), cells with fibrous extensions (Plate 5, fig. 1) and also elongated cells were observed (Plate 4, fig. 2). A cytopathic effect began to develop in the cells (Plate 4, fig. 3), and haemadsorption showed a further increase. At 24–36 hr. nuclear fluorescence was again observed (Plate 5, fig. 2), presumably due to secondary infection since antiserum had not been added after virus had been allowed to infect the cells, and from 48 to 72 hr. the fluorescence was granular and cytoplasmic. Cells were beginning to detach from the cover-slips. Multiple patches of haemadsorption were now visible.

As with other influenza viruses, the nucleoli were prominent throughout the infection with influenza C virus, and fluoresced brightly during the nuclear stages of multiplication. Throughout the experiments only a small percentage of the cells were infected, usually in groups of 2 or 3.

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Using anti-V serum on infected secondary monkey kidney cells and staining with SWAR only cytoplasmic fluorescence was observed from 10 to 12 hr. onwards.

Control with influenza virus A/Hong Kong

Primary monkey kidney cells inoculated with influenza A/Hong Kong were treated after 4, 6 and 8 hr. with convalescent antiserum and counter-stained by SWAR. At 4 hr. fluorescence was weak, but at 6 hr. strong nuclear fluorescence was present.

DISCUSSION

It has been shown by immunofluorescence of infected cells that soluble antigen of influenza A and B viruses is first synthesized in the nucleus and moves later to the cytoplasm (Watson & Coons, 1954; Lui, 1955; Breitenfeld & Schäfer, 1957; Franklin, 1958), whereas the antigen of myxovirus group 2 and paramyxoviruses has been detected only in the cytoplasm of cells (Wheelock & Tamm, 1959; Traver *et al.* 1960).

The influenza C/Paris virus investigated in the present paper conforms to the immunofluorescent pattern produced by influenza A and B viruses. Soluble antigen was first detected in the nucleus 10 hr. after infection. This is considerably later than the time of 3 hr. first reported for fowl plague virus (FPV) by Breitenfeld & Schäfer (1957) and 3 hr. reported for influenza A and B viruses by Watson & Coons (1954) and many subsequent workers. Our influenza A/Hong Kong control tests also took longer than 3 hr. (5–6 hr.) to develop strong nuclear immunofluorescence. The nucleoli exhibited strong fluorescence at 10 hr., as had been observed in FPV and influenza A and B infections. By 17–21 hr. only cytoplasmic fluorescence was found. Again, the time is much later than the 5–6 hr. for influenza A and B viruses reported by Breitenfeld & Schäfer (1957) and Watson & Coons (1954). No nuclear fluorescence was observed when specific anti-V serum was used. V antigen, also, first appeared in the cytoplasm 10 hr. after infection. At this time, too, weak haemadsorption confined to individual cells was noted. The appearance of considerable fluorescence in the amnion of infected embryo at 4 hr. approaches the time of development of antigen as reported by Breitenfeld & Schäfer (1957) and Watson & Coons (1954). Clearly, influenza C grows much more slowly in tissue culture than in eggs.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Section of chick amnion 4 hr. after inoculation with influenza C/Paris. Outer cells of membrane (section between arrows) fluorescing. (Blue light, 15 sec.)

Fig. 2. Section of chick amnion 45 hr. after infection with influenza C/Paris. Entire membrane fluorescing. (Blue light, 10 sec.)

PLATES 2–5. Magnification × 400

PLATE 2

Fig. 1. Monkey kidney cells 6 hr. after infection with influenza C/Paris. Individual fluorescing granules. (Blue light 25 sec.)

Fig. 2. Single infected monkey kidney cell exhibiting bright nuclear and cytoplasmic fluorescence. (Blue light, 25 sec.)

Fig. 3. Central group of infected monkey kidney cells showing nuclear and cytoplasmic fluorescence. One cell (arrowed) has a perinuclear halo. (Blue light, 30 sec.)

PLATE 3

Fig. 1. Influenza C/Paris infected monkey kidney cell; 17 hr. Large central cell with granular fluorescing cytoplasm. The nucleolus is unstained and the nucleus contains granules of weakly staining remnant S antigen. (Blue light, 30 sec.)

Fig. 2. Influenza C/Paris infected monkey kidney cells at 21 hr. Five cells with bright cytoplasmic fluorescence; the nuclei are unstained. (Ultraviolet, 1 min.)

PLATE 4

Fig. 1. Influenza C/Paris infected monkey kidney cells; 36 hr. Large binucleate (nuclei arrowed) cell – bright cytoplasmic fluorescence, the nucleoli and nucleus are unstained. (Blue light, 15 sec.)

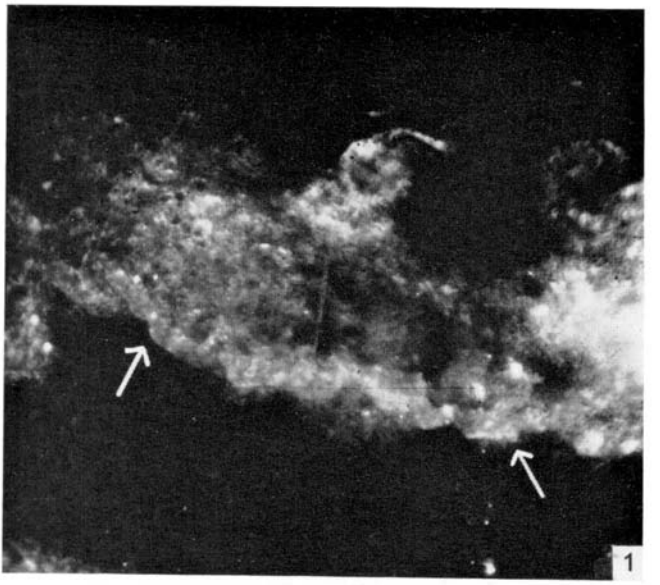
Fig. 2. Influenza C/Paris infected monkey kidney cells; 21 hr.; 1 central extended cell with cytoplasmic fluorescence only. (Ultraviolet, 45 sec.)

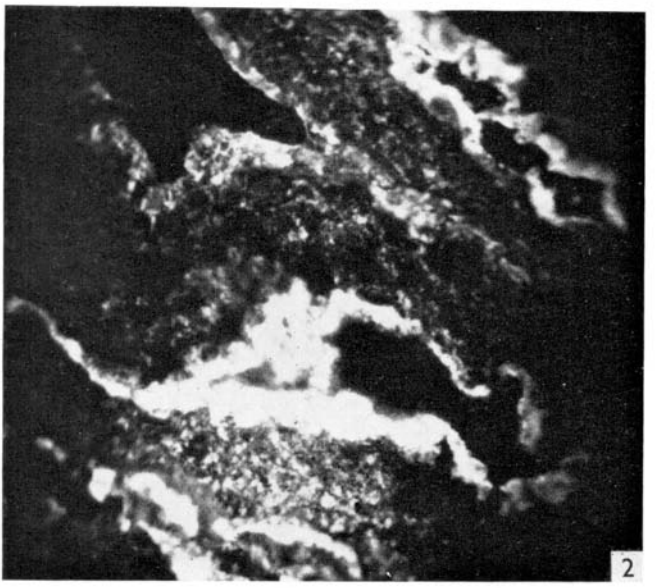
Fig. 3. Influenza C/Paris infected monkey kidney cells; 21 hr. Cytoplasmic fluorescence. Early cytopathic effect. Group of rounded cells – some with cytoplasmic fluorescence. (Ultraviolet, 80 sec.)

PLATE 5

Fig. 1. Influenza C/Paris infected monkey kidney cells; 21 hr. Large cell with fibrous extensions. Cytoplasmic fluorescence. (Blue light, 30 sec.)

Fig. 2. Influenza C/Paris infected monkey kidney cells; 36 hr. Strong nuclear and cytoplasmic fluorescence in two central cells. (Blue light, 15 sec.)





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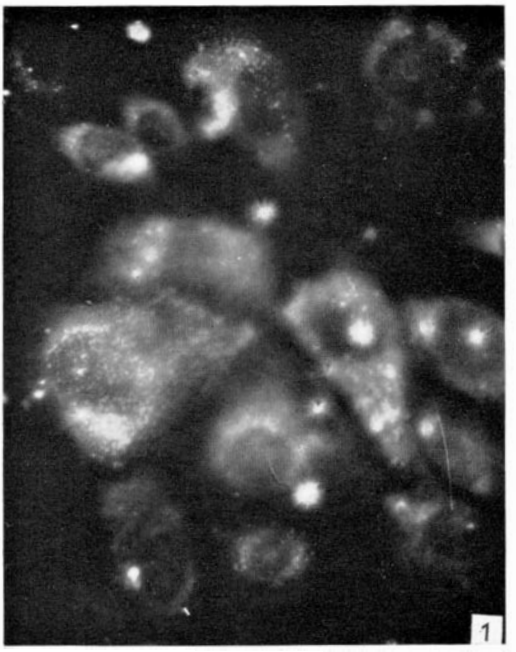

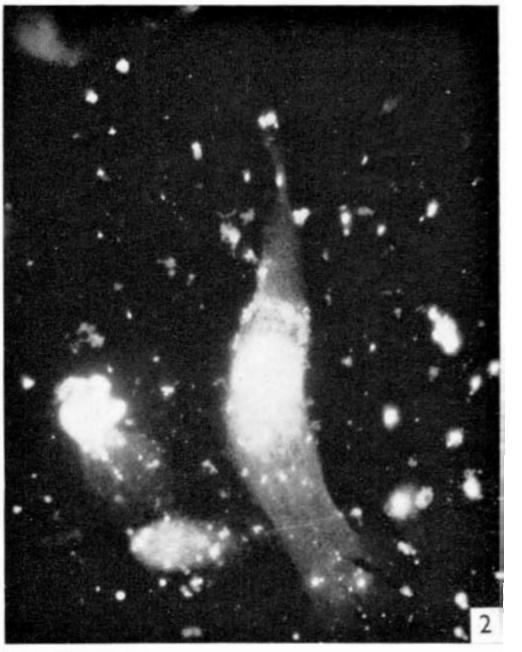

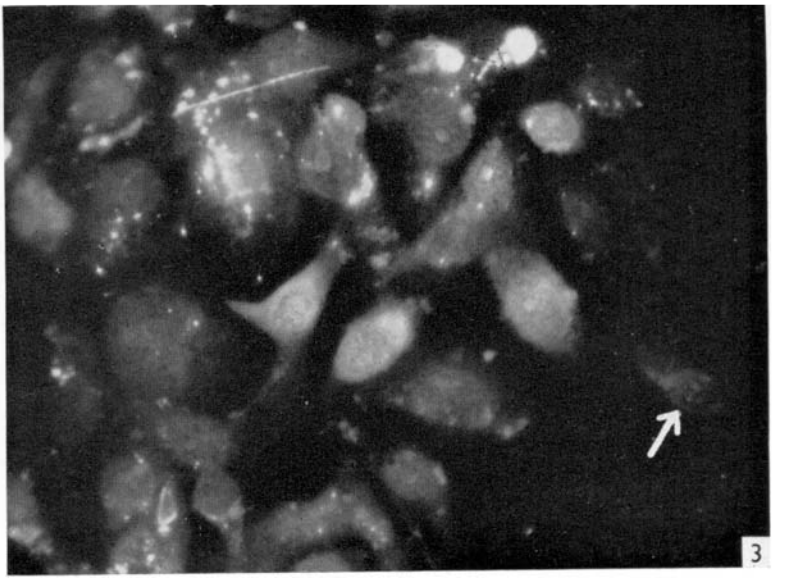

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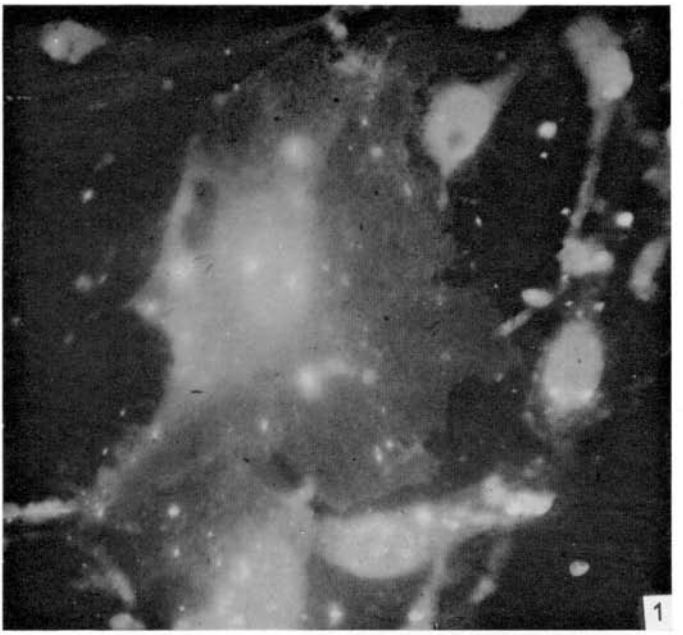

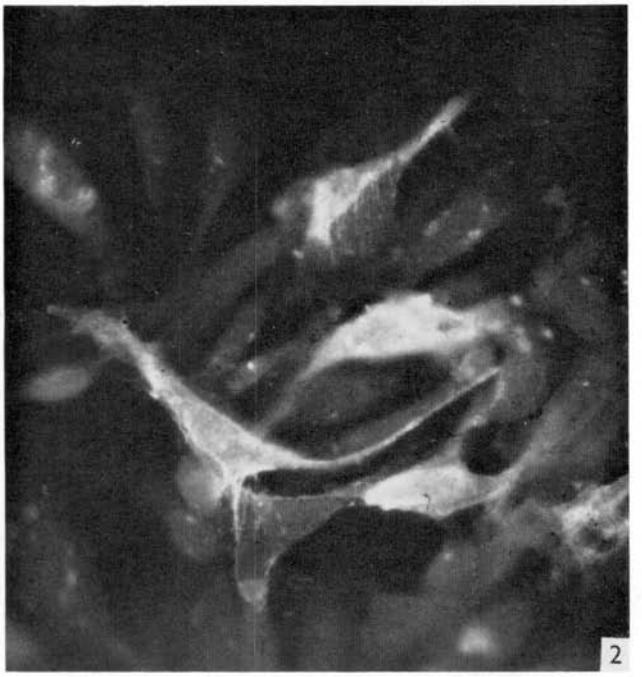

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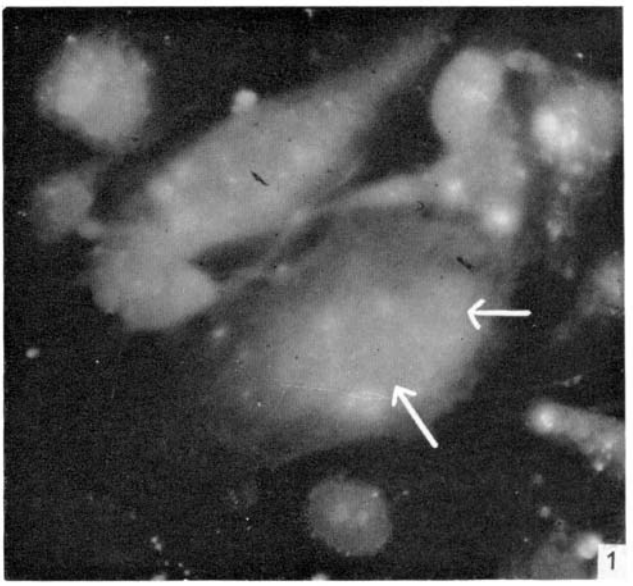

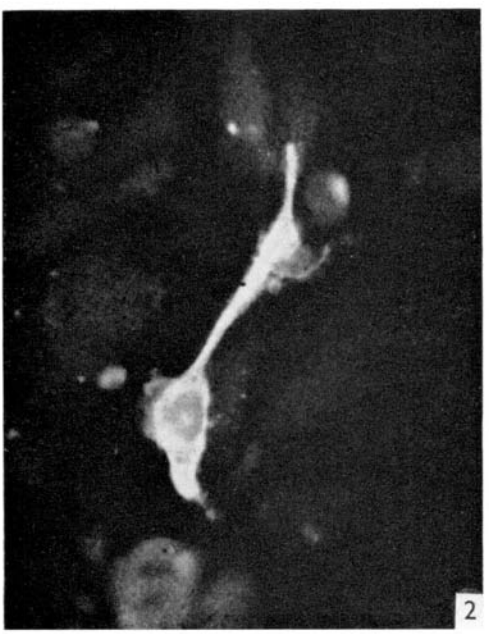

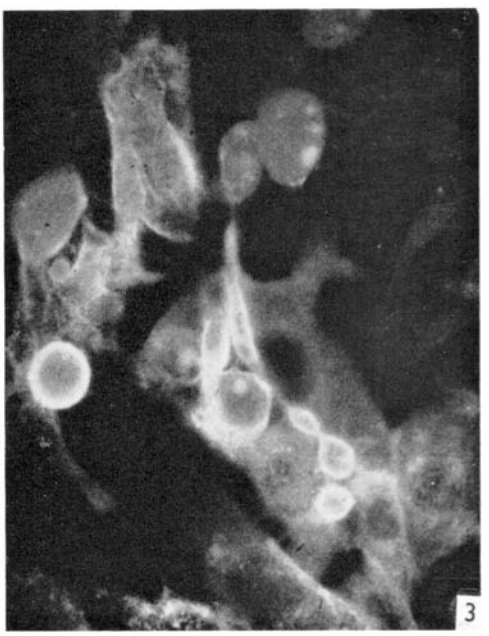

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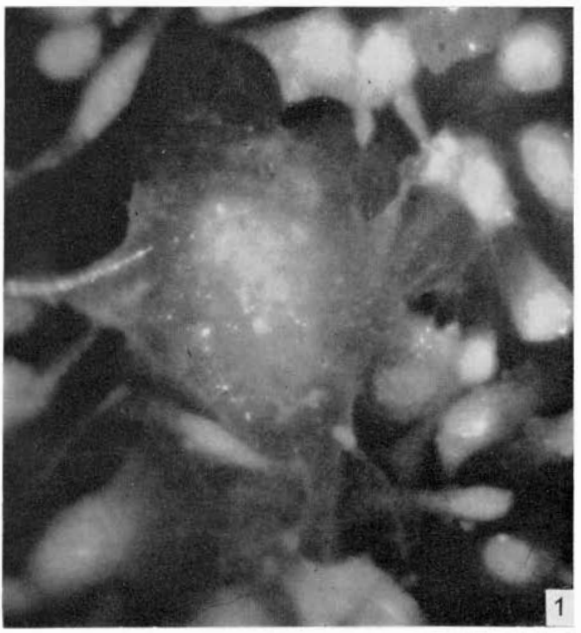

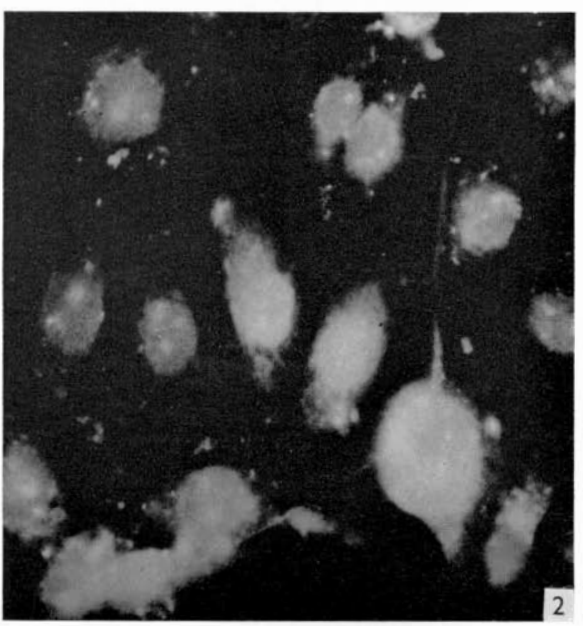

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