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## Virological Studies on Measles Virus

### II. Growth of Toyoshima Strain in Four Established Cell Lines

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#### SUMMARY

The measles virus, isolated and established in FL cells, readily grew in three different kinds of established cell lines of human origin (HeLa, hepatic and conjunctival) when large inocula were used.

The earliest appearance of CF-antigen in FL cells was 24 hours after inoculation, which is earlier than that previously reported by Black (1959). In FL cells cytopathic change and cytoplasmic inclusion bodies were also observed as early as 24 hours after infection and intranuclear inclusion bodies were present 2 days after infection.

In other cells cytopathic change appeared a little later and the two types of inclusions were not so easily detected as in FL cells. However, in HeLa cells cytoplasmic inclusion bodies were observed without difficulty 3 days after infection.

The susceptibility of the three cell lines to the virus, however, were 2 to 3 log units lower than that of FL cells in the 1st passages. The susceptibility of hepatic cells did not exceed that of FL cells during a few passages of virus in hepatic cell line.

#### INTRODUCTION

Adaptation of laboratory strains of measles virus to continuous cell lines was accomplished with some difficulty. The appearance of cytopathogenic effect of Edmonston strain passed in human amnion cultures, required 4 to 5 day in a KB cell line (Dekking and McCarthy, 1956) and much longer period in other cell lines (Black *et. al.*, 1959). On the other hand Edmonston strain already adapted to a Hep-2 culture grew readily in the FL strain (Frankel and West, 1958) and in other human or simian cell lines (Girardi, 1957). The virus yields and the cytopathic changes were different in each virus-cell system. In some systems high virus yields could be obtained (Black *et. al.*, 1956; Mutai, 1959).

To select cell strains suitable for virus growth and for virus titration, four established cell lines were tested for their sensitivity and virus yield using a strain isolated in our laboratory.

#### MATERIALS AND METHODS

##### 1. *Virus*

Toyoshima strain of measles virus was used at 10th passage in FL cells. This strain

was isolated and established in FL cells (Toyoshima *et al.*, 1959).

## 2. *Tissue culture cells*

A human amnion cell line (FL strain), HeLa cells, a hepatic cell line (Chang, 1954) and a conjunctival cell line (Chang, 1954) were used. The last two lines were kindly given to us by Dr. Yamada (N.I.H. Japan).

## 3. *Medium*

LE with 15 per cent ox serum for the growth of cells and LE with 3 per cent ox serum for the maintenance medium were used.

## 4. *Titration*

The method described in the preceding paper was employed. Unless otherwise specified, tubes of FL cells were used.

## 5. *Infection with a large inoculum*

Tubes containing nearly  $1 \times 10^6$  cells and 1 ml of growth medium were prepared. After removing the growth medium, 1 ml of virus were added to each tube and incubated for one hour at 37°C. Fluid was decanted and the culture was washed 3 times with 4 ml of phosphate buffered saline (PBS), then 1 ml of maintenance medium was added and the tubes were reincubated. The medium was changed on the 4th day after infection. At the appointed time, a pair of tubes were centrifuged and the supernatant fluids were pooled. Then, 1 ml of maintenance medium per tube was added to each tube. They were stored at -20°C. Immediately before titration, the fluid from pairs of tubes containing infected cells was mixed.

The fluid of slide cell cultures were replaced by an equal volume of virus sample to the growth media. After one hour's incubation the slides were washed and reincubated before morphological observations.

## 6. *Infection with a small inoculum*

0.2 ml of the virus sample was inoculated into a tube. 1 ml of maintenance medium was added to each tube after one hour's incubation.

The medium was changed at 2 to 4 day intervals.

## 7. *Complement fixation test*

Kolmer's method was employed for the test.

## 8. *Fixation and staining*

Slides were fixed with Bouin's fixative and stained with hematoxylin and eosin.

## 9. *EDTA treatment*

After washing with PBS (Ca and Mg free), 0.02 per cent EDTA in PBS (Ca and Mg free) was added to the cultures and after 20 minutes' incubation at 37°C, cells were dispersed with shaking.

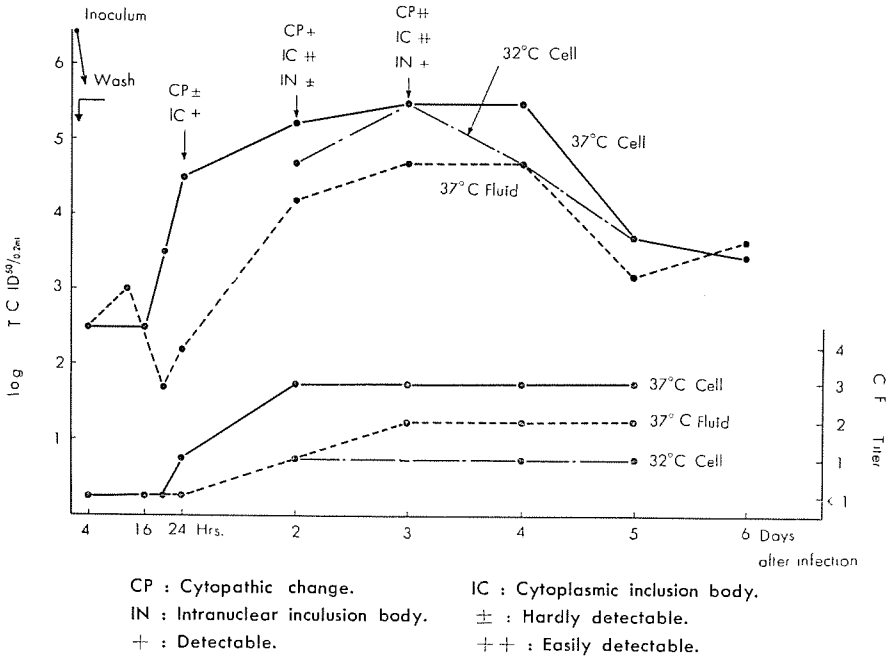
# RESULTS

## 1. *Growth of the virus in FL cells*

Samples were collected at 4 hour intervals until 24 hours after infection, and at one day intervals thereafter (Fig. 1).

The earliest rise of virus titer was observed 20 hours after inoculation in cells and the maximum titers were detected 2 to 4 days after inoculation. The virus titer in the supernatant was lower than in the cells and its increase could not be detected until the 3rd day of incubation.

Fig. 1. Growth curve of measles virus established in FL cells in the same cell line.



CF-antigen was found in cells first as early as 24 hours after infection, and reached a maximum at the 3rd day, keeping a constant titer thereafter until the 6th day. The appearance of CF-antigen in fluid delayed and the maximum titer was also lower.

The cytopathogenic effect with few giant cells was first detected at 24 hours, and became clear 2 days after infection. Cells began to decrease in number at the 3rd day and relative small number of cells including many giant cells were seen after 4 days.

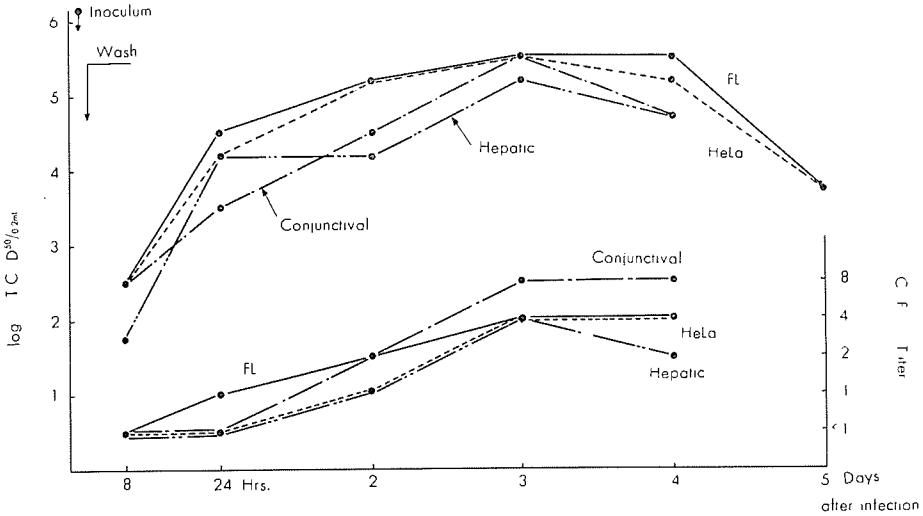
Cytoplasmic inclusion bodies were detected in the stained preparations at 24 hours, and clearly seen 2 days after infection. Intranuclear inclusion bodies were found in the 3rd day. After that, the number of inclusion-bearing nuclei increased gradually.

2. Comparison of virus growth in four different tissue cultures (FL cells, HeLa cells, hepatic cells and conjunctival cells) infected with a large inoculum of the FL cell passed virus

Paired tissues were frozen at 8 and 24 hours after infection and then at one day intervals. Titrations were made on total infected cultures including cells and fluid (Fig. 2).

An increase in virus titer was observed at 24 hours and the maximum titer was obtained 3 days after infection in all 4 cultures. The appearance of CF-antigen was first detected in FL cells 24 hours after infection and in the 3 other cells

Fig. 2. Growth of measles virus established in FL cells in FL, HeLa, conjunctival and hepatic cells.



after 2 days. Nearly equal maximum titers of virus were obtained in all 4 cell lines. At the earlier stages, the titer of virus was a little higher in FL cells than in the 3 others.

A cytopathogenic effect appeared earliest and most clearly in FL cells (Fig. 3, 4) and was detectable in the 3 other cells one or two days later than in FL cells.

Both cytoplasmic and intranuclear inclusion bodies were present in all the lines 3 days after infection. In HeLa cells cytoplasmic inclusion bodies appeared first after 2 days, and one day later, intranuclear inclusion bodies could be seen and the cytoplasmic inclusion bodies increased in their number. Inclusion bodies in hepatic and conjunctival cells could be observed when they were seen in the HeLa cells, though they appeared in fewer cells. (Fig. 5-11)

### 3. Susceptibility of the 4 tissue cultures to virus passed in FL cells

Ten fold serial dilutions of virus samples harvested from FL cells were inoculated in triplicate into tissue culture cells of 4 different kinds of cell lines.

The appearance of cytopathic change at all dilutions was a few days earlier in FL cells than in the 3 other cell lines.

As shown in Table 1, the maximum virus dilution which caused much cytopathic change within 2 weeks was also 2 to 3 log units higher in FL cells than in the three other cell lines, and number of cultures showing cytopathic change did not increase in the next two days.

### 4. Serial passage of virus in hepatic cells

Virus established in FL cells was inoculated and passed twice in hepatic cells (Table 2a).

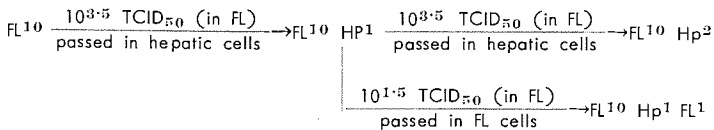
Table 1. Susceptibility of FL, HeLa, conjunctival and hepatic cells to FL passaged virus.

Cell line \ Virus dilution	FL	HeLa	Conjunctival	Hepatic
10 <sup>-2</sup>	3/3	3/3	3/3	3/3
10 <sup>-3</sup>	3/3	3/3	3/3	3/3
10 <sup>-4</sup>	3/3	9/3	1/3	2/3
10 <sup>-5</sup>	3/3	9/3	9/3	9/3
10 <sup>-6</sup>	3/3	9/3	9/3	9/3
10 <sup>-7</sup>	9/3	9/3	9/3	9/3
Susceptibility	1	10 <sup>-3</sup>	10 <sup>-2.8</sup>	10 <sup>-2.3</sup>

Results are expressed as CP positive tubes per total tubes. Susceptibility of cell line is expressed as TCID<sub>50</sub> of virus in each cell line/TCID<sub>50</sub> of the same virus in FL cells.

The susceptibility of hepatic cells to FL established and hepatic cell passed virus was compared with that of FL cells using tubes of each type of cells infected with 5 steps of ten-fold dilution of the viruses up to 10<sup>-7</sup> in triplicate. The virus passed once in FL cells after single passage in hepatic cells was tested for its susceptibility in both cell lines. (Table 2b) The susceptibility of hepatic cells to measles virus infection did not exceed that of FL cells during these passages.

Table 2 a. Passages of the virus



5. EDTA treatment

Virus passed once in hepatic cells was diluted in two steps at about the minimum required for infection and each dilution was inoculated in cultures of FL cells and of hepatic cells in triplicate.

After two weeks neither FL cells nor hepatic cells showed cytopathic change at the higher dilution. At the lower dilution, although FL cells showed much changes, one of three hepatic cell cultures only exhibited a slight change, and the other two showed so little change that the presence of virus was dubious. Cells were dispersed by EDTA treatment in cultures in triplicate except for the three FL cultures in which remarkable cytopathic changes were observed. After mild centrifugation the cells in each culture were resuspended in a double volume of maintenance medium to the volume of preceding culture fluid and seeded into tubes in duplicate.

Table 2 b. Susceptibility of FL and hepatic cells in various passages.

Virus	FL <sup>10</sup>		FL <sup>10</sup> Hp <sup>1</sup>		FL <sup>10</sup> Hp <sup>2</sup>		FL <sup>10</sup> Hp <sup>1</sup> FL <sup>1</sup>	
Passage No.	11		12		13		12	
Cell line	FL	Hp	FL	Hp	FL	Hp	FL	Hp
Virus dilution		1st Pass.		2nd Pass.		3rd Pass.		
10 <sup>-2</sup>	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
10 <sup>-3</sup>	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
10 <sup>-4</sup>	3/3	2/3	3/3	3/3	3/3	3/3	3/3	3/3
10 <sup>-5</sup>	3/3	0/3	3/3	1/3	3/3	3/3	3/3	3/3
10 <sup>-6</sup>	3/3	0/3	0/3	0/3	3/3	0/3	3/3	0/3
10 <sup>-7</sup>	0/3	0/3	0/3	0/3	0/3	0/3	1/3	0/3
Susceptibility	1	10 <sup>-2,3</sup>	1	10 <sup>-0.7</sup>	1	10 <sup>-1</sup>	1	10 <sup>-1,3</sup>

Results are expressed as CP positive tubes per total tubes. Susceptibility is expressed as TCID<sub>50</sub> of virus in hepatic cells/TCID<sub>50</sub> of virus in FL cells.

Cytopathic change became evident 2 days after this treatment in the pair of tubes containing hepatic cells which had shown a slight change, and 6 days after in the two pairs of hepatic cell tubes from the cultures which had represented doubtful cytopathic changes. The three remaining pairs of tubes of hepatic cells and all pairs of FL cell tubes showed no evidence of virus multiplication during observation for further eight days.

Table 3. The effect of EDTA treatment

Dilution	Observation	1st	2nd
	Cell		
lower	FL	+	none // //
	Hp	± ? ?	T. + 2 days* T. + 6 days* T. + 6 days*
higher	FL	- - -	T.- T.- T.-
	Hp	- - -	T.- T.- T.-

+ : Distinct CP change.

± : Little CP change.

? : Difficult to determine whether + or -.

- : No CP change.

\* : Days after EDTA treatment.

## DISCUSSION

Although an increase of virus in FL cells was observed 20 hours after infection with a large inoculum, the earliest appearance of progeny virus was uncertain since the remaining virus titer was as high as  $10^3$  TCID<sub>50</sub> after washing. When more thorough washing was performed the proportion of infected cells was one in ten, as determined by titration of the living cells suspension. In this case, the initial rise of virus titer could be observed only 4 hours later than that in the first experiment. This was probably because of the difference of inoculum size, which was 10 times larger in the first experiment. The infectivity of the virus was higher in the cells than in culture fluid in each case.

CF-antigen in infected FL cells was detected 24 hours after infection and at this time cytopathic changes with giant cell formation were also observed though not distinct. Very few cytoplasmic inclusion bodies could be found after 24 hours and intranuclear inclusions were seen after 2 days. After 3 to 4 days of infection, cells were apparently falling off from the walls of the tubes.

The earliest appearance of CF-antigen and cytological change is earlier than previously reported (Black, 1959) and this suggests a striking affinity of this strain to FL cells.

The susceptibility of other cell lines to virus isolated and established in FL cells were 2 to 3 log units lower than that of FL cells, in spite of the fact that nearly the same virus yield could be obtained in all cell lines including FL cells when cytopathic changes were maximal. The delay in appearance of CF-antigen, the maximum infective titer and the cytopathic changes in the three cell lines other than FL cells, were supposedly due to a difference between their susceptibility to the virus.

Cytoplasmic inclusion bodies were seen in relative small proportions of conjunctival, hepatic and HeLa cells on the 3rd day after infection, and in a large proportion of HeLa cells 3 days after infection. Intranuclear inclusion bodies could be observed in all of the three cell lines, but were few in number. The differences of morphological change in the various cell lines producing nearly the same virus yield has been discussed by Black *et al.* (1959).

Serial passage of virus was performed using the hepatic cell line because of this line showed the highest sensitivity to the virus and the most distinct cytopathic changes in unstained cultures of the three cell lines tested. During three serial passages of virus in hepatic cells, the susceptibility was a little lower in hepatic cells than in FL cells. There is a slight possibility that the susceptibility might increase if observations had continued over a longer period. Occasionally hepatic cell cultures infected with very small amounts of the virus showed so little cytopathic change that the presence of the virus was doubted even by careful observation with a light microscope. Such cells showed remarkable cytopathic changes within several days after treatment with EDTA. This supports the suggestions mentioned above and it suggests that the isolation in tissue culture cells might be increased by using EDTA.



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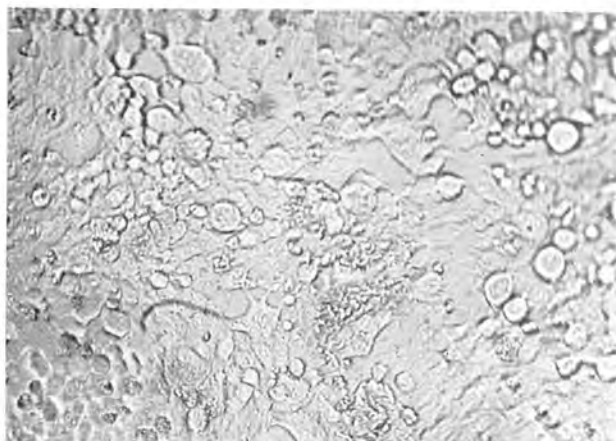


Fig. 3. CPC in FL cells 2 days after infection.  $\times 100$ .

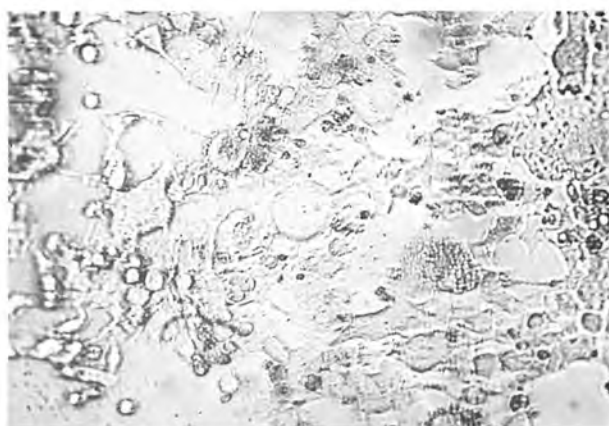


Fig. 4. Remarkable CPC in FL cells 4 days after infection.  $\times 100$ .

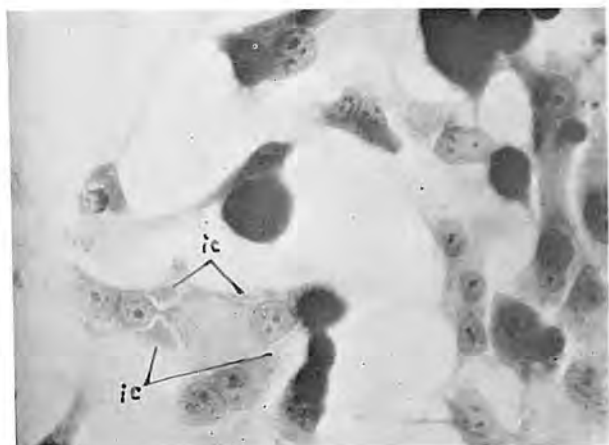


Fig. 5. FL cells 2 days after infection. H-E stain.  $\times 400$ .  
ic: cytoplasmic inclusion body.  
in: intranuclear inclusion body.

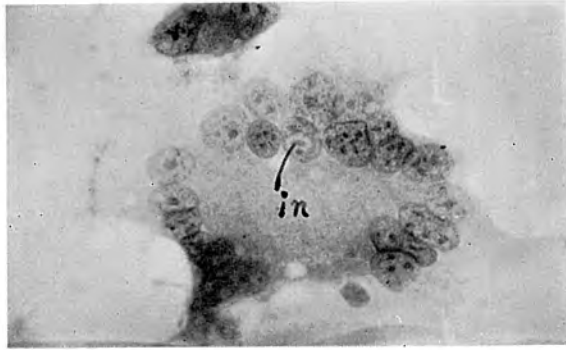


Fig. 6. FL cells 3 days after infection. H-E stain.  $\times 400$ .

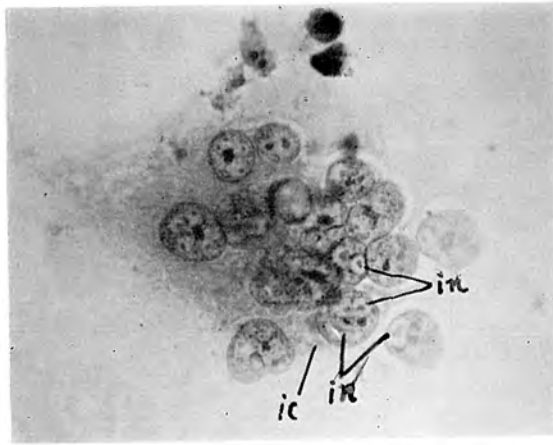


Fig. 7. FL cells 6 days after infection. H-E stain.  $\times 400$ .

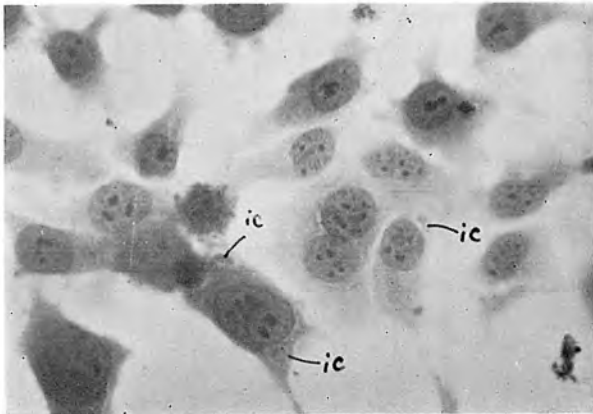


Fig. 8. HeLa cells 2 days after infection. H-E stain.  $\times 400$ .

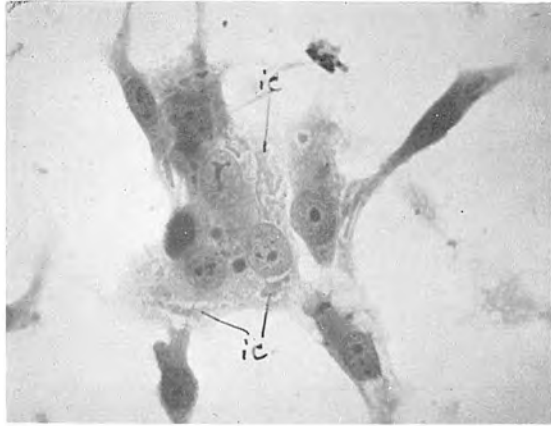


Fig. 9. HeLa cells 3 days after infection. H-E stain.  $\times 400$ .



Fig. 10. Hepatic cells 3 days after infection. H-E stain.  $\times 400$ .

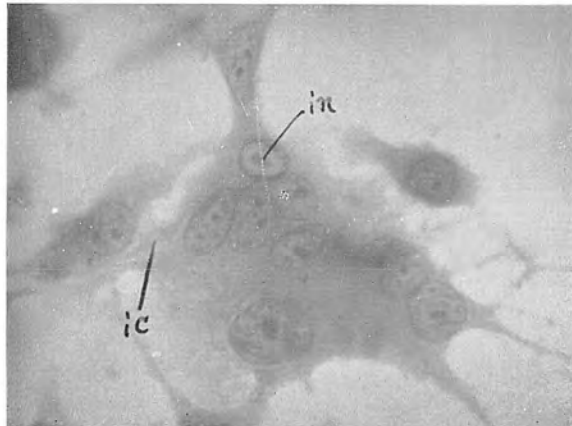


Fig. 11. Conjunctival cells 4 days after infection. H-E stain.  $\times 400$ .