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A Study on the Multiplication of Rabbit Myxoma Virus with the Fluorescent Antibody Technique

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SUMMARY

FL cell cultures infected with rabbit myxoma virus were studied by the fluorescent antibody technique and by light microscopy. The findings were correlated with the growth curve of the infectious virus, and development of complement fixing antigen determined from companion cultures.

By restaining the fluorescent cells with Giemsa solution, it was shown that "B" type inclusion bodies of this virus consisted of viral antigens.

Furthermore the appearance of fluorescent cells and inclusion bodies just before the increase in infectivity suggested that these inclusion bodies might be the main site of virus synthesis in the cells.

No distinct fluorescence was seen in the nucleus at any stage of infection implying that the virus synthesis took place exclusively in the cytoplasm.

INTRODUCTION

Although pathological studies on rabbit myxoma have long been made ever since the discovery of transmissible agents of this tumor, no coincidental evidence has been obtained as regards the localization and the stainability of the inclusion bodies of these tumor cells. Besides, no work on inclusion bodies has not been done from the standpoint of virus multiplication.

Chaproniere (1956) recently depicted the growth curve of myxoma virus in rabbit tisssue culture and described the appearance of eosinophilic inclusions in the cytoplasm and some alterations in the nuclei. Human amnion cells were found to be susceptible to the myxoma virus by Kato and Cutting (1959). They found inclusion bodies in human amnion cells as well as in the monocytes from the abdomen and the tumor tissue of infected rabbits. These inclusion bodies are Feulgen positive, and stained reddish purple with Giemsa solution, taking on a slight hematoxylin tinge with H.E. stain. The morphological and histochemical characteristics of these inclusion bodies of myxoma virus are the same as those of "B" type inclusion bodies of ectromelia, fowlpox, cowpox, canarypox, rabbit fibroma and vaccinia virus as reported before by Kamahora, Kato and their colleagues (Kato *et al.*, 1955; Kamahora *et al.*, 1955; Kamahora *et al.*, 1958; Kato and Cutting, 1959; Kato *et al.*, 1959).

The fluorescein-labelled antibody technique, by which intracellular viral antigen can be localized, provided a suitable way of gaining information on the antigenicity of inclusion bodies (Coons and Kaplan, 1950).

We have already reported in the preceding article (Takahashi *et al.*, 1958) that the "B" type inclusion bodies of myxoma virus have much viral antigen employing this method.

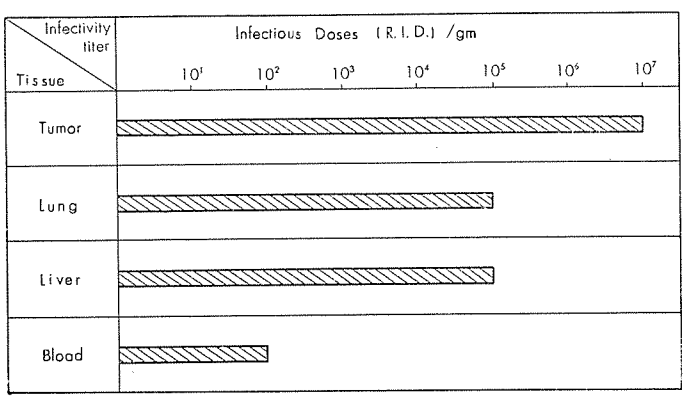
In this paper, this observation was confirmed in a sequential study of FL cells infected with myxoma virus. The results were correlated with the production of complement fixing antigen and infectious virus in companion cultures, to elucidate the role of "B" type inclusion bodies in the multiplication of myxoma virus.

MATERIALS AND METHODS

1. *Virus*

Myxoma virus obtained from American type cultures was used throughout. When this virus material was injected intradermally into albino rabbits, the infectious virus doses of various tissues of moribund tumor-bearing animals were observed. The results are shown in Fig. 1. It was apparent that tumor tissues contained the largest amount of virus, so that suspension of tumor tissue was used as the virus material in further experiments. The tumor tissue was removed and ground in a mortar, and diluted with Hanks' solution to 20 per cent. Then it was added with penicillin and streptomycin and centrifuged for 15 min at 3,000 rpm. The supernatant was used as the virus material.

Fig. 1 VIRUS DOSES OF VARIOUS TISSUES



R. I. D. = Rabbit Infectious Doses

2. *Cells and Cell Culture*

Human amnion cells (FL cells) obtained from the National Institute of Health, Japan, were used. The cell sheet was treated with 0.25 per cent trypsin and washed twice with P.B.S. After centrifugation, it was diluted with maintenance medium and about 10^5 number of cells were introduced into a series of tubes.

For cytological studies, the cells were grown on 8 × 50 mm strips of coverglass in Portor tubes. Companion cultures for virus growth curve tirations were carried out in tubes without coverslips. The handling of the cultures and inoculation of virus were the same in both series.

Maintenance medium was Hanks' balanced salt solution containing 0.5 per cent Lactalbumin Hydrolysate, 0.1 per cent Yeast Extract and 5 per cent calf serum.

3. *Antibody*

1.5 ml of virus material was irradiated in a Petri dish, 9 cm in diameter, with a 20 W

UV-light at the distance of 10 cm with constant shaking.

The exposure time was 20, 10, 5 and then 2 minutes, and 1 ml of the each batch of irradiated material was given intraperitoneally to the rabbits at weekly intervals. Finally 0.5 ml of the active virus material diluted to 1:100 was injected intravenously after further 10 days and blood was removed by cardiac puncture after 2 weeks. The titer of this immune serum was 1:640 in the complement fixation reaction.

4. *Preparation of the Conjugate*

The rabbit antiserum was concentrated by precipitation with half saturated ammonium sulphate in the cold, and dialysed to remove ammonium sulphate. It was then conjugated with fluorescein-isothiocyanate by the method of Marshall *et al.* (1958).

After removing non-bound fluorescein in the cold by dialysis, the conjugate was absorbed twice with 100 mg of acetone-precipitated mouse liver powder per ml of conjugate. This procedure removed material responsible for non-specific staining.

The titer of conjugated antibody was 1:80 in the complement fixation reaction and the neutralizing index was about 2.

5. *Infection of Tissue Culture Tubes*

Cultures were inoculated with 0.1 ml of virus material 2 or 3 days after cell distribution.

After a period of several hours to allow viral adsorption, the virus containing fluid was removed, and the cell sheet was washed two or three times with Hanks' solution to remove residual virus. Then 2 ml of maintenance medium was added in each tube.

6. *Infectivity Titrations*

At intervals after infection, the infectivity of both the medium and the cells was measured. The medium was harvested and centrifuged for 5 min. at 1,500 rpm and the supernatant was used as the original material.

After removal of the medium, the cell sheet was washed with P.B.S. and 2 ml of new medium were added. Pipetting was repeated 10 times to scrape off the cell sheet, and the cells were destroyed by freezing and thawing 10 times.

Virus was titrated in adult rabbits, 0.2 ml of ten fold dilutions of virus material in P.B.S. were inoculated intradermally along the shaved flanks of each rabbit.

The virus titer was expressed at the highest dilution which produced appreciable tumor before generalization of the disease usually on about the 7th day or later after inoculation.

7. *Titration of Complement Fixing Antigen*

The routine complement fixation test used in our institute for syphilis was used. For the hemolytic system, 3 per cent cow red cells, seven units of hemolysin, and 2 full units of complement were used.

0.25 ml of two-fold serial dilutions of the antigenic tissue material were mixed with 0.25 ml of immune sera diluted so as to contain 10 units of antibody and the same quantity of complement. The mixture was kept overnight at 4°C, then added with 0.25 ml of hemolytic system and incubated at 37°C for 30 min. The antigen titer was expressed at the highest dilution which gave 50 per cent hemolysis.

8. *Preparation of Cells for Microscopic Study*

The coverslip in the Porter tube was washed with P.B.S. as described above. It was withdrawn gently from the tube and air dried. One half of the cells were used for the fluorescent antibody test, and the other half were fixed with methanol and stained with Giemsa solution.

9. *Staining with Fluorescent Antibody*

The direct fluorescent antibody technique described by Coons and Kaplan was used for the visualization of viral antigens. The coverslip was fixed in acetone for 10 min. at room temperature and air dried.

The coverslip was flooded with fluorescein-labelled antibody, covered with a Petri dish containing moist paper to prevent evaporation, and incubated for 60 min. at 37°C. After incubation, the coverslip was washed for 10 min. in three changes of P.B.S. and mounted in buffered glycerin (PH 7) and examined under the fluorescent microscope. The specific yellow-green fluorescence was readily discriminated from greyish blue autofluorescence of the cells.

After a suitable area of fluorescence was located and photographed, the slide was removed and stained with Giemsa solution. The slide was then replaced under the microscope and a photograph of the same field was taken with visible light. In this manner, by comparing photographs, fluorescent objects could be correlated with their color-stained counterparts.

10. *Fluorescent Microscopy and Photography*

Fluorescence Microscope: An AH-6 water-cooled mercury vapor lamp modified in our laboratory was used as the light source. Source filters included a standard No. 5850 and a half thickness of a 5840 Corning glassfilter.

Fluorecence Photomicrography: All photomicrographs were taken with a 35 mm Canon camera and SSS film (Fuji) and the exposure time was about 3 min.

11. *Specificity of the Fluorescent Staining*

The specificity of the fluorescent staining was established in the following way.

1. Uninfected cultured cells did not exhibit any staining when stained with fluorescent antibody.

2. Pretreatment of infected cultured cells with non-conjugated rabbit antiserum inhibited staining by fluorescent antiserum, whereas pretreatment with normal rabbit serum did not inhibit the staining.

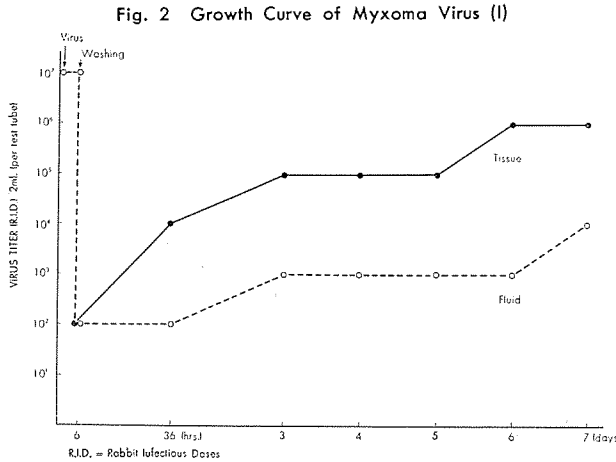
RESULTS

1. *Comparison of Fluorescent Cells with the Same Cells restained with Giemsa solution*

A study was made of the growth of myxoma virus in FL cells and of whether the fluorescent areas correspond to inclusion bodies.

6 hours after inoculation of the virus material, medium was removed and the cell sheet was washed twice with Y.L.H. medium. 36 hours, 3 days, 4 days, 5 days

Fig. 2 Growth Curve of Myxoma Virus (I)



and 6 days after infection, the virus titers of both medium and cells were measured.

As shown in Fig. 2, the virus titer in the cell just after washing was 10^3. It gradually increased as the infection proceeded, finally reaching the titer of 10^6 after 7 days.

The virus titer in the medium wasd 10^2 or 10^3 lower than that of the cell at each time. This might be due to rapit heat inactivation and the slow rate of virus release from the cell.

2 days after infection, the cell in which a large oval fluorescent area was present adjacent to nucleus, as Fig. 1, was restained with Giemsa solution, and it was shown that the fluorescent area corresponded to the "B" type inclusion body, as seen in Fig. 2. No fluorescence was observed in the nucleus. On restaining with Giemsa solution, the stainability of the cytoplasm became very weak probably due to the long exposure to UV-light and some distortion was noted in the inclusion bodies owing to contraction and replacement. Fluorescent spot which was partly in contact with the nucleus in another cell was likewise shown to be localized in the "B" type inclusion body (Fig. 3, 4). Detailed study of the cells of other group showed that all fluorescent areas corresponded with "B" type inclusion bodies.

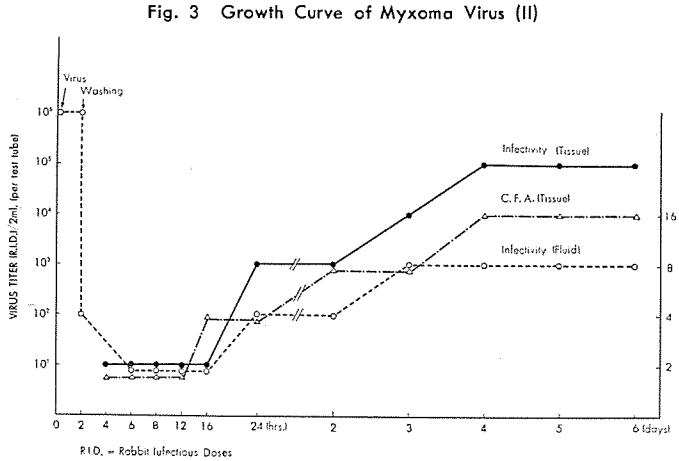
It has thus become more evident that "B" type inclusion bodies have much viral antigen.

2. *Correlation of Growth Curve with Appearance of Inclusion Bodies and Fluorescent Cells*

The appearance of intracellular viral antigen and "B" type inclusion bodies was correlated with the growth curve of infectious virus and development of complement fixing antigen, as determined from companion cultures.

To remove the residual unadsorbed virus as completely as possible, and to find out the exact time when the virus titer first increased, an adsorption time of only 2 hours was allowed and the cells were then washed three times. The infected cultures were harvested for virus titration and companion coverslip cultures were prepared for micorscopic examination.

Fig. 3 Growth Curve of Myxoma Virus (II)



The titer of infective virus and complement fixing antigen were measured from 2 hours to 7 days after infection. Early stage of infection were especially studied.

The virus titer did not rise for the first 16 hours, but then rose sharply 24 hours after infection. It gradually increased to 10^5 after 4 days, thereafter remaining constant as shown in Fig. 3.

The virus titer in the fluid first increased 24 hours after infection. The complement fixing antigen was first detected after 16 hours, increasing in proportion to the infectious virus titer thereafter.

On staining with Giemsa solution, a few small round compact "B" type inclusion bodies had already appeared after 16 hours (0.5 per cent). The number of inclusion cells increased considerably after 24 hours and somewhat larger ones than previously were seen. Many diffuse inclusion bodies were noted after 48 hours. Occasionally 2 or more inclusion bodies were found in one cell.

The alteration of the nucleus was hardly ever detected at early developmental stages of the inclusion bodies.

In a comparative study by the fluorescent antibody method, small round compact fluorescent spots were seen near the nucleus in a few cells after 16 hours and these cells had increased at 24 hours. Therefore it has become evident that a close correlation exists between the time of the appearance of fluorescent cells and inclusion bodies and the time when newly synthesized infectious virus was first detected.

After 48 hours, the fluorescent cells looked like an colony, showing various type of fluorescence in the cytoplasm ranging from a compact to a diffuse type. Occasionally several distinct fluorescent spots were noted in one cell, which seemed to agree with the result of Giemsa staining mentioned above.

In no cell was any distinct fluorescence detected in the nucleus at any stage of infection.

Even after a prolonged period of infection, *i. e.* 10 days, there existed many nonfluorescent normal looking cells among colonies of fluorescent cells implying that many noninfected cells were present with the infected cells even at this later stage.

This phenomenon developed into a carrier culture as reported previously (Kato *et al.*, 1959), showing marked contrast with other pox viruses, ectromelia and vaccinia (IHD strain), which caused rapid degeneration of all cells.

The carrier culture of the myxoma and fibroma viruses have been lasting for more than 10 months. Details of these cultures will soon be reported in this Journal.

DISCUSSION

The pox group virus seems to be the most advanced of all viruses with regard to the morphological study of the cytoplasmic inclusion bodies.

As already reported, Kamahora and Kato and their colleagues have shown that ectromelia, fowlpox, cowpox and vaccinia produce the same kind of inclusion bodies, the "B" type inclusion bodies, which have common morphological and cytochemical characteristics. As to the inclusion body of rabbit myxoma virus, Rivers (1930) stated that acidophilic material with basophilic rod appeared in

cytoplasm, and Hurst (1937) described that Feulgen weak-positive basophilic spherules were seen besides azurophilic granules in the cytoplasm and basophilic spherules in oxyphilic spheres in the nucleus. Recently Chaproniere stated that an eosinophilic mass appeared in the cytoplasm in rabbit tissue culture.

More recently Kato and Cutting have found that "B" type inclusion bodies are present in myxoma and fibroma infected rabbit tumor cells and the peritoneal cells of infected rabbits. They showed that established cultured human amnion cells (FL cells) are also susceptible to these viruses and have "B" type inclusion bodies.

Therefore all pox viruses including myxoma and fibroma viruses have been shown to form Feulgen positive "B" type inclusion bodies with common morphological and cytochemical characters. It was therefore important to examine the antigenic nature of these "B" type inclusion bodies. We have previously shown that the "B" type inclusion bodies of myxoma virus have much viral antigen. Furthermore the "B" type inclusion bodies of other pox viruses, such as ectromelia, vaccinia, cowpox and fibroma, were stained with the fluorescent antibodies of the myxoma virus, implying that all these "B" type inclusion bodies were composed of the same antigenic material (Takahashi *et al.*, 1959).

In the present study of myxoma virus, it was further shown that the brilliant fluorescent areas corresponded to the inclusion bodies, indicating that "B" type inclusion bodies were aggregations of virus antigen.

It has also been shown that inclusion bodies and fluorescent cells appeared just before the rise in infectivity. From this finding and the fact that the "B" type inclusion bodies are Feulgen positive, it is assumed that these "B" type inclusion bodies might be the site of virus multiplication, although more strict quantitative analysis of them is desirable. It is conceivable that concurrent synthesis of protein and DNA of the virus may occur in the cytoplasm forming "B" type inclusion bodies, and then gradual maturation of virus particle may ensue. Detailed analysis of viral antigen will render possible a much more critical analysis of the phenomenon.

The fact that no distinct fluorescence could be seen in nucleus in any stage of infection suggests that the synthesis of viral antigen takes place exclusively in the cytoplasm. This is in marked contrast with the case of RNA type viruses, such as influenza and fowl plague, in which distinct fluorescence was seen both in the cytoplasm and nucleus during the course of infection and it was presumed that the nucleus might also participate in the synthesis of virus particles.

ACKNOWLEDGEMENT

We are greatly indebted to Prof. K. Fukai of our Institute for his helpful suggestions and technical assistance, and also to Dr. Saiwald of San Francisco University, who kindly supplied us with the fluorescein isothiocyanate.

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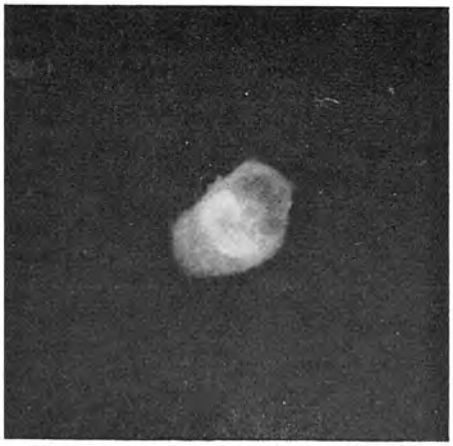


Fig. 1. Fourty eight hours after infection. The brilliant fluorescence (a) is located in the cytoplasm beside the nucleus.

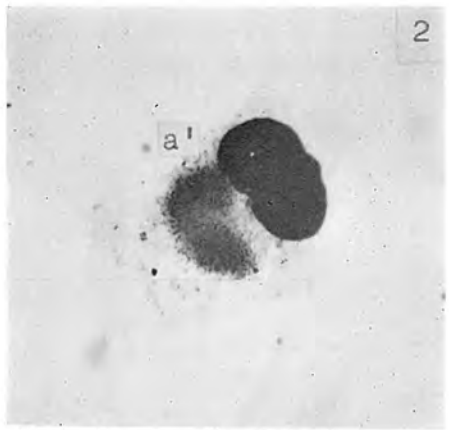


Fig. 2. The same cell as Fig. 1. after restaining with Giemsa solution. The fluorescent area (a) corresponds strictly to the inclusion body (a') showing that this inclusion body is composed of much viral antigen.

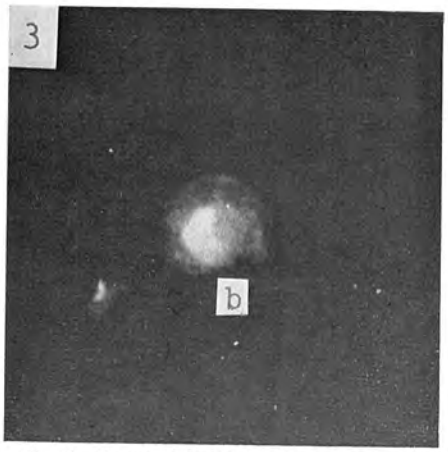


Fig. 3. Fourty eight hours after infection. The fluorescent area (b) is present in the cytoplasm partly in contact with nucleus.

Fig. 4. The same cell as Fig. 3. after restaining with Giemsa solution. The fluorescent area(b) corresponds precisely with the inclusion body(b') in this cell.

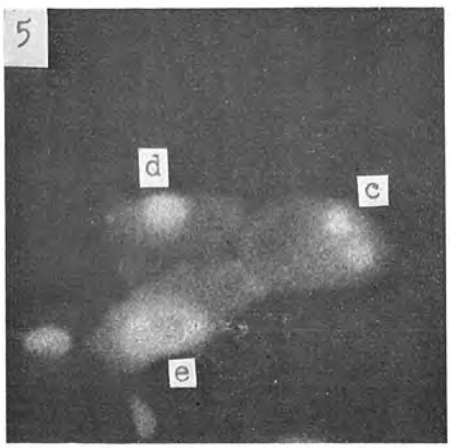


Fig. 5. Seventy two hours after infection. The fluorescent areas (c, d, e) are visible in each cell of the group.

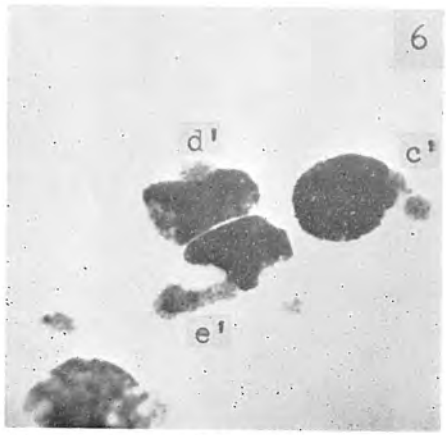


Fig. 6. The same field as Fig. 5. restained with Giemsa solution. Every fluorescent area (c, d, e) evidently proved to be localized in the inclusion bodies (c', d', e').

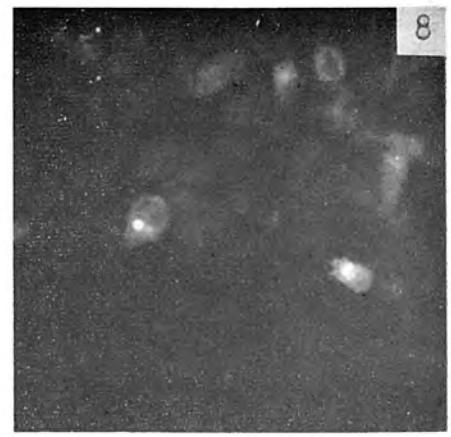
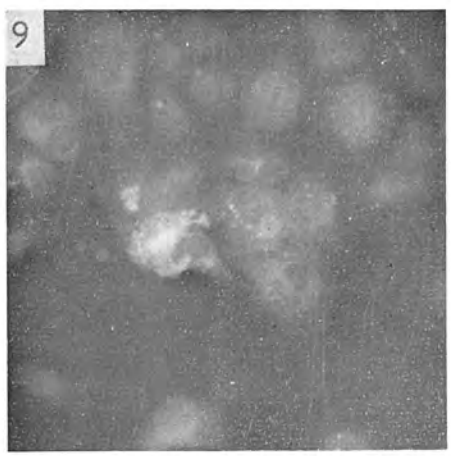


Fig. 7-8. Sixteen hours after infection. Small round compact fluorescent spots are seen beside the nucleus in a few cells.



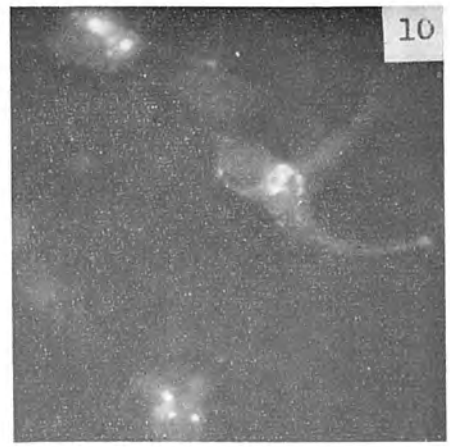
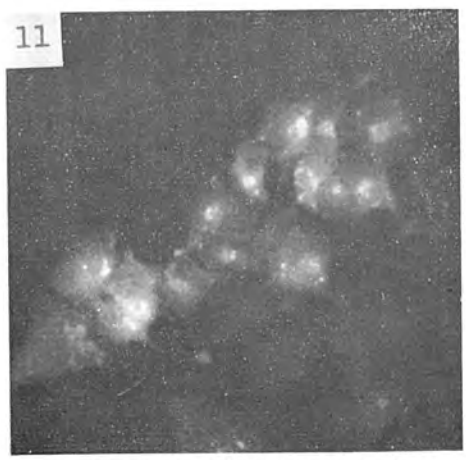


Fig. 9-10. Twenty four hours after infection. Rather diffuse fluorescent areas are also seen in the cytoplasm.



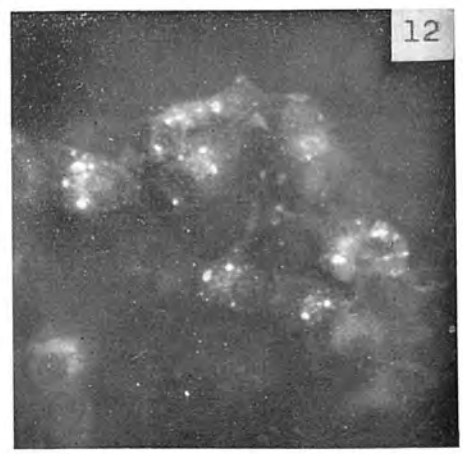
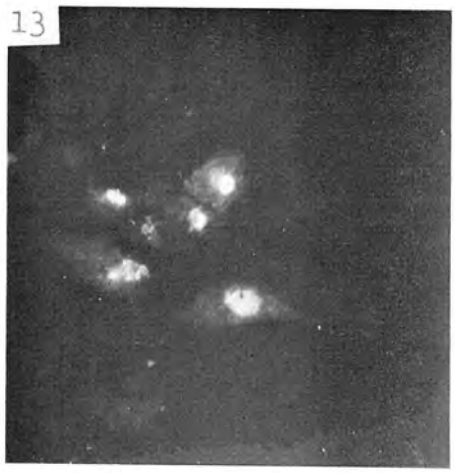
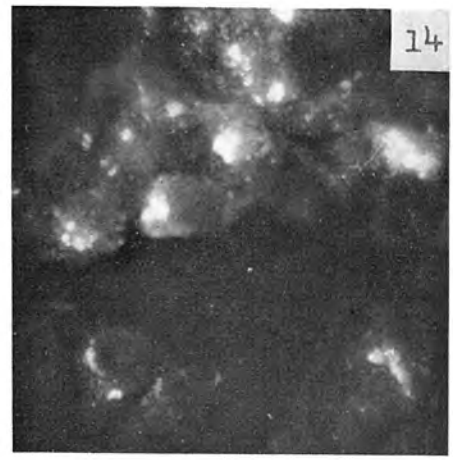


Fig. 11-14. Fourty eight to seventy two hours after infection. Fluorescent cells increase at this stage of infection, showing various types of flucrescence in the cytoplasm. Several distinct fluorescent spots are sometimes visible in a single cell.





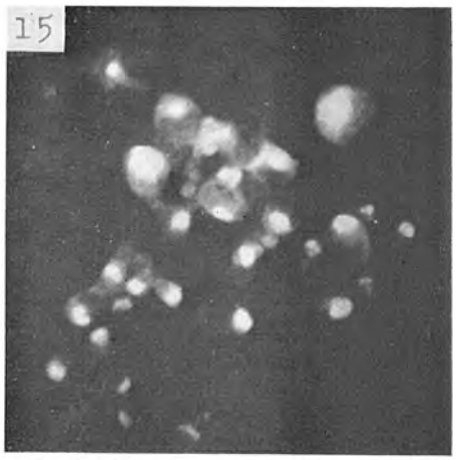


Fig. 15. Six days after infection. Small compact fluorescence is still visible at this stage.

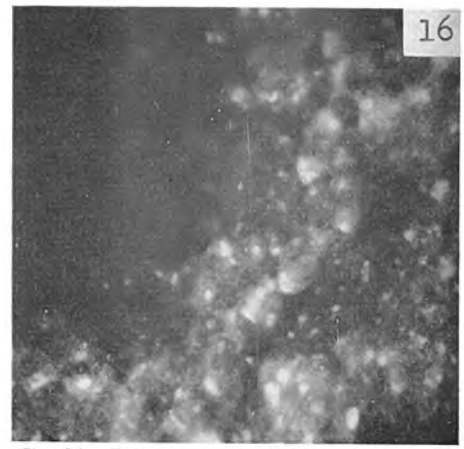


Fig. 16. Ten days after infection. Considerable numbers of nonfluorescent cells are still present among the block of fluorescent cells at this later stage of infection.

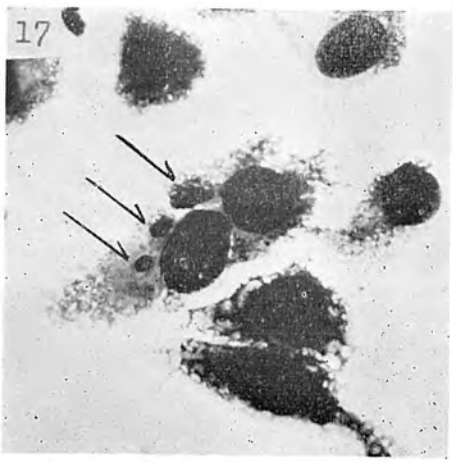


Fig. 17. Compact "B" type inclusion stained with Giemsa solution. Arrow marks indicate "B" type inclusion.

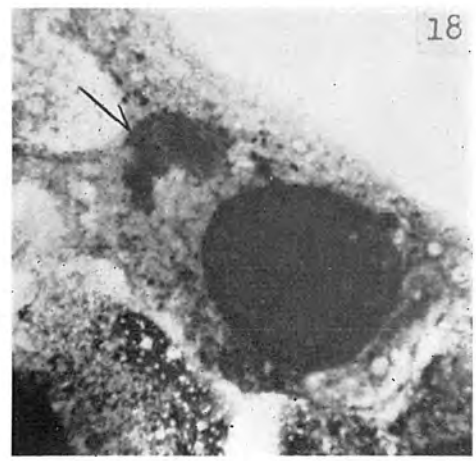


Fig. 18. Diffuse "B" type inclusion stained with Giema solution.