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Shope Fibroma and Rabbit Myxoma Viruses
I. Autoradiographic and Cytoimmunological Studies on "B" Type Inclusions

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SUMMARY

The inclusions of Shope fibroma and rabbit myxoma were investigated by the fluorescent antibody technique and autoradiography with ^{3}H-thymidine. The cytoplasmic inclusions produced in tissue culture cells and in tumor cells, with characteristics in common with "B" type inclusions of other poxviruses in their stainability and developmental form, were found to be the site of viral antigen and viral DNA synthesis.

INTRODUCTION

During studies on the inclusions of poxviruses, it became apparent that all poxviruses produce a common type of intracytoplasmic inclusion named the "B" type whenever multiplication of the virus occurs. Some poxviruses also produce another type of inclusion named the "A" type (Kato *et al.*, 1959, 1962). The characteristics of the "B" type inclusions of all poxviruses are alike, while the characteristics and appearance of the "A" type inclusions vary in different kinds, and sometimes even in different strains of virus and probably also with the kind of host cell. "B" type inclusions have been shown to be the site of synthesis of viral DNA and viral protein (Kato *et al.*, 1959, 1960a, 1960b).

There have been few detailed reports on the morphology of the inclusions of the Shope fibroma and rabbit myxoma viruses, and in these reports there have been only speculations on the nature of inclusions. Shope (1932) noted the existence of eosinophilic inclusions in fibroma tissue. Hyde (1936), utilizing spread preparation, fixing with methanol and staining by Wolbach's modification of Giemsa stain, saw bipolar bodies which stained red in the blue cytoplasm of fibroma cells. Furthermore, he noted that the bodies present in stellate myxoma cells described

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by Hyde and Gardner (1933). In other words, Hyde (1936) noticed the similarity of the inclusions of fibroma and myxoma viruses. Ahlström (1938) found that fibroma inclusions could be clearly demonstrated with Heidenhein's azan procedure, which stained them blue. He noted also that they were stained positively by Weigert's methyl violet procedure for fibrin and had a red-violet color with Mallory's phosphotungstic acid hematoxylin stain.

Kilham and Fisher (1953) and Fisher (1953) reported unique fibroma inclusions which had a glycoprotein rather than a nucleoprotein composition. These inclusions failed to stain either with Feulgen's nuclear procedures, or in Giemsa solution, or hematoxylin-eosin. The same author (Kilham, 1956) reported acidophilic intracytoplasmic fibroma inclusions which were irregular in size and shape and were surrounded by a clear zone or halo, in tissue culture cells fixed with Bouin's solution, stained with hematoxylin-eosin. The inclusions closely resembled those described by Feller, Enders and Weller (1940) in their study of vaccinia virus in chick fibroblasts tissue culture. To our interest he also noted the formation of intracytoplasmic vacuoles with a ground-glass appearance apparently due to mucin-like contents in tissue culture preparations of cottontail testis tissue which had been inoculated 3 weeks previously. The vacuoles were associated with the inclusions mentioned above.

Our previous reports (Kato and Cutting, 1959) revealed that the inclusions produced by Shope fibroma virus and rabbit myxoma viruses were the "B" type inclusions of these poxviruses and these two viruses did not produce secondary inclusions, at least not in FL cells or in the tumor tissues in normal domestic rabbits. Cytoimmunologically both fibroma inclusions and myxoma inclusions stained well with anti-myxoma virus rabbitglobulin coupled with fluorescein-isothiocyanate (Takahashi *et al.*, 1958, 1959a, 1959b,).

This paper reports further investigations on the nature of these inclusions, in tissue culture cells, by autoradiography with ^{3}H-thymidine and by fluorescent antibody technique, using antifibroma virus rabbitglobulin coupled with fluorescein-isothiocyanate.

MATERIALS AND METHODS

1. *Virus*

For convenience, the strain of Shope fibroma virus employed in this study was designated as the ATCB strain. This name signifies that the virus was originally supplied from American Type Culture in 1958 and passaged many times in the skin of domestic rabbits in our Institute, named Biken, which is the abbreviation of the Japanese name. Stock virus was prepared as follows. Fibroma tumors produced on the flank of domestic rabbits were excised and minced with scissors. Then they were ground in the Vir Tis "45" homogenizer and the suspension adjusted to 10 per cent with LE medium (Earl's balanced salt solution containing 0.5 per cent lactalbumin hydrolyzate). After centrifugation at 3,000 rpm for 15 minutes to eliminate most of the cell debris, material was purified by three treatments with fluorocarbon, a treatment which scarcely affected the infectivity of the virus.

Rabbit myxoma virus which was also obtained from American Type Culture in 1958 was employed in this study. The procedure for preparing stock virus was the same as that for Shope fibroma virus.

2. *Cell culture*

Human amnion cells (FL cells) and embryonal rabbit kidney cells (ERK cells) were used. These cells were dispersed in a Leighton tube containing a 11×45mm coverslip. The medium was composed of 90 parts of Earl's solution containing 0.5 per cent of lactoalbumin hydrolyzate, and 10 parts of inactivated bovine serum and antibiotics (100 units of penicillin and 100μg of streptomycin per ml).

3. *Isotopes*

^{3}H-thymidine (nominally 6-T) was purchased from the Radiochemical Centre, Amersham, England. The specific activity of the thymidine was 4.4 C/mM. The concentration of ^{3}H-thymidine used was 5 μC/ml throughout the experiments.

4. *Autoradiography*

The coverslips in the culture tubes were removed and carefully washed with abundant Hanks' solution. Then the preparations were fixed with Carnoy's fluid and treated with two per cent perchloric acid at 4°C for 40 min to remove acid soluble materials. Autoradiographs were made, using NTB2 Kodak liquid emulsion. After 3 days exposure to the emulsion the preparations were developed, fixed and stained with Giemsa solution. Simultaneously a part of the preparation was treated by Feulgen reaction procedure and autoradiographed.

5. *Fluorescent antibody staining*

Antiserum for fibroma virus was obtained in the following way.

Fibroma bearing domestic rabbits were kept for a month during which time the tumors regressed entirely and became transformed into a mere crust. One further intradermal inoculation of fibroma virus was carried out on these rabbits. Blood was collected by cardiac puncture 10 days later. The titer of immune serum was 1:640 in the complement fixation reaction.

The direct fluorescent antibody method was used in this study. The fixation was carried out with aceton at room temperature for 10 min. The identification of fluorescent area was done in the following way. After a suitable area of fluorescence had been 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 stained counterparts.

RESULTS

1. *Fluorescent antibody staining*

1) *FL cells or ERK cells infected with Shope fibroma virus*

Fibroma virus material, having a titer of 10^6 RID (Rabbit Infectious Doses) was introduced into FL cell or ERK cell tubes containing coverslips. Coverslips were with-drawn at some intervals, one half being used for the fluorescent antibody staining and the other half for Giemsa staining.

On staining with Giemsa solution, a few small round compact "B" type inclusions had appeared at 24 hours. The number of inclusion bearing cells increas-

ed in groups at 48 hours, in which somewhat larger inclusion bodies than previously were seen. More diffuse inclusion bodies were noted after 72 hours, and occasionally two inclusion bodies were found in one cell.

In comparative study by fluorescent antibody, small round compact fluorescent spots were seen near nuclei in a few cells 24 hours after infection. At 48 hours, fluorescent cells increased in groups, looking like colonies, and fluorescent spots became larger. The more increased and extensively grouped cells were observed at 72 hours, showing various type of fluorescence in the cytoplasm ranging from compact to diffuse type. By restaining with Giemsa solution, these fluorescent areas were shown to coincide closely with the "B" type inclusions (Fig. 1,2).

The fluorescent areas in cytoplasm were generally more compact and smaller in shape and size than those of other poxviruses, *i.e.* vaccinia, ectromelia *etc.* This might suggest that inclusion bodies of fibroma virus develop slowly than other poxviruses.

After a prolonged period of infection, many nonfluorescent cells were still observed among colonies of fluorescent cells, which readily developed into carrier state as we reported previously (Kato *et al.*, 1959).

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

2) *Scratch preparation of fibroma tumor of rabbit*

Two tenth ml of fibroma virus materials were inoculated into rabbit intradermally. When the tumors were well developed, about on the 7th day, they were dissected and scratch preparations were made.

On fluorescent antibody staining, many fluorescent spots, predominantly compact round or oval shape, could be seen in cytoplasm of wandering cells and fibroblasts. One or two fluorescent spot was usually present polarly in one cell. However nonfluorescent cells were also observable abundantly.

By restaining with Giemsa solution, fluorescent spots were proved to correspond exactly to "B" type inclusions (Fig. 3,4).

No fluorescence was ever seen in nucleus.

2. *Autoradiography with ^{3}H-thymidine*

Shope fibroma virus and rabbit myxoma virus were inoculated respectively into Leighton tubes containg FL cells or ERK cells. After infection proceeded, when inclusion bodies were readily detectable, culturem edium was exchanged with medium containing 5 μc/ml of ^{3}H-thymidine. After two hours of exposure time, the coverslip was withdrawn and subjected to autoradiography.

In preparations examined, all "B" type inclusions were proved to be labelled, whereas other areas of cytoplasms were entirely free from silver grains. The incorporation of ^{3}H-thymidine into nuclei of inclusion bearing cells was mostly suppressed.

DISCUSSION

The significance of inclusion bodies with respect to the multiplication of virus has been uncertain until recently, although they have occasionally been employed in diagnosis of viral infection. During about the last ten years, the development of the fluorescent antibody technique has contributed much to the elucidation of the role of inclusion bodies in the synthesis of viral protein. Moreover, autoradiography with ^{3}H-thymidine, which was introduced more recently in the study of viral infection, provides a decisive means of study of DNA metabolism.

By taking advantage of these methods, the inclusion bodies of poxgroup viruses has been investigated in detail. Kato *et al.* (1955, 1959, 1962) found that ectromelia, fowlpox, vaccinia and cowpox viruses produced inclusion bodies with the same morphology and stainability and named these "B" type inclusion bodies. They stained red with Giemsa solution and gave a hematoxylin tinge with hematoxylin-eosin. This type developed from small compact to large diffuse inclusions in cytoplasm as infection proceeded. The classic "Guarnieri body" found by Guarnieri was shown to correspond to this "B" type inclusion body. Further it was found by Kato et al. that ectromelia, cowpox and fowlpox viruses produced another kind of inclusion body and they named it the "A" type inclusion body. Classic Marchal and Bollinger bodies are classified as "A" type inclusion bodies. In further studies, they found that these "B" type inclusion bodies contained much viral antigen and also that intense incorporation of ^{3}H-thymidine occurred in them. On the contrary, no viral NP antigen or incorporation of ^{3}H-thymidine was observed in the basic substance of "A" type inclusions. Thus it was concluded that "B" type inclusion bodies were the true site of synthesis of viral protein and DNA, and "A" type inclusions played no direct role on virus multiplication. Rabbit myxoma and fibroma viruses, classified in the poxvirus group, have another aspect of tumor forming character and provided a suitable object for study of the virus-tumor relationship. However, the descriptions of the inclusion bodies in these two viruses varies somewhat from author to author, and no coincidental or convincing evidence has been presented from studies of these inclusion bodies. Kato and Cutting (1959) found that human amnion cells are susceptible to these viruses and inclusion bodies produced in these cells, as well as in tumor cells, are quite similar to the "B" type inclusions of vaccinia, cowpox, ectromelia and fowlpox viruses in their stainability and developmental forms. Takahashi *et al.* (1958, 1959a) demonstrated that the inclusions of myxoma virus contained much viral antigen. From parallel studies of morphology and viral growth they concluded that these inclusions were the site of viral multiplication.

In the present study, it has also become evident that the Shope fibroma inclusions in cytoplasm of FL cells, ERK cells and tumor cells, are the site of viral antigen and synthesis of viral DNA. Thus it has been established that Shope fibroma and

myxoma virus produced the same kind of inclusions, the "B" type inclusion, as has already been found in other poxviruses, in which viral protein and DNA are produced.

Then the discrepancy between the descriptions of Kilham and Fisher and of us about inclusions must be considered. One possible explanation may be that different kinds of inclusions were studied. There are several other possible explanations. First, the animals used in this study was the domestic rabbit which is a different species from the cottontail rabbit Kilham and Fisher employed. As discussed in the next paragraph, tumor formation in the cottontail rabbbit was evidently slower than in the domestic rabbit. It must also be noted that they studied the inclusions only in sections while we studied them both in sections and scratch preparations.

As we have reported repeatedly (Kato *et al.*, 1959; Kato and Kamahora, 1962), poxgroup viruses without exception produce a common type of inclusion in infected cells, named the "B" type inclusion. From the point of view of formation of "B" type inclusions, Shope fibroma virus could be classified in the poxvirus group, as shown in other ways, such as by immunology and electronmicroscopy of the virus particles. We have also mentioned that several poxviruses produce secondary inclusions besides the "B" type inclusions, which have been designated as "A" type. However, the appearance and the characteristics of the "A" type inclusions depends entirely upon the kind and sometimes even upon the strain of virus, and probably also upon the kind of host cell. Therefore it is possible that the polysaccharide positive inclusion formed only in cottontail rabbits is an "A" type inclusion of Shope fibroma virus.

Then it must be considered why no inclusions corresponding to "B" type inclusions were observed in the work of Kilham and Fischer. Most of the reports on "A" type inclusions of other poxviruses, such as those of Marchal body, Bollinger body, did not mention the existence of "B" type inclusions, which were later found to be the actual site of virus multiplication. As mentioned before, it is not easy to recognize "B" type inclusion formation in sections and it is particularly difficult when "A" type inclusion formation is the dominant and striking phenomenon. Hence it might be presumed that the existence of "B" type inclusion, with the "A" type inclusions, was not noticed in their work. Unfortunately we have no way to confirm this suggestion, because cottontail rabbits are not obtainable in this country.

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EXPLANATION OF FIGURES

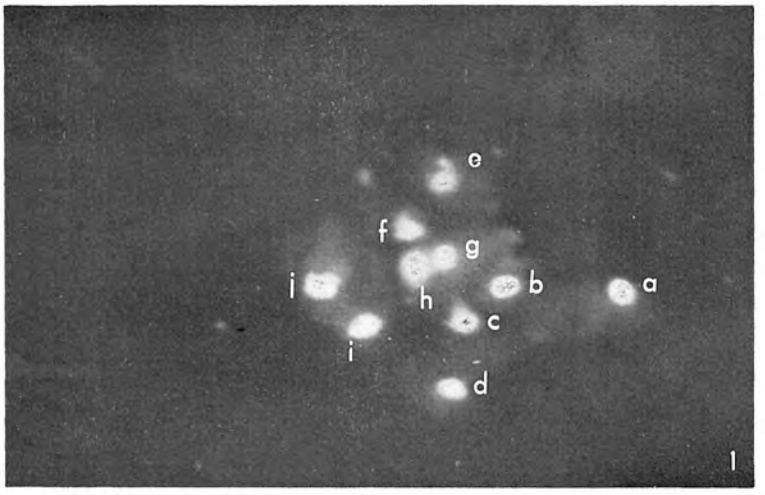
Fig. 1. ERK cells infected with Shope fibroma virus, stained with fluorescent anti-Shope fibroma virus rabbit serum. Many distinct fluorescent spots can be seen.

Fig. 2. The same field that in Fig. 1, restained with Giemsa solution. Every fluorescent spot was found to correspond with "B" type inclusions of Shope fibroma virus.

Fig. 3. Fibroma cells in the scratch preparation of Shope fibroma of domestic rabbit, stained with fluorescent Shope-fibroma virus antibody. Two distinct fluorescent spots were observed bipolarly in cytoplasm.

Fig. 4. The same field that in Fig. 3, restained with Giemsa solution. Two fluorescent spots correspond with bipolar "B" type inclusions in the fibroma cell. The nucleus is free from fluorescence.

Fig. 5. Autoradiogram of Shope fibroma virus-infected FL cells exposed to ^{3}H-thymidine. Two sites of aggregation of silver grains were found in cytoplasm. These sites correspond to the bipolar "B" type inclusions of Shope fibroma virus. Incorporation of ^{3}H-thymidine into the nucleus of cell with inclusions was suppressed.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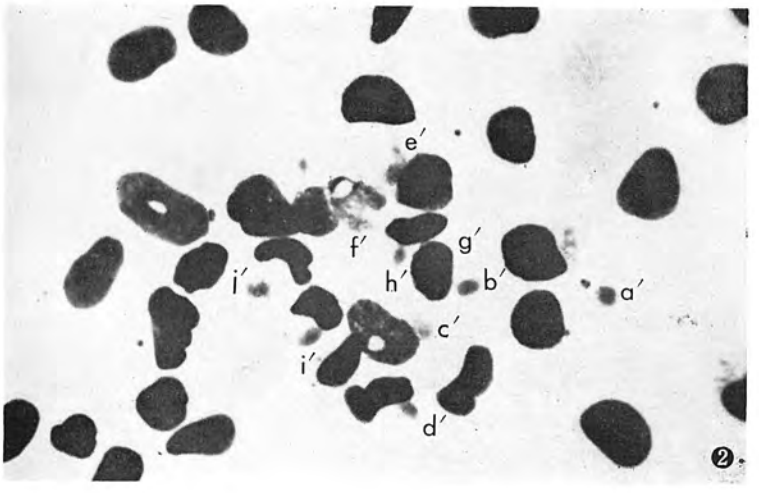
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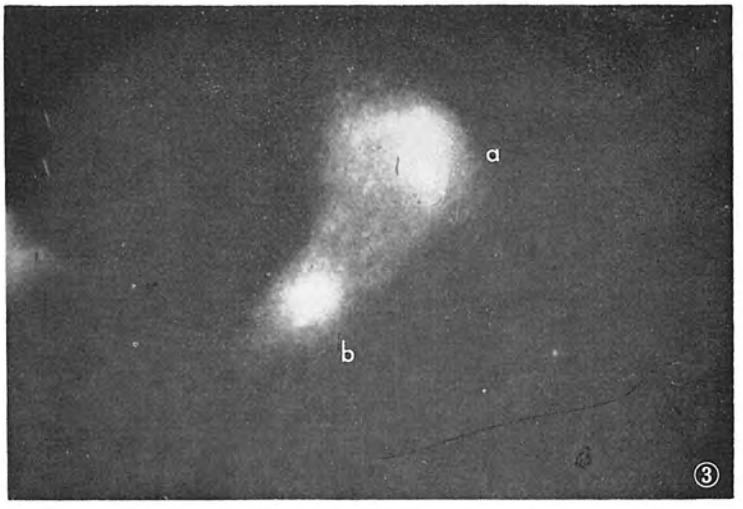
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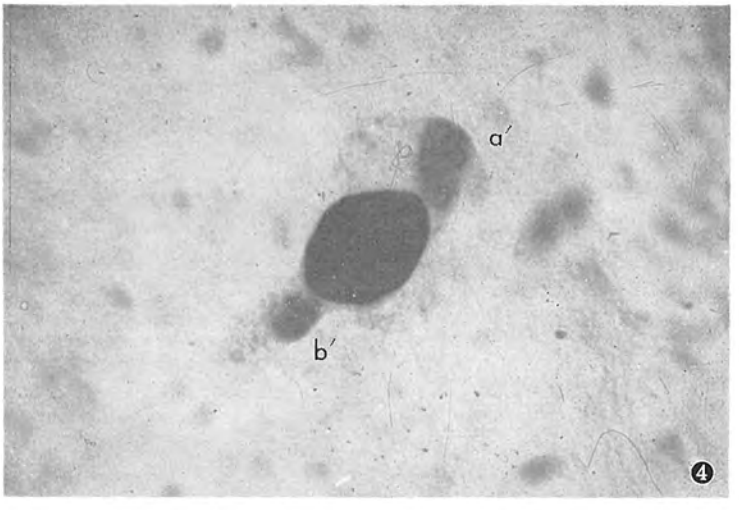
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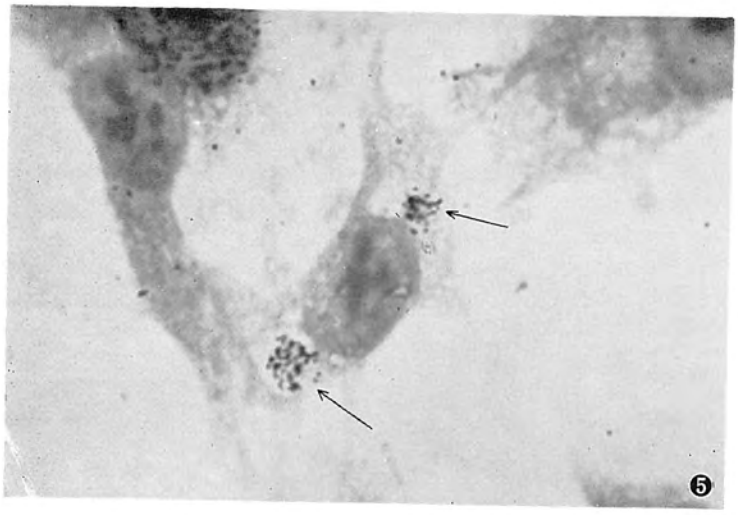
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