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# Shope Fibroma and Rabbit Myxoma Viruses
# II. Pathogenesis of Fibromas in Domestic Rabbits

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SUMMARY

The correlation between tumor formation, virus multiplication, inclusion formation and antibody response was studied in domestic rabbits inoculated with Shope fibroma virus. When $10^6$ RID/ml of virus was inoculated intracutaneously, a palpable tumor appeared on the 4th day, reaching a maximum size on about the 8th day and disappearing completely in a month. The virus titer of the tumor gradually increased for 10 to 12 days, occasionally up to $10^7$ RID/gr and then rapidly decreased to zero on the 25th day.

Inclusions staining reddish purple with Giemsa stain were exclusively found in the cytoplasm, and shown to be rich in viral antigen. The developmental form of the inclusions was similar to that of "B" type inclusions of other poxviruses, for they developed from a compact to a diffuse form, which seemed to cause degeneration of infected cells. Inclusion bearing cells appeared and reached a maximum far earlier than did the infectivity titer or tumor formation. The percentage of inclusion bearing cells in the total tumor cells in scratch preparations was rather low, being 40 per cent at most and these decreased to zero as early as the 10th day.

Complement fixing antibody and neutralizing antibody showed a parallel increase from the 4th to the 20th day.

From these results, a possible mechanism of tumor formation of fibromas is discussed.

## INTRODUCTION

The relation of Shope fibroma and rabbit myxoma viruses to poxviruses has gradually been clarified from several points of view, which include the morphology of the virus particles, immunology, reactivating activity for other poxviruses and development of "B" type inclusion bodies. Extensive studies on the mechanism of growth of poxviruses has been carried out in our laboratory during the past few years, and our interest has been extended to the problem of the similarity and dissimilarity in the mechanism of growth of these two tumor viruses to other poxviruses. The previous report showed that one of the most unique characteristics of

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these two tumor viruses with regard to established tissue culture cells (FL cells) was the formation of a carrier culture state. This has never been observed with other poxviruses (Kato *et al.*, 1959). This state is supposedly due to a balance between destruction of cells infected with the virus and multiplication of uninfected cells which are not necessarily resistant cells. The results obtained from tissue culture studies have aroused our interest in studies on the mechanism of tumor formation *in vivo*. This paper reports on the pathogenesis of Shope fibroma, especially with respects to the relationship between tumor formation, virus growth, inclusion formation and antibody response in domestic rabbits. The pathogenesis of fibromas in cottontail rabbits was reported by Kilham and Fisher (1954) from similar points of view. There were some dissimilarities in the pathogenesis observed with the cottontail rabbits they employed and the domestic rabbits used in our laboratory. The possible mechanism of tumor formation of this virus is discussed.

## MATERIALS AND METHODS

### 1. *Virus*

Shope fibroma virus (ATCB strain) was used. The details of preparation of stock virus was described in the preceding paper.

### 2. *Rabbits*

Domestic white rabbits, weighing approximately 2kg, chosen from litters, were used in this study. Prior to inoculations, the fur was removed on the sides and back of the animals with electric scissors.

### 3. *Virus titration*

Excised tumors were minced with scissors and ground in a Vir Tis "45" homogenizer, and the suspension was adjusted to 10 per cent with LE medium. Serial 10-fold dilutions were made with LE medium and aliquots of 0.5 ml were inoculated intracutaneously in duplicate into domestic rabbits using a 26-gauge needle.

The results of these titrations were noted after 10 days by the presence or absence of palpable lesions, as shown by Kilham and Fisher (1954). The end point was expressed as Rabbit Infectious Doses (RID).

### 4. *Counting of inclusion bearing cells*

As shown in previous papers (Kato and Cutting, 1959; Takahashi *et al.*, 1959;Kato *et al.*, 1963), the "B" type inclusion is the only type of inclusion found both in tumor cells in the domestic rabbit and in FL tissue culture cells. The "B" type inclusion can not be clearly seen in tissue sections stained with hematoxylin-eosin. Therefore, scratch preparations of fibroma tissue were made, fixed with methanol and stained with Giemsa solution. A number of degenerating cells were noticed in the preparation, which probably resulted from mechanical damage and some other cause. To study the general tendency of inclusion formation in tumor tissues, approximately 1,000 cells were counted and the percentage of inclusion bearing cells was calculated in scratch preparations.

5. *Fluorescent antibody staining*

The direct method, using anti-fibroma virus rabbit serum-globulin coupled with fluorescein-isothiocyanate was employed. The procedures used were described in the previous paper (Takahashi, *et al.*, 1959; Kato *et al.*, 1963).

6. *Titration of complement Fxing antibody*

The routine Kolmer's method was employed. Sera obtained by cardiac bleedings were stored at −20°C, and inactivated at 56°C for 30 minutes before dilution. As virus antigen, the partially purified virus stock described above was used, as there was scarcely any detectable anticomplementary effect. For the hemolytic system, 3 per cent ox red cells, seven units of hemolysin and 2 full units of complement were used. 0.25 ml of two-fold serial dilutions of serum was mixed with 0.25 ml of virus antigen and the same quantity of complement. The mixture was kept overnigth at 4°C, and then 0.25 ml of the hemolytic system was added and the mixture was incubated at 37°C for 30 minutes. The antibody titer is expressed as the highest dilution which gave 50 per cent hemolysis.

7. *Serum neutralization test*

Sera were diluted serially 10-fold with LE medium. An equal volume of purified virus suspension from stock virus and serial dilutions of the serum were well mixed and stood for 60 minutes at 37°C. Then, 0.5 ml aliquots were inoculated intracutaneously into domestic rabbits. The neutralizing antibody index was obtained by noting tumor formation in 10 days.

## RESULTS

### 1. *Distribution of fibroma virus in various tissues of rabbit inoculated intracutaneously and intratesticularly*

To test whether the virus of tumor tissues definitely resulted from multiplication at the inoculated site, the virus titers of various tissues of 7 day-old-tumor bearing rabbits, which had been inoculated both intracutaneously and intratesticularly with virus were measured. As shown in Fig. 1, no virus could be found in any tissues other than the skin and testicular tumors produced at the sites inoculated. It is thus safe to assume that the inoculated tissues were the only sites for virus multiplication in the body when inoculations were by the intracutaneous and intratesticular routes.

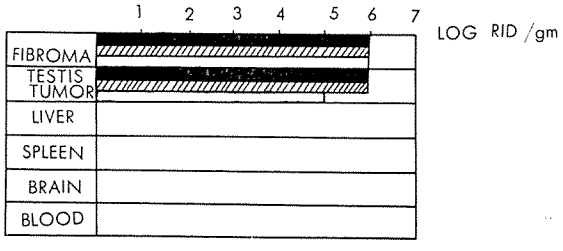


**Fig. 1. Rabbit Infectious Doses in Various Organs of Shope Fibroma Bearing Rabbits, 7 Days after Infection**

2. *Correlation between tumor formation, virus growth, cyto-morphological and-immunological observations and the antibody response in domestic rabbits inoculated intracutaneously*

To study the correlation between tumor formation, virus growth, inclusion formation and antibody response, the following study was carried out. Twenty domestic rabbits were used in one serial study. Each rabbits were inoculated intracutaneously on its side with the same stock virus preparations having a titer of $10^6$ RID/0.5 ml. Rabbits were bled to death by cardiac punture at about 2 day intervals, and the blood samples were used for the complement fixation and neutralization tests. Inoculated tissues were also taken for virus titration, for inclusion bearing cell counts and for fluorescent antibody staining.

1) *Gross appearance of developing tumor*

Skin thickening at the site of inoculation was first detectable 3 or 4 days after inoculation, and this progressed subsequently to tumor development. After about 8 days, the tumors ceased to increase in size, the maximum size being usually 30 to 50 mm in diameter. A reddish purple area appeared after about 10 days at the middle of the surface of the tumor and began to spread outwards. This area soon began to degenerate and had transformed into a crust after 18 to 25 days. After a month, the crust desquamated and finally disappeared completely. The schematic process of the fibroma development is shown in Fig. 2.

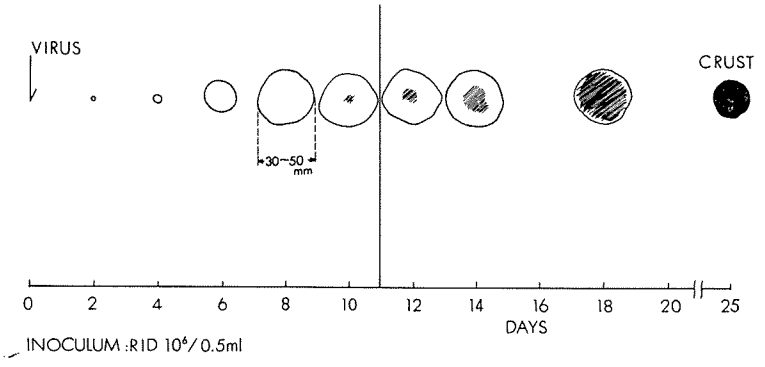


**Fig. 2. Development of Shope Fibroma at the Skin of the Domestic Rabbit**

2) *Growth of fibroma virus in the skin of the domestic rabbit*

Three series of experiments were carried out and the results are plotted in Fig. 3.

The titer usually decreased slightly on the 3rd day and then increased

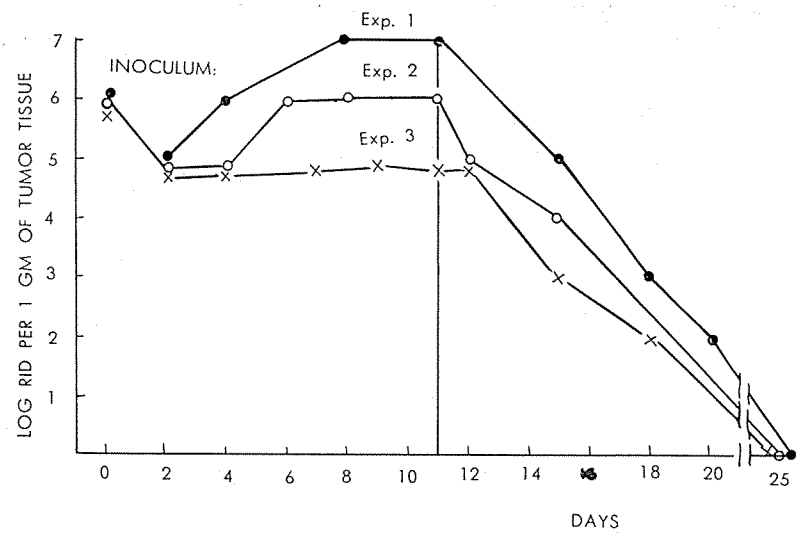


**Fig. 3. Virus Growth Curve in the Shope Fibroma**

gradually, occasionally rising to $10^7$ RID per gm of tissue after 8 days. After 11 days the titer had greatly decreased and was zero after 25 days.

3) *Morphological and cytoimmunological studies on cells in scratch preparations*

Morphological and cytoimmunological studies on the cells were carried out in parallel with the first series of viral growth experiments. Inoculated tissues were

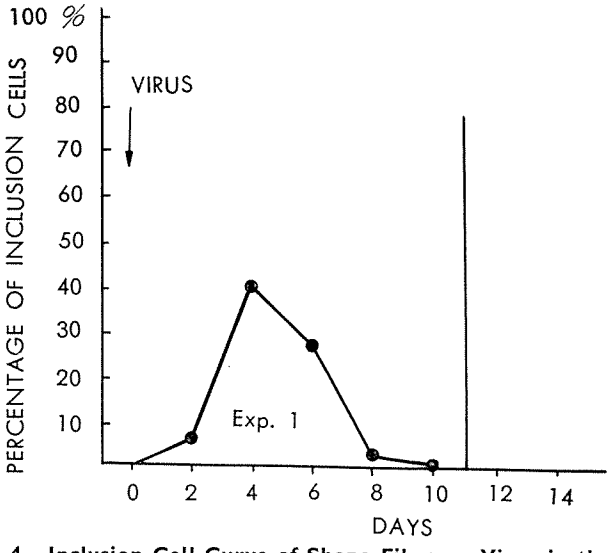


**Fig. 4. Inclusion Cell Curve of Shope Fibroma Virus in the Rabbit Tumor Tissue**

taken serially, a part being used for viral titration and another for scratch preparations. Some of the scratch preparations were fixed with methanol for Giemsa staining and others were fixed acetone for fluorescent antibody staining.

Inclusions stained reddish purple with Giemsa stain, the characteristic staining property of "B" type inclusions of poxviruses. Cells appearing at the beginning of infection were mostly round though a few were spindle type. Inclusions in the cell at this early stage were usually round and either unipolar or bipolar, and more than three inclusions in the same cell were very rarely seen. The number of inclusion bearing cells gradually increased, reaching a maximum of 40 per cent of the total cells on the 4th day after inoculation, when the tumor was still not well developed. Then the number of inclusion bearing cells decreased rapidly and none were found on the 10th day (Fig. 4). The later the preparations were taken, the more diffuse the inclusions appeared. Inclusions invariably gave a positive reaction with Feulgen stain. It was very rare to see mitotic figures and inclusions in the same cell, and many cells with diffuse well developed inclusions looked degenerate. This suggests that inclusion bearing cells degenerate and do not multiply.

On fluorescent antibody staining, fluorescent spots were seen exclusively in the cytoplasm. These were shown by restaining with Giemsa solution to correspond to inclusions. The shape, number and developmental form of fluorescent spots was in agreement with those of inclusions observed in Giemsa stained preparations.

4) *Responses of complement fixing antibody and neutralizing antibody*

The titer of complement fixing antibody showed a slight increase as early as

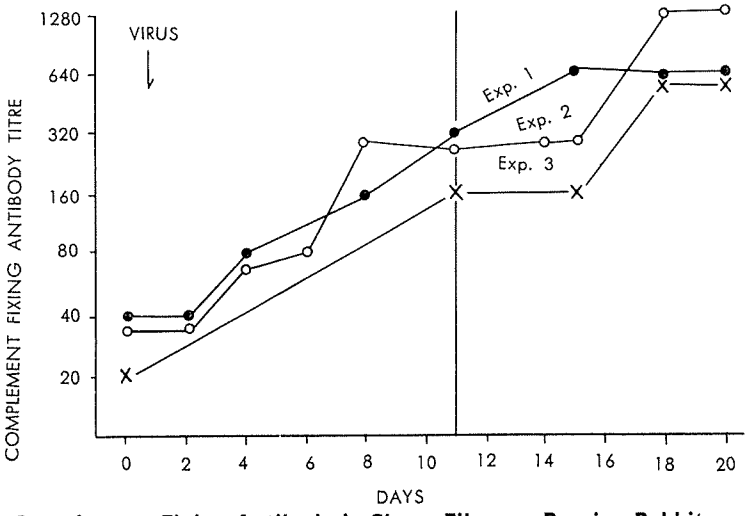


**Fig. 5. Complement Fixing Antibody in Shope Fibroma Bearing Rabbits**

4 days after inoculation. Then it gradually increased until about 18 days after inoculation, reaching a maximum titer of 1:1280 (Fig. 5).

Neutralizing antibody showed a parallel increase with the complement fixing antibody (Fig. 6).

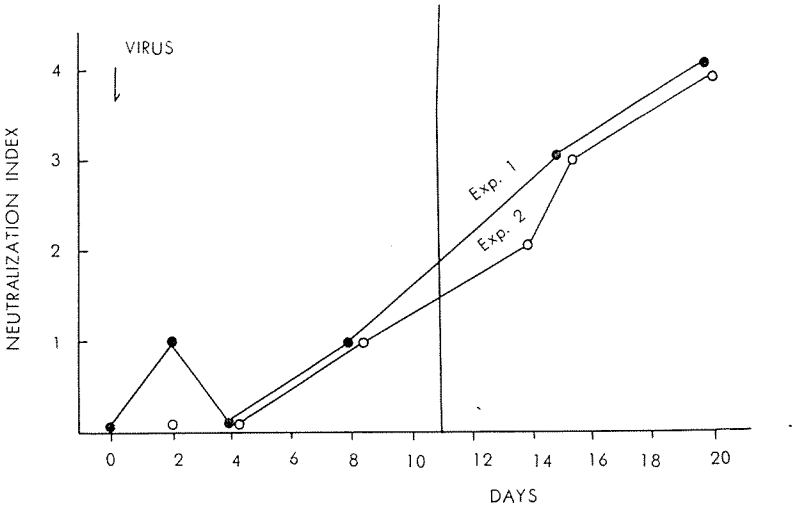


**Fig. 6. Neutralization Antibody in Shope Fibroma Bearing Rabbits**

## DISCUSSION

Summarizing the results in this study, the possible sequence of events seems to be as follows: Virus inoculated intracutaneously infects the tissue cells. In the infected cells "B" type inclusions are produced as a pool of viral DNA and antigenic protein, and maturation and release of viral particles ensue (Kato *et al.*, 1963; Kato *et al.*, 1963). Meanwhile neutralizing antibody appeared and increased, especially 10 days after inoculation. This decreases the rate of reinfection of neighboring cells with released virus. In this way, most of the observations obtained in this study could reasonably be connected. However the reasons why cells multiply abnormally forming a tumor, and why the tumor then ceases to grow and finally regressed are unknown. The former phenomenon is probably related to the virus multiplica tion and the latter probably to the presence of neutralizing antibody. How could these phenomena be plausibly connected? If the infected cells multiply at the same time as the virus in them, all the tumor cells would contain "B" type inclusions. It was found that the percentage of inclusion bearing cells was rather low throughout tumor formation, being 40 per cent at most. Furthermore, inclusion bearing cells very rarely showed mitotic figure and many cells with diffuse well developed inclusions appeared to be degenerating. This suggests that inclusion bearing cells do not multiply but rather degenerate.

Further, the cessation of development of the tumor when the neutralizing antibody increases rapidly seems to exclude ideas on lysogenization of the virus.

The coexistence of inclusion bearing cells and non-inclusion bearing cells in the tumor reminds us of the carrier state in tissue cultures, which was found to depend on a balance between destruction of infected cells and multiplication of noninfected cells (Kato *et al.* 1959b). Utilizing antibody, the carrier state could be blocked and the infection completely cured.

Menkin (1956) suggested from biochemical studies on inflammation that mildly damaged cells liberate a kind of growth factor. It is plausible that inclusion bearing cells release some factor which causes the multiplication of fibroblasts. Based on results of both in vivo and in vitro experiments, a possible hypothesis of tumor formations is presented in Fig. 7. The cycle of virus growth must proceed

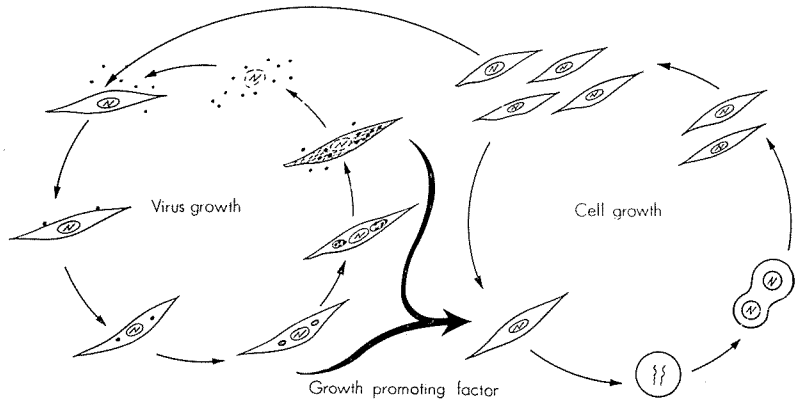


**Fig. 7. Possible Scheme of Tumor Formation by Poxvirus**

as in other poxgroup viruses, though the growth rate and yield of virus is not so rapid or high. Some growth promoting factor, with a rather transient action, might be liberated from infected cells and cause the multiplication of other non-infected cells. Infected cells themselves would have no ability to multiply. With the progress of infection, neutralizing antibody increases rapidly, which would suppress reinfection to noninfected cells, so that the supply of the growth promoting factor would decrease.

The working hypothesis presented here serves to explain the event until the fibroblasts cease to multiply. However, another event, the regression of the fibroma still remains unexplained. Kilham and Fisher (1954) suggested two factors which might help to explain why fibromas eventually regress. One is that the steady enlargement of fibroma cell inclusions, whether they represent byproducts or cell meta bolism or not, may put such pressure on the remaining cytoplasm that normal cellular activities are inhibited. However, this may not apply to the events occurring in domestic rabbits, because inclusions such as they described were not

found, and inclusion-bearing cells could not be found in the stage of regression. Secondly, scratching and bruising the tumors may lead to bacterial infections unfavorable to the persistence of the tumor. In our experiments with domestic rabbits, the regression of the fibromas did not seem to depend on bacterial infection, because antibiotics were used and the period was short. It is recalled, however, that infection with ectromelia virus causes necrosis in the legs of mice, which finally leads to the entire absorption of the granulomatous tissues (Dohi 1952). Either allergic vascular change or some other factor causing this would be probably essentially the same one which leads to regression of the fibroma.

## ACKNOWLEDGEMENTS

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