BIKEN'S JOURNAL 2, 77-84 (1959)

Transformation Phenomena in the Pox Group Viruses.

I. Transformation of Ectromelia into Vaccinia Virus in Tissue Culture

TERUKO HANAFUSA, HIDESABURO HANAFUSA AND JUNTARO KAMAHORA

Department of Pathology, Research Institute for Microbial Diseases, Osaka University

(Received for publication, July 20, 1959)

SUMMARY

The transformation of ectromelia into vaccinia virus has been performed in tissue culture. When heat killed vaccinia and live ectromelia were inoculated on L cells, live vaccinia appeared after 48 hours. Inactivation of vaccinia was carried out at 56°C for 2 hours and experiments showed this heat killed preparation did not contain any surviving virus. The purified transformed virus was identical with vaccinia-IHD in the following characters: (1) pathogenicity in rabbits and mice (2) heat stability (3) behavior in tissue culture (4) plaque size. The specific characters of new-born virus could be transmitted continuously in tissue culture. The transformation can also take place in HeLa, FL cells and CAM of eggs in which both viruses can be propagated. Many separate experiments gave the same results.

Some properties of the transforming agent are discussed.

INTRODUCTION

The Berry-Dedrick phenomenon (Berry and Dedrick, 1936) is well known as the first instance of what is called genetic interaction between animal viruses. These workers inoculated rabbits with a mixture of active fibroma virus (Shope) together with a suspension of heat killed myxoma virus (Sanarelli) and produced in the animal the symptoms and pathological lesions characteristic of infectious myxomatosis. These observations have been confirmed by other investigators (Gardner and Hyde, 1942; Smith, 1952). Recently Kilham has found that this transformation can occur in cultures of rabbit tissues with greater regularity than *in vivo* and reported on the several properties of the transforming agent of myxoma (Kilham *et al.*, 1957, 1958 a, 1958 b, 1959).

Both ectromelia (poxvirus muris) and vaccinia virus (poxvirus officinale) are classified in the pox group, and the characteristics of their multiplication in tissue culture are similar in many respects. Hitherto, differentiation of these two viruses has been made mainly on the basis of their host range. As shown in the preliminary communication (Hanafusa *et al.*, 1959 a) in studies on transformation of these two viruses advantage was taken of these differences. Transformations are also possible with other members of the poxvirus group in tissue culture (Hanafusa *et al.*, 1959 b). The present paper describes details of experiments with ectromelia and vaccinia virus.

Materials and Methods

Cells

Earle's L cells were grown in Hanks' saline to which had been added 0.1% yeast extract, 0.5% lactalbumin hydrolysate and 5% bovine serum. The growth medium for HeLa and FL (established cell line of human amnion) cells consisted of Earle's saline plus 0.5% lactalbumin hydrolysate and 15% bovine serum. Cellular monolayers in 200 ml bottles were used throughout these experiments, containing on the average 5×10^6 cells / bottle of L cells and 1×10^7 cells / bottle of HeLa and FL cells.

Viruses

Vaccinia IHD strain and ectromelia Biken strain (which was isolated in our Institute in 1945) were used after serial passages in L cells. Such fluids contain on the average about 10^4 $TCID_{50}$ / ml (10^4 LD_{50} / ml in mice) for ectromelia and 10^6 $TCID_{50}$ / ml for vaccinia. Both viruses induced the formation of polynucleated giant cells and cytoplasmic inclusion bodies. However, when the same titer of virus was used, vaccinia destroyed the cell sheet more rapidly than ectromelia. In addition, ectromelia differs from vaccinia in the following two characters which were used for the discrimination of the two viruses. First, when inoculated intraperitoneally into mice, only ectromelia causes death within 8 days. Second, on intradermal injection into rabbits, vaccinia virus produces flat indurated lesions, whereas ectromelia causes no visible change.

Virus Assay

Infectivity titrations of the virus preparations were carried out in L cell cultures by the end point method or the plaque assay method. The plaque system used for both viruses was in principal like that reported by Nii (1958). For convenience this method was modifed as follows. To the monolayers of L cells was added 1 ml of suitably diluted virus suspension and after 3 hours the cells were washed twice with Hanks' saline to which were added 8 ml of growth medium without an agar overlay. The number of foci was counted after two or three days. When a suitable dilution was used several primary foci were formed, having equal sizes and the number of the foci was proportional to the infectious units. Thus these titration values coincided with the PFU (plaque forming units) within 10% error. This titration method is useful only for viruses which produce focal degeneration in the limited area where they had been adsorbed.

Experimental Animals

Rabbits. About 3 month old rabbits were used. Intradermal inoculations were made under the clipped skin with 0.1 ml of inoculum.

Mice. Four week old Swiss albino mice were inoculated intraperitoneally with 0.2 ml of inoculum.

EXPERIMENTAL RESULTS

Heat inactivation of vaccinia IHD

The pooled suspension of vaccinia IHD (10^6 $TCID_{50}$) was sealed in an ampoule and submerged in a water bath at 56°C for two hours. It was important to see whether heated preparations contained infectious surviving virus. The inactivated virus preparations (I. V.) were not infectious either in tissue culture or in rabbit skin, and no active virus was recovered from I. V.-inoculated culture cells disrupted by freeze-thawing. As shown in Fig. 3., vaccinia virus can be inactivated at 56°C to a survival of less than 10^{-6} within thirty minutes. It seems certain that the two hours period used was ample for complete inactivation. However, interference between the inactivated virus and the surviving one might suppress the appearance of surviving active virus in this system. To test this possibility the following experiments were performed. The I. V. preparations were diluted ten times with growth medium and inoculated into ten bottles of L cell culture. Viral growth was not found in any bottle. On the other hand, the presence of undiluted I. V. resulted in no apparent decrease in the number of plaques produced by a suspension containing 10^1 and 10^2 PFU of vaccinia virus as shown in Table 1. From these results it was obvious that the possibility mentioned above could be excluded under the condition used.

Table 1. Effect of interference between I. V. and vaccinia

	Number of plaques			
	Exp. 1	Exp. 2	Exp. 3	Exp. 4
Vaccinia + I. V.*	65	5	4	2
Vaccinia (control)	80	5	3	3

* The monolayers were infected with 0.5 ml of I. V. and 0.5 ml of vaccinia simultaneously.

Transformation experiments

Monolayer cultures of L cells were exposed to a mixture of 1 ml of 10^4 infectious ectromelia and 1 ml of heat inactivated vaccinia virus which had been added to 8 ml of the growth medium. Cellular degeneration occurred at a similar rate to that of a monolayer infected only with ectromelia. After 48 hours, the infected cells were harvested and centrifuged at 3000 rpm for 5 minutes. Test rabbits were inoculated intradermally at four separate sites with 0.1 ml samples of the supernatant from this centrifugation, similarly treated ectromelia, I. V. preparation and active vaccinia virus. Of the four inoculations only active vaccinia and the supernatant of the test material produced large indurated lesions, while the other had no effect. The positive result was confirmed by the demonstration under the microscope of specific inclusion bodies in specimens from the lesion.

The supernatant fluid of L cells 24 hours after inoculation of the mixture gave a negative skin reaction.

The transformation took place after 3 hours adsorption, though little is known about the mechanism of adsorption of the heat inactivated virus. Thus a mixture

of 1 ml of ectromelia and 1 ml of heat inactivated vaccinia was added simultaneously to the cell culture, and after 3 hours the fluid was removed. Then the cells were washed 3 times with Hanks' saline to which were added 8 ml of fresh medium. The supernatant fluid after 48 hours incubation gave a positive skin reaciton. When the heat inactivated virus was inoculated 24 hours prior to the addition of ectromelia, transformation was also successful. These experiments suggest that the heat killed virus could get reach and possibly penetrate the cells by itself.

Purification of transformed virus

In the present studies the characters of transformed virus were mainly investigated. However, the viral preparation thus obtained might have been a mixture of viruses; at least containing active ectromelia and transformant. As described in the sections on Materials and Methods, the rate of propagation of vaccinia was greater than that of ectromelia and the virus titer in the supernatant fluid after serial passages of the former was always higher than that of the latter. If the transformed virus had the same growth rate as that of vaccinia, the separation of transformant from ectromelia would be possible by several passages of the mixture on L cells. The procedure is shown in Fig. 1. The apparently degeneration of the cells during these successive cycles of multiplication was a

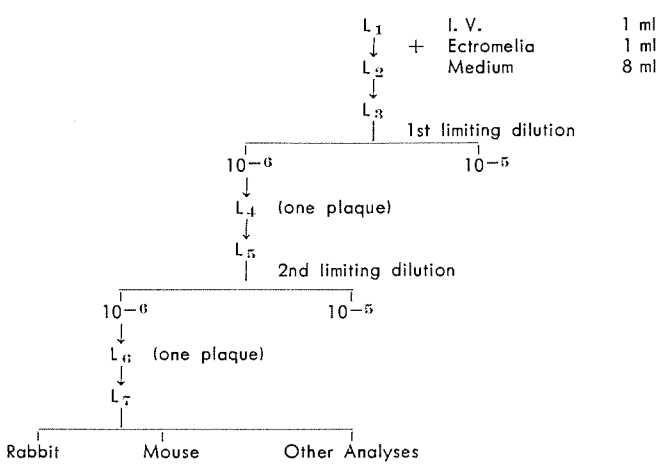

Fig. 1. Flow diagram showing steps in the procedure for the purification of transformed virus. L_1, L_2..... designate the passages on L cells.

proof that the ectromelia had been transformed. The supernatant fluid was diluted to 10^{-6} with Hanks' saline and 1 ml aliquots of the diluent was put into a series of bottles containing monolayers of L cell. The monolayers producing only one focus were incubated at 37°C for several days. When the bulk of the cell sheets had been destroyed, the supernatant fluid was passaged once or twice on L cells. The same limiting dilution procedure was repeated as shown in Fig. 1. Some of the characters of the transformed virus were studied with this purified virus (10^6 $TCID_{50}$ / ml).

Properties of purified transformed virus

1) Pathogenicity in experimental animals

As described previously, the purified transformed virus could induce skin lesions in rabbits, whereas it was not pathogenic in mice when injected intraperitoneally. The mice inoculated with this virus fluid remained healthy, while cetromelia killed them within five days. So this new virus behaved as if it were a vaccinia virus.

2) The size of the plaques

Vaccinia virus usually produces plaques of a larger size than those of ectromelia on L cells, the diameter of a vaccinia plaque being about 2 mm and ectromelia plaque 1 mm on the 3 rd day. To produce plaques, virus suspensions of vaccinia, ectromelia and transformed virus were suitably diluted and added to the monolayers. Fig. 2 shows photographs of the plates from these experiments. The size of the plaques of the transformed virus was indistinguishable from those by vaccinia.

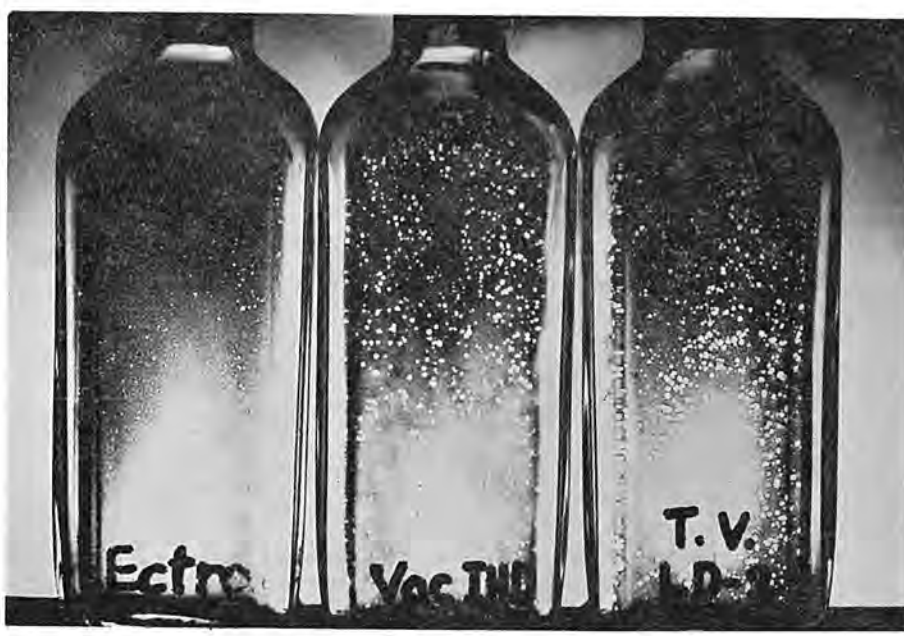

Fig. 2. Comparison of plaque size of vaccinia, ectromelia and transformed virus. All platings were made on the same lot of L cell monolayers. Three days after inoculation.

3) Heat stability

Heat inactivation curves of the virus preparations at 56°C are shown in Fig. 3. Viruses suspended in growth medium of L cells (pH 7.0) were sealed in ampoules and completely immersed in a water bath at 56°C. After a few minutes of incubation one ampoule of each preparation was put into ice water. This sample was taken as a zero-time sample. At various intervals from this zero-time the ampoules were successively removed from the water bath. Virus survival

after such treatment was measured by plaque counting. Each point on the curve represents the mean of two independent determinations. From the results obtained, it is evident that vaccinia is more resistant to heat than ectromelia and that its viable titer declines by a factor of 10^{-2} every 10 minutes. Infectivity of ectromelia dropped sharply to an undetectably low titer after 10 minutes at 56°C. It was clear that the rate of loss of infectivity of transformed virus completely coincided with that of vaccinia.

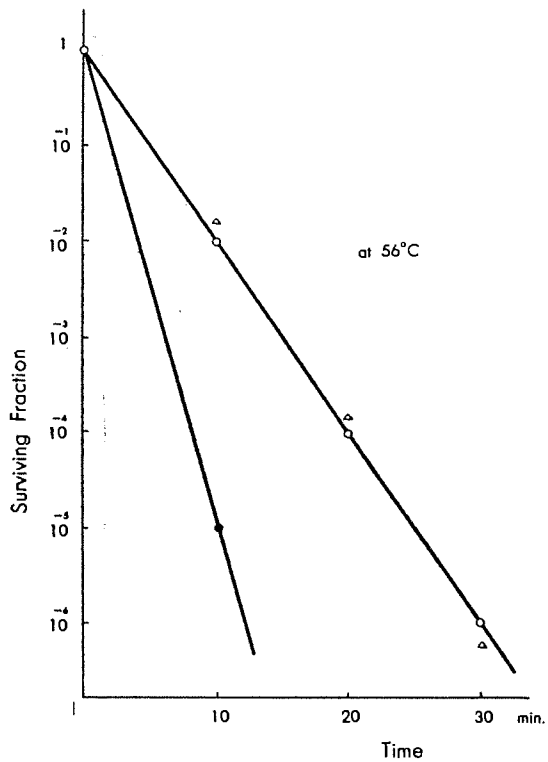

Fig. 3. The survival of vaccinia, ectromelia and transformed virus exposed to 56°C as a function of time. ○——○ Vaccinia, ●——● ectromelia, △——△ transformed virus.

Transformation in HeLa, FL cells and CAM of eggs

Both vaccinia and ectromelia could be propagated on HeLa, FL cell cultures and chorioallantoic membranes of hatching eggs. So transformations in these cells were attempted. The procedures used for tissue culture were almost the same as those in L cells. In the experiments *in ovo*, the chorioallantoic membranes of 11 days old embryonated eggs were inoculated with 0.1 ml of inoculum and the eggs were incubated at 36°C. After 3 days, the membranes were harvested, ground with alundum in Hanks' saline, and centrifuged at 3000 rpm for 10 minutes. The supernatant fluids were passed on L cells and tested on rabbits. All three

kinds of cells gave the same positive skin reaction. There seems to be no difference between the three host cells.

DISCUSSION

It appears from these results that the transformation from ectromelia to vaccinia takes place in tissue culture, and that the transformant acquires at least some of the characters of the vaccinia virus. The experiments showed that these characters of transformant were continuously transmissible to their progeny in tissue culture. Further, a virus of the same character could also be obtained from transformation experiments between ectromelia and the heat killed transformant when the transformed virus was heat inactivated again under the same conditions used for vaccinia. These facts seem to indicate that the characters of the transformant are rather stable and permanent, and that this phenomenon is due to a genetic interaction of the two viruses. No attempt has yet been made to isolate any recombinant from the mixed infection material. The reproducibility of this experiment was exceedingly high, under a variety of conditions.

Kilham (1957) reported on the role of serum in the transformation between fibroma and myxoma. This observation that the presence of serum in the virus suspension during heat inactivation was essential to retain the transforming activity of the heat killed preparation was true in our experiments also. Experiments are now under way to elucidate the role of serum with the transforming agent. Vaccinia virus inactivated by exposure to ultraviolet light lost its transforming activity when the virus survival was less than 10^{-6}.

The Berry-Dedrick phenomenon has been studied by many investigators. Recent works by Kilham (1958 b, 1959) have thrown light on the properties of the transforming agent of myxoma. This phenomenon has been considered a specific interaction between fibroma and myxoma. The present studies therefore, offer another example of viral transformation. We have also succeeded in accomplishing transformations with various combinations of tumor viruses and other pox group viruses. (Hanafusa *et al.*, 1959 b). These observations may give strong support to Berry-Dedrick phenomenon and indicate that this phenomenon is not specific between fibroma and myxoma but general among other pox group viruses.

Although the present studies can hardly answer the problem on the true mechnism of this phenomenon, it is assumed that the heat killed viruses might attach to or possible enter the cells where they interact with active viruses to cause the transformation. The term " transformation " has been used genetically for those systems in which cell-free and phage-free desoxyribonuclease sensitive material (i. e., DNA), genetically representative of the cell from which it was extracted, is used to transfer genetic characters to recipient bacteria (Hartman, 1957). Since both in the present experiment and the Berry-Dedrick phenomenon all the events occur in living animal cells and virus particles, after entering the cells, supposedly break down to their components such as nucleic acids and protein, the situation is in the strict sense of the word quite different from bacterial transformation. It may merely be reactivation of inactive virus by active virus that coexists with it. Therefore the term " transformation " may be

inadequate for a phenomenon of this type, but as described by Kilham (1958 a) it seems preferable to retain the general term "transformation", until definite information on the mechanism involved is available.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to Dr. K. Fukai for his helpful suggestions and discussion. They also wish to thank Dr. K. Kanazawa for generously supplying the IHD strain of vaccinia used.

REFERENCES

Berry, G. P., and H. M. Dedrick (1936) A method for changing the virus of rabbit fibroma (Shope) into that of infectious myxomatosis (Sanarelli) *J. Bacteriol.*, **31,** 50-51.

Gardner, R. E., and R. R. Hyde (1942) Transformation of rabbit fibroma virus (Shope) into infectious myxomatosis (Sanarelli). *J. Infectious Diseases,* **71,** 47-49.

Hanafusa, T., H. Hanafusa and J. Kamahora (1959 a) Transformation of ectromelia into vaccinia virus in tissue culture. *Virology,* **8,** 525-527.

Hanafusa, H., T. Hanafusa and J. Kamahora (1959 b) Transformation of myxoma into vaccinia or ectromelia virus in tissue culture., Nature, To be published.

Hartman, P. E. (1957) Transduction: a comparative review, The Chemical Basis of Heredity, Baltimore, The John Hopkins press, 408-409.

Kilham, L. (1957) Transformation of fibroma into myxoma virus in tissue culture. *Proc. Soc. Exptl. Biol. Med.,* **95,** 59-62.

Kilham, L. (1958 a) Fibroma-myxoma virus transformation in different types of tissue culture. *J. Natl. Cancer Inst.,* **20,** 729-739.

Kilham, L., E. Lerner, C. Hiatt and J. Shack (1958 b) Properties of myxoma virus transforming agent. *Proc. Soc. Exptl. Biol. Med.,* **98,** 689-692.

Nii, S., (1958) Plaque formation and some cytopathogenic characteristics of ectromelia virus (Hampstead strain) and vaccinia virus (IHD strain) on monolayers. Presented at the 6th annual meeting of Japanese virologists held in October, 1958. To be published in this journal.

Shack, J. and L. Kilham (1959) Relation of myxoma deoxyribonucleic acid to fibroma-myxoma virus transformation. *Proc. Soc. Exptl. Biol. Med.,* **100,** 726-729.

Smith, M. H. D. (1952) The Berry-Dedrick transformation of fibroma into myxoma in the rabbit. *Ann. N. Y. Acad. Sci.,* **54,** 1142-1152.