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Transformation Phenomena in the Pox Group Viruses.

II. Transformation between Several Members of Pox Group

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SUMMARY

Transformations between some members of the pox group viruses have been performed in tissue cultures and the chorioallantoic membranes of embryonated eggs. From experiments with various combinations of viruses, using vaccinia IHD, vaccinia Biken, ectromelia, myxoma, fibroma and fowlpox as active forms and vaccinia IHD, vaccinia Biken and ectromelia as heat killed forms, it was shown that transfomation was a general phenomenon in pox group viruses. Two viruses, vaccinia Biken and ectromelia, are interconvertible under suitable experimental procedures. None of the viruses used, other than the pox group, can be transformed with heat killed vaccinia. Some of the conditions favorable to transformation and some information on the mechanism of this phenomenon are discussed.

INTRODUCTION

In the preceding paper, it was shown that transformation between ectromelia and vaccinia can take place in tissue culture with high reproducibility and that, in several respects, the transformant has the same character as vaccinia (Hanafusa *et al.*, 1959 a, c). Kilham (1958) reported that the transformation of fibroma to myxoma virus was not always dependent on the virus of the Shope rabbit fibroma, for squirrel fibroma could also lead to transformation to live rabbit myxoma. These results suggest that phenomena of this type occur generally among other members of the pox group. A short report has already been given on experiments using myxoma or fibroma as the active virus and vaccinia or ectromelia as the inactive form (Hanafusa *et al.*, 1959b). This paper reports further studies on this subject with various combinations of pox group viruses, namely vaccinia (IHD), vaccinia (Biken), ectromelia, myxoma, fibroma and fowlpox. Since the attitudes of these viruses in tissue culture were different from one another, several kinds of host cells were used for the transformation experiments with each combination of virus strains.

Materials and Methods

Details of *tissue culture method*, *virus assay* and *the experimental animals* used were described in the previous publication by Hanafusa *et al.* (1959 c).

Cells

A monolayer culture of L, HeLa and human amnion (FL) cells and the chorioallantoic membranes of 11 day old embryonated eggs were employed.

Viruses

The following virus strains were used.
1. Ectromelia (Ect); The Biken strain, isolated in our Institute, was used after serial passages on L cells. 2. Vaccinia IHD (Vac IHD); A neurotropic strain of vaccinia was received from Dr. K. Kanazawa (Research Laboratories, Takeda Pharmaceutical Industries, Ltd., Osaka) and maintained in our laboratory by successive passages in mice brain, Ehrlich tumor cells and L cells. The virus stocks used in the present work obtained from infected L cell culture. 3. Vaccinia Biken (Vac B); A dermotropic strain of vaccinia used as the vaccine, was grown once on eggs and then many times in FL cells. 4. Myxoma (Myx); Myxoma virus was received from American Type Culture and passaged many times in rabbit skin and then in FL cells. 5. Fibroma (Fib); Fibroma virus was passaged in FL cells in the same way as myxoma virus, sometimes the carrier cultures were used as the cells (Kato *et al.*, 1959 b). 6. Fowlpox (Fowl); Homogenized suspensions of chorioallantoic membranes infected with fowlpox virus were used as the virus stocks. 7. Influenza NWS 8. HVJ Z; Chorioallantoic fluids infected with influenza NWS or HVJ Z were used. 9. Poliomyelitis; HeLa cell supernatant of a Brunhilde strain of poliovirus type I. 10. Measles; FL cell supernatant of an Edmonston strain of measles virus. 11. Herpes simplex; Herpes simplex virus isolated in our laboratory (Nii, unpublished) and passaged in FL cells.

Virus assay

The titration of infectivity was carried out in L cell cultures by the end point and plaque assay methods for both ectromelia and vaccinia IHD. Virus titers of vaccinia Biken, myxoma and fibroma were determined on rabbit skins by the end point method and expressed as RID (rabbit infectious doses).

Heat inactivated virus preparations

Heat inactivation was carried out with ectromelia, vaccinia IHD and vaccinia Biken. The virus fluids (10^6 PFU or RID) from culture cells infected with these viruses were heated at 56°C for two hours (Vac IHD, Vac B) or one hour (Ect) respectively. These heated preparations were not infectious to either tissue culture or test animals. Details of inactivation procedures and further studies on the heat killed virus were described in the preceding paper (Hanafusa *et al.*, 1959 c).

Outline of transformation experiments

The procedures for transformation experiments varied somewhat with the combinations of active and inactive viruses. A typical procedure was as follows. A mixture of one ml each of active and heat killed virus was put on the cell layer adhering to the glass surface of a large culture bottle. After 3 hours of adsorption the inoculum was removed and the cells were washed with Hanks'

saline to which were added 8 ml of growth medium. Sometimes, the inoculum was added together with the growth media and the cells were kept at 37°C without washing. At suitable time intervals, the cells were disrupted by freeze-thawing and centrifuged at 3000 rpm for 5 minutes. The supernatant of this centrifugation was inoculated onto other cells, which could support the propagation of transformed virus. After successive passages on cell cultures, the transformant was purified twice by the limiting dilution method. Some characters of this purified transformant were compared with that of original virus strain. More details and additional methods are presented under the various headings below.

RESULTS

Properties of poxviruses used.

In the present experiments, it is essential that the characters of the two viruses used as genetic markers are stable and differ clearly from each other. Several properties of the pox viruses were described by Fenner (1957, 1958), but in his articles the viral growths in tissue culture are not mentioned. Differences in susceptibility of a tissue culture cell to various virus strains offered a convenient tool in the present experiments. The attitudes of these viruses on cluture cells are summarized in Table 1. together with pathogenicity to experimental animals. These properties are stable except for the giant cell formation, which seems to depend upon, not only the strain of virus used but also on the metabolic state of the host cells. Since differences in pathogencity to experimental animals are very distinct and clear, they were used for the determination of the presence or absence of transformation.

Table 1. Properties of poxviruses

		Attitudes in Tissue Culture			Pathogenicity for Experimental Animals	
Virus Strain	Viral Growth	Cytoplasmic Inclusion Bodies	Giant Cell Formation	Destruction of Cell Sheets	Rabbits (Intradermally)	Mice (Intraperitoneally)
ectromelia	L, HeLa, FL	+	+	+	none	lethal
vaccinia IHD	L, HeLa, FL	+	+	+	skin lesions not lethal	none
vaccinia Biken	L, HeLa, FL	+	−	+	skin lesions not lethal	none
myxoma	HeLa, FL	+	−	−	skin tumors lethal	none
fibroma	HeLa, FL	+	−	−	skin tumors not lethal	none
fowlpox	——	−	−	−	none	none

Transformation experiments

1) Myx - Vac IHD, Fib - Vac IHD, Myx - Ect

The two tumor viruses, myxoma and fibroma can be propagated in FL cells (Kato *et al.*, 1959 a) with cytoplasmic inclusion bodies. Seven days after inoculation, the titer of intracellular virus reached about 10^6 RID (Takahashi *et al.*, 1958). These viruses did not destroy the cell sheets in contrast to vaccinia and ectromelia.

The transformation of Myx-Vac IHD (between active myxoma and heat killed vaccinia IHD) was attempted in FL cells. After monolayers had been exposed to a mixture of active and inactive virus, the culture media were replaced twice weekly by fresh media containing 1 ml of heat inactivated vaccinia IHD. Seven days after inoculation, infected cells were disrupted by freeze-thawing and centrifuged. One ml of the supernatant was transferred to monolayers of L cells. Focal degeneration similar to that of vaccinia was seen after two days. In some experiments the same focal degeneration was found in an FL cell culture used for transformation. This would indicate the appearance of the transformant, probably vaccinia virus. The purified transformed virus had the same pathogenicity to rabbits as vaccinia virus. In L cells where myxoma virus cannot propagate, transformation failed to occur.

By a similar procedures, transformation could take place with Myx-Ect and Fib-Vac IHD in FL cells.

2) Vac B - Ect, Ect - Vac B, Vac B - Vac IHD

Multiplication of vaccinia Biken on L cells was reported by Kanazawa (1957). When a dilute virus suspension was inoculated, it could be propagated and passaged successively in L cells. On the contrary, undiluted suspension of this virus (10^{6-7} RID) induced degeneration of the whole cell sheets within 24 hours. On passage in other L cells however, only a few focal degenarations were produced in the same way as with diluted virus. When vaccinia Biken was used after serial passages on L cells, but not when undiluted vaccinia was used, transformation with heat killed ectromelia could take place. Further studies on the attitude of vaccinia Biken in L cells are now in progress.

Transformation with this combination always occurred in FL cells. Vaccinia Biken can be propagated in FL cells more rapidly than ectromelia. The reverse is true in L cells. So the supernatant fluid of FL cells which had been exposed to a mixture of vaccinia Biken and heat killed ectromelia, was transferred to monolayers of L cells to allow the transformant to grow sufficiently. When injected intraperitoneally the purified transformed virus was pathogenic to mice.

Transformation of Ect-Vac B, was successful in FL and HeLa cells. The appearance of transformed virus was shown by a positive skin reaction in rabbits.

Transformations between two strains of vaccinia virus were attempted on FL cells using active Biken strain and heat killed IHD strain. Supernatant fluids of FL cells used for transformation experiments induced a rapid degeneration and the giant cell formation in L cells characteristic of vaccinia IHD. Therefore there seemed to be a positive result, although further examinations of the properties of the transformant were not performed.

3) Fowl - Vac IHD

As shown in the preceding paper, transformation of ectromelia into vaccinia IHD could also be performed in the chorioallantoic membranes (CAM) of embryonated eggs. As not all of the cell cultures used in the present studies could support the multiplication of fowlpox virus, experiments with Fowl-Vac IHD were carried out in the CAM of eggs. The procedure was the same as those with Ect-Vac IHD in the earlier paper. The CAM were each inoculated with

0.1 ml of Fowlpox (10^{-1} dilution of a 10% suspension of infected CAM) and of a heat killed vaccinia IHD suspension. After 3 days, the membranes were harvested, ground with alundum in Hanks' saline, and centrifuged at 3000 rpm for 10 minutes. The supernatant fluid of this centrifugation produced focal degeneration in monolayers of L cells. After several passages in L cells, test rabbits inoculated with this transformant gave skin test as for vaccinia IHD.

4) Other viruses used as the active virus

With heat killed vaccinia IHD, no virus tested other than pox group such as poliomyelitis, measles and herpes simplex led to transformation in cells where they and the vaccinia could be propagated. Influenza NWS and HVJ induced cytopathic changes on L cells, but transformation also failed. Results of experiments with various combinations of viruses are summarized in Table 2.

Table 2. Transformation among poxviruses

Cell	Live Virus	Heat Killed Virus	Transformation
L	ectromelia	vaccinia IHD	+
	myxoma	vaccinia IHD	−
	HVJ Z	vaccinia IHD	−
	influenza NWS	vaccinia IHD	−
	vaccinia Biken	ectromelia	−(+)
FL	ectromelia	vaccinia IHD	+
	myxoma	vaccinia IHD	+
	fibroma	vaccinia IHD	+
	vaccinia Biken	vaccinia IHD	+
	measles	vaccinia IHD	−
	herpes simplex	vaccinia IHD	−
	myxoma	ectromelia	+
	vaccinia Biken	ectromelia	+
	ectromelia	vaccinia Biken	+
HeLa	ectromelia	vaccinia IHD	+
	poliomyelitis	vaccinia IHD	−
	ectromelia	vaccinia Biken	+
CAM	ectromelia	vaccinia IHD	+
	fowlpox	vaccinia IHD	+

DISCUSSION

From the above data it is concluded that transformation of Berry-Dedrick's type occurs widely among members of the pox group. Further some combinations seem to be interchageable, if the following conditions are satisfied. First, both viruses forming a combination of active and inactive forms should be able to grow in the cells used for the transformation. Thus myxoma or vaccinia Biken could not lead to transformation in L cells, while they were transformed into vaccinia in FL cells (Table 2). Second, for transformation, the use of cells in

which the virus to be inactivated can be propagated more rapidly than the virus used as the active form, may facilitate the growth of the transformant. For example, as vaccinia grows more rapidly than myxoma, transformation can take place with a combination of Myx-Vac IHD in FL cells. But if attempts were made to transform vaccinia into myxoma, the culture cells are destroyed by active vaccinia sooner than transformed myxoma virus is propagated.

Kilham (1958) showed that it was easier in such experiments to demonstrate an increase rather than a decrease in virulence. However, from our experience, the transformant, even if it is less virulent, is isolated easily, when it is obtained with a higher titer than that of the active form in the tissue culture. In experiments with a combination of Vac B-Ect in FL cells, the transformed virus could be grown selectively by passages in different cells, that is L cells. Positive results were also obtained with the reverse combination of Ect-Vac B in FL cells. These facts indicate that transformation can take place reversibly between the two viruses.

Present studies also offer some informations on the mechanism of viral transformation. Negative results with Myx-Vac IHD in L cells indicate that active virus must multiply in the cells used for the transformation. Moreover, it is evident that this phenomenon is responsible for some interaction of the viruses in the cells they have entered. These concepts are supported by results given in the earlier paper on the ability of heat inactivated virus to enter cells. Viruses tested, other than pox group, could not lead to transformation with heat killed vaccinia. It may be conceivable that transformation can take place only between two closely related viruses in the mechanism of viral synthesis, and that the heat killed virus can be reactivated by utilization of some common activities of the active form, which have been lost by heat inactivation.

Recently Takahashi *et al.* (1959) found deep crossings of complement fixing antigens among several pox viruses. It is very interesting to see whether or not this phenomenon can take place only between serologically related viruses, and if so, the relationship between the transformation and cross-immunity may be an important problem.

Comment: After this manuscript was submitted similar results were reported independently by F. Fenner *et al.* (1959). Although the details of the experiments are not given in the short communication, their findings agree essentially with ours.

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