GENETIC ANALYSIS OF POLYKARYOCYTOSIS BY HERPES SIMPLEX VIRUS

I. EXPERIMENTS USING RECOMBINANTS FROM INTERTYPIC CROSS

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Strain HF (type 1) produces large plaques with a single flat polykaryocyte, whereas strain KY (type 2) produces small ones with round cells. Four recombinants were obtained by cross of HF $\times$ KY which possessed the characters intermediate between the parents with respect to plaque size and cytopathogenic effect. Further crosses between these recombinants produced HF-type virus. The crosses between the one recombinant and fresh isolates or mutant viruses also produced HF-type virus at different ratios.

The occurrence of parental type by such the crosses suggests that plural genes are involved in the processes of polykaryocytosis by herpes simplex virus.

The previous studies showed that type 1 and type 2 herpes simplex virus could easily recombine with each other if cultured cells were mixedly infected with both types of virus³⁾. The high frequencies of recombination were confirmed with respect to such the traits as antigenicity, plaque size, cytopathogenicity and IDU (5-iodo-2'-deoxyuridine)-resistance. The tests of progeny viruses isolated from single plaques revealed that a number of recombinants were produced which involved those possessing characteristics intermediate between parents.

Widly reported earlier in interstrain crosses between markers affecting pock character and virulence that four out of ten clones isolated from progeny were recombinant types possessing degrees

of virulence intermediate between those of the parents⁷⁾. Timbury et al. also reported one recombinant antigenically different from parent viruses which had been obtained from a cross between ts-mutants of type 1 and type 2⁶⁾.

The facts of the production of viruses with intermediate characters between parents through recombination probably mean that any of these characters used for genetic marker is determined by two or more cistrons in the viral genome. If this is the case, the viruses having the same characters as parents should be produced by further cross between such the recombinants.

This report investigates the occurrence of parental virus, with special reference to polykaryocytosis, by genetic interaction between recombinants

possessing intermediate characters obtained from intertypic cross, and also between the recombinant and the other virus strains with different origins.

MATERIALS AND METHODS

Cells

Cells cloned from GMK (continuous cell line of African green monkey kidney) cells were used throughout the present experiments. The cells were grown in Eagle's medium (MEM) supplemented with 10 % heated (56 °C, 30 minutes) bovine serum.

Viruses

The original parent viruses, strain HF (type 1) and KY (type 2), from which the recombinant viruses used in this study have been produced by their cross, have distinct genetic characteristics in plaque size and cytopathogenic effect (CPE). HF is characterized by large plaque (3 to 5 mm) consisted with a single large flat polykaryocyte (syncytium), while KY forms small plaques (0.8 to 1.2 mm) and renders the infected cells round.

Four recombinant viruses were isolated and purified by plaquing the progeny which were produced by cross between HF and KY. These recombinants designated R1, R2, R3 and R4 have the characters intermediate between parents, that is, they form plaques of 1.5 to 2 mm in size which are composed of mixtures of round cells and polykaryocytes with various size (Fig. 1).

Various type 1 and type 2 strains were also used for cross experiments with a recombinant R3, whose origins and characters will be described later.

Titration and characterization of viruses

Viruses were titrated by plaque technique. GMK cell monolayers in 2-oz bottles were washed twice with phosphate-buffered saline (PBS) and inoculated with 0.1 ml virus dilution. After adsorption for two hours, the cultures were overlaid with four ml of MEM containing 2.5 % bovine serum, 1.5 % bacto-agar and protamine sulfate at a concentration of 400 µg/ml. The monolayers were stained after three days incubation at 37 °C by secondary overlay with three ml of agar medium containing neutral red and counted the following day.

The size of plaques formed was measured after four days incubation, and the CPE was checked microscopically on each plaque.

Cross experiment

GMK cells were simultaneously infected with two different viruses and the resulted progeny were plaqued and characterized.

In practice the cell monolayers washed twice with PBS were inoculated with 10 plaque forming units/cell of each virus. After adsorption for two hours at 37 °C the cells were washed seven times with PBS in order to eliminate unadsorbed virus. Four ml of MEM were added to the cultures and incubated at 37 °C for 24 hours. The progeny viruses were harvested by freeze-thawing followed by centrifugation.

RESULTS

Cross experiments between different recombinants

As reported previously³⁾, a cross between type 1 (strain HF) and type 2 (strain KY) herpes simplex viruses resulted in the production of a large number of recombinant viruses in the

Fig. 1 Cytopathogenic effects and plaques formed by parent and recombinant viruses.
A ; strain HF (type 1)
B ; strain KY (type 2)
C ; R2 (a recombinant)

progeny including those possessing the characters intermediate between parents. Four among the recombinants were se-

lected which had possessed the intermediate characters in both plaque size and CPE. These recombinants, R1, R2,

R3 and R4 could not be phenotypically differentiated between each other and the characters of them were genetically stable as they were not modified even after ten cycles of passages in GMK cells.

Crosses have been made with all pairwise combinations of the four recombinants. The progeny viruses from each cross were plaqued to obtain the total yield and to characterize the plaques with respect to the size and CPE. It was found that the cross produced progeny viruses with various combinations of two characters including intermediate nature, but only the ratios of large plaques consisted of a large flat polykaryocyte (HF-type plaques) were scored. The results are shown in Table 1 and demonstrate that the progeny contained HF-type viruses despite that every cross was between the intermediate characters, and the ratios of HF-type viruses produced were different with the cross. No HF-type virus was produced by single infection with any recombinant.

It should be noted also that the progeny contained such the viruses with very low frequencies that formed small plaques consisted with a single flat polykaryocyte. The plaques formed by progeny from the crosses were illustrated in Fig. 2.

TABLE 1

Ratios of HF-type viruses in the progeny from crosses between recombinants

Cross	Plaques counted	HF-type plaques	% of HF-type viruses
R 1 $\times$ R 2	460	50	10.9
R 1 $\times$ R 3	1130	27	2.4
R 1 $\times$ R 4	1400	21	1.5
R 2 $\times$ R 3	1216	27	2.2
R 2 $\times$ R 4	1180	41	3.5
R 3 $\times$ R 4	526	90	17.1

Fig. 2 Plaques formed by progeny from crosses between recombinants.

A ; progeny from cross of R 1 $\times$ R 3
B ; progeny from cross of R 2 $\times$ R 3

Production of HF-type polykaryocyte formers by crosses between recombinant R3 and various virus strains

Preceding fact that every combination of crosses between four recombinants resulted in the production of progeny viruses which induced HF-type polykaryocytes leads us to make an attempt to carry out the cross between the recombinants and several virus strains with different origins. For this purpose only one recombinant, R3, was selected among four recombinants mentioned above.

Virus strains used for crosses with R3 were as follows: UW268, MS, 196, Ouchida and KY were type 2 viruses originally isolated from genital lesion except KY which was isolated from the lesion of finger and was one of the parent viruses from which R3 was derived by the recombination with strain HF

TABLE 2

Production of HF-type viruses by crosses between R3 and different strains

Cross of R3 with	Plaque size in mm	CPE cells	Plaques counted	HF-type plaques	% of HF-type viruses
Type 1					
YH	0.2-0.8	round	481	7	1.5
J	0.2-0.8	"	1955	45	2.3
K	0.2-0.8	"	1262	9	0.7
KB	0.2-0.8	"	1031	46	4.5
M	2.0	"	2323	63	2.7
Z	2.2-2.5	"	2735	44	1.6
Type 2					
UW268	0.2-1.2	"	2732	3	0.1
MS	0.2-0.8	"	1859	2	0.1
196	0.2-1.2	"	1169	3	0.3
OUCHIDA	0.2-0.8	"	4353	4	0.1
KY	0.2-1.2	"	1125	1	0.1

Fig. 3 Production of HF-type viruses by cross between R3 and fresh isolate.

A ; plaques formed by fresh isolate, strain YH (type 1).

B ; plaques formed by R3 (a recombinant from cross of HF (type 1) × KY (type 2).

C ; plaques formed by progeny from cross of R3 × YH.

of type 1. The other virus strains belonged type 1; YH, J and M were the isolates from labial lesion, Z from dorsal one, KB from one on the head, and K from generalized eczema. The plaque-purified virus from every strain was used for cross experiment.

The progeny from the crosses between these strains and a recombinant R3 were plaqued and only HF-type plaques were scored, although the plaques of medium size with polykaryocytes were also occasionally found. The results are shown in Table 2 together with the plaque and CPE characters of each strains used, and demonstrate: (a) that every cross produced more or less HF-type polykaryocyte formers; (b) that relatively small ratios of HF-type virus were found in the progeny from the crosses with type 2 viruses; and (c) that the ratios of HF-type virus produced were different with crosses with different type 1 strains. The plaques formed by YH, R3 and the progeny from the cross

between them were illustrated in Fig. 3.

Crosses of R3 with mutants of strain YH and their recombinants

We previously described the spontaneous mutations with strain YH which belonged to type 1 and also genetic interaction between them^{8) 9) 10)}. Fig. 4 illustrated the sequence of mutations and recombinant viruses obtained from two sets of cross and also shows the ratios of HF-type virus produced by the cross between these viruses and R3. The ratios were obtained by inspection of approximately 2,000 plaques formed by progeny from each cross. As illustrated in the figure, S is a clone which has the same characteristics as original fresh isolate, strain YH. The sequential spontaneous mutations occurred like as $S \rightarrow L \rightarrow G$. IDU-resistant mutants designated I^r were isolated from single plaques formed in the presence of IDU

Fig. 4 Sequence of mutation with type 1 strain YH and recombinant viruses obtained from crosses of $S \times GI^r$ and $SI^r \times G$, and ratios of HF-type virus produced by crosses between these viruses and R3.

* % of HF-type viruses produced by cross with R3.

** plaque size in mm and CPE character, R; round cells,

P; polykaryocyte.

--> mutation.

at the concentration of 500 $\mu\text{g/ml}$ in agar overlay. L, m3, L1 and C15-18 usually cause cell rounding by infection but rarely form very small polykaryocyte containing only few nuclei. L, m3 and C5-18 cannot be phenotypically differentiated between each other but L1 forms slightly larger plaques than these three viruses in spite of the lower yield (1/5 to 1/10) of progeny virus. G forms the plaque consisted of polykaryocyte with slightly larger size than that of L. The plaques of GI^r cannot be discriminated from those of HF, so that the cross experiment between this mutant and R3 was not attempted. The results demonstrate with the cross between these viruses and R3: (a) that the yield of HF-type virus does not change after the mutation with respect to IDU-resistance ($S \rightarrow SI^r$); (b) that mutations in plaque and CPE characters ($S \rightarrow L \rightarrow G$) increase the ratio of HF-type virus produced; and (c) that the two recombinants m3 and L1 apparently differ in the ratio of HF-type virus produced despite that they derived from the same parents.

DISCUSSION

The stepwise spontaneous mutation in relation to CPE and recombination between the mutants were previously reported⁸⁾⁻¹¹⁾. Strain YH (type 1) mutates in the sequence of $S \rightarrow L \rightarrow G$ as illustrated in Fig. 4. The cross experiments between S and G revealed that the L-type viruses were produced through recombination⁸⁾. Moreover, the recombinants possessing characters intermediate with respect to CPE between parents were obtained by cross between type 1 (strain HF) and type 2 (strain KY) which induce polykaryocyte and rounded cells, respectively. Brown et al.¹⁾ also found a mixed morphology plaque type with a phenotype that is

partly syncytial and partly non-syncytial in the progeny from crosses between ts-mutants of type 1. These phenomena suggest that the factor determining the formation of polykaryocyte, especially a large flat polykaryocyte, is not under the control by a single gene or multiple alleles associated with CPE but under the controls by two or more genes. The present study gave an additional evidence for the association of plural genes with induction of polykaryocyte by herpes simplex virus. The further evidence for the involvement of plural genes has been obtained from the cross experiments using S and G mutants of strain YH and the resultant recombinants. The details of the experiments will be described elsewhere.

That crosses between the intermediate recombinants from $HF \times KY$ produced the HF-type viruses, that is, a large flat polykaryocyte formers, at the different frequencies with the different combinations of crosses between the recombinants probably means the presence of more than two genes which responsible for polykaryocyte formation.

The interesting is the production of HF-type virus by the cross between the intermediate recombinant and freshly isolated or mutant viruses which forms rounded cells by infection. This strongly suggests that even such a virus as fresh isolate, especially of type 1, which never form polykaryocyte involves some genes which affect polykaryocytosis.

It is now considered that the alteration of the cell surface after infection with herpes viruses causes cell fusion to lead polykaryocytosis. The experiments using several inhibitors suggest that the fusion process is an early event during virus replication²⁾⁴⁾⁵⁾, but the mechanism involved in the fusion process is not yet known.

Our present experiments indicate that

several genes are possibly associated with the cell fusion, at least with the production of a large flat polykaryocyte. Thus, a hypothesis is that the so-called fusion factor is produced by several sequential processes which are controlled by different genes. This hypothesis will be especially available for the experiments suggesting the involvement of some genes affecting polykaryocytosis in freshly isolated viruses which cause no fusion (Table 2). If this is the case, however, the question remains to be explained for the occurrence of the viruses by mutation or recombination which have an ability to form a number of small polykaryocytes. Therefore, it is probably reasonable to consider that several kinds of fusion factors takes place in polykaryocytosis, each of which is determined by separate genes. Thus, a virus having a complete set of genes will produce a large flat polykaryocyte and that lacking one or other gene will cause the formation of mixtures of rounded cells and small polykaryocytes or those of various types of small polykaryocytes.

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