MUTANTS OF HERPES SIMPLEX VIRUS

II. IDU-RESISTANT MUTANTS AND A PRELIMINARY EXPERIMENT ON GENETIC RECOMBINATION

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The IDU (5-iodo-2'-deoxyuridine)-resistant mutants (SI, LI and GI) obtained from S (small plaque formers), L (large plaque formers) and G (polykaryocyte formers) of herpes simplex virus have a similar character in antigenicity and the same heat stability as the original types of viruses. Mixed infection with SI and L produced a recombinant virus at relatively high frequencies. In the mixed infection with SI and G, unexpected recombinant types such as L and LI were produced in addition to GI recombinant. This suggests that G character may be determined by a combination of at least two cistrons which exhibit the L character when they function separately.

In previous studies two mutants, designated L and G, were isolated from YH strain of herpes simplex virus. In contrast to the small plaque formation on GMK cells by parental virus (S), both mutants produced the large plaques and the G was in particular characterized by the formation of polykaryocytes⁷⁾. There was no antigenic difference to be detectable among S, L and G although they differed in the feature of the CPE (cytopathogenic effect) caused by infection to the cells.

It may be reasonable to consider that such a variety of the CPE is primarily determined by the genetic factor(s) in the herpes virus genome, despite the appearance of the CPE may be due to the interaction between virus and cells. It was necessary to obtain another stable marker in order to investigate the genetic relationship

between the different characters of the CPE.

The present report consists of the characterization of IDU-resistant mutants obtained from each plaque- or CPE-mutant and the preliminary studies as to whether the recombination could occur between plaque or CPE characters and IDU resistance.

MATERIALS AND METHODS

Cells

Cells cloned from GMK (continuous cell line of African green monkey kidney) were used for virus titrations and plaque purification. The cells were grown in 2-oz culture bottles using the mixture of 7 parts of Hanks saline containing 0.5% lactalbumin hydrolysate and 3 parts of Eagle's medium (MEM),

supplemented with 10 % heated (56°, 30 minutes) bovine serum. RK cells (serially passaged cells derived from rabbit kidney) were grown in MEM supplemented with 10 % bovine serum, and used for preparation of the virus stocks.

Viruses

The parental virus (S) and the mutants (L and G) isolated from YH strain were used ⁷⁾. The viruses were propagated in RK cells in MEM. The plaque assays of the viruses were carried out according to the procedure previously described ⁷⁾.

Isolation of IDU-resistant mutants

The procedures according to Buthala ¹⁾ were used with a slight modification for isolating the mutant resistant to IDU. S, L and G viruses were plaqued on GMK cells with 500 µg/ml of IDU in agar overlay medium. The well-isolated plaque formed in the presence of IDU was picked, suspended in the medium and centrifuged after freeze-thawing. The diluted supernatant fluid was inoculated on the cell monolayers and

overlayed with agar medium with the same concentration of IDU as above. After five cycles of this procedure the cloned virus was propagated in RK cells for preparation of virus stock and passaged in the same cells for inspection of the stability of IDU resistance. The IDU-resistant mutants obtained as above from S, L and G will be referred to as SI, LI and GI, respectively.

RESULTS

Plaquing efficiency of IDU-resistant mutants

The characters of plaque and CPE yielded by the IDU-resistant viruses were the same as those by the original viruses from which the resistant mutants were derived. The effect of IDU on the number and the size of the plaques formed by each virus was shown in Table 1. The original virus, S, L and G were greatly reduced in number of plaques as showing the plaquing efficiencies to be below 0.1 %. It may be thought that there were the IDU-resistant virus particles at such the

TABLE 1
Plaquing efficiency of different mutant viruses in the presence of IDU

virus	exptl no.	Concentration of IDU, µg/ml				
		300 µg	500 µg	virus	300 µg	500 µg
SI (0.1-0.3)	1	26.8	13.8	S (0.2-0.8)	0.09	0.07
	2		13.4			
	3	33.2	17.1			
	4		16.0			
LI (1-1.5)	1	99.6	91.0	L (1.5-2)	0.05	0.04
	2	80.8	80.0		0.05	
	3		105.0		0.06	
	4	88.7				
GI (0.5-2)	1	81.2	87.6	G (1.5-2.5)	0.03	0.03
	2	83.9	61.2			

The numbers in parenthesis represent the plaque size in mm.

ratio in the population of S, L or G viruses. The plaquing efficiencies were 80 to 100 % with LI, and 60 to 80% with GI. In contrast to these IDU-resistant mutants, SI showed the value to be approximately 30 and 15 % in the presence of 300 and 500 $\mu\text{g/ml}$ IDU, respectively. The low efficiencies may indicate that SI always contains the IDU-sensitive virus particles in the population. An attempt was therefore made to isolate some clones from plaques of SI and reestimate the plaquing efficiencies on them. Table 2 shows that the plaquing efficiencies of every clone obtained from the plaques formed in the presence or absence of IDU in agar overlay medium are essentially the same as that of original SI virus. Therefore, it is not considered that there is the possibility of the existence of a significant amount of IDU-sensitive viruses in the SI population. The plaquing efficiencies of each mutant remained unchanged on passage, so that the IDU-resistance of virus can be considered as a genetically stable character.

TABLE 2
Plaquing efficiency of clones obtained from SI

Clone	origin*	plaquing efficiency**
1	+	30.3
2	+	21.5
3	—	21.9
4	—	25.5
5	—	30.8
6	—	28.1
7	—	34.9
SI		25.7

* Clone obtained from a plaque formed in the presence (+) or in the absence (—) of IDU.

** Plaque efficiency in the presence of 300 $\mu\text{g/ml}$ IDU.

Heat stability

Inactivation at 50° were carried out by the procedure previously described⁷⁾. As shown in Fig. 1, the results in the previous report was again confirmed, that is, S and L were inactivated at a similar rate, whereas G mutant was inactivated more rapidly than S and L. Furthermore, the IDU-resistant mutants showed the same inactivation rates as the viruses from which the mutants were derivd.

Fig. 1 Heat inactivation at 50° of different mutant viruses

Antigenic properties

The antigenic properties of SI, LI and GI were compared with their original viruses, S, L and G by neutralization kinetics using antisera prepared in rabbits against S, L and GI. The procedures were already described⁷⁾. Table 3 shows the results expressed by the values of normalized neutralization rate constant (NK-values). It was shown that the IDU-resistant mutants and their original viruses cannot be

TABLE 3
NK-values of different mutant viruses

anti-serum	virus					
	S	SI	L	LI	G	GI
S	100	101	87	103	101	95
L	117	94	100	99	114	108
GI	116	110	84	120	80	100

significantly differentiated from each other by the neutralization rates.

Detection of recombinant

GMK cells were mixedly infected with SI and L (or G). The cells were washed twice with PBS (phosphate buffered saline) and inoculated with a mixture of SI and L (or G). After adsorption for two hours, the cells were washed seven times with five ml of PBS and added four ml of MEM. The progeny viruses were harvested after 21 hours incubation by freeze-thawing followed by sonication at 20 kc/sec for two minutes. For detecting the re-

combinant LI (or GI), the progeny viruses were plaqued on GMK cells with or without 500 μ g/ml IDU in agar overlay medium. The recombination frequency in the yield was expressed as the ratio; number of large plaques formed in the presence of IDU/number of large plaques formed in the absence of IDU $\times 100$.

Recombination between SI and L

The results of mixed infection with SI and five L mutants were shown in Table 4. Infections were done at multiplicities of infection (MOI) of 3 and 5 PFU/cell with SI and L mutant virus, respectively. For the controls, each mutant were singly infected to cells at the same MOI. The results shown in Table 4 demonstrated the fairly high frequencies of recombination between SI and L. The progeny from single infection with L mutants contains LI virus at a low ratio comparable to that of the parent L as shown in Table 1. The frequencies of recombinant produced

TABLE 4
Recombination frequency between SI and L

viruses inoculated	progeny				Recombination frequency
	yield, PFU/ml		no. of large plaques		
	L-type	S-type	without IDU	with IDU	
SI+L	2.8×10^8	8.5×10^7	7000	505	7.22
SI+L ₁	3.5×10^8	1.0×10^8	7830	365	4.66
SI+L ₂	2.1×10^8	1.3×10^8	7250	371	5.12
SI+L ₃	3.0×10^8	1.0×10^8	6175	409	6.62
SI+L ₄	2.8×10^8	7.0×10^7	6475	366	5.66
SI		4.9×10^7			—
L	4.1×10^8		7680	8	(0.104)
L ₁	4.3×10^8		7050	3	(0.043)
L ₂	2.2×10^8		5550	3	(0.054)
L ₃	2.8×10^8		6625	1	(0.015)
L ₄	4.3×10^8		4825	1	(0.021)

The numbers in parenthesis represent the ratio of LI in the progeny from single infection with L.

in the progeny from mixed infection are so high that the low ratios of LI in the progeny from single infection with L mutant may be negligible in the recombination frequencies obtained.

Recombination between SI and G

Cells were mixedly infected with SI and G mutant at the MOI of 3 and 1, respectively. It should be noted that the mixed infection caused cell rounding which was characteristic to the CPE by SI, despite a single infection with G always produced polykaryocytes. In this experiment, as shown in Table 5, none of the IDU-resistant viruses out of 6425 large plaques from single infection with G could be detected and the higher recombination frequency (10.9%) in the mixed infection was obtained than in the case of mixed infection of SI and L. Unexpectedly, the large plaques that produced the CPE of rounding cells were found by microscopic observation of plaques formed by the progeny viruses from the mixed infection. Therefore, in order

to confirm the production of recombinant type such as L or LI, an attempt was made to isolate the clones from the large plaques formed by the progeny viruses in the presence and absence of IDU and determined the CPE character and IDU resistance. Out of 10 clones, 7 produced the CPE which is characterized by cell rounding and rarely small giant cells including only few nuclei, and 3 produced the polykaryocytes as well as the parent G mutant. Four of the 7 L type and 1 of the 3 G type were IDU-resistant. Every clone obtained from G (14 clones) and progeny viruses (11 clones) from single infection with G was polykaryocyte formers and IDU-sensitive as same as G parent virus. Although the ratios of these unexpected types of viruses in the progeny were not determined, it was confirmed that, by mixed infection with SI and G, the recombinants such as L and LI of which characters were not detected in the parent viruses could be produced.

TABLE 5
Recombination frequency between SI and G

viruses inoculated	progeny				Recombination frequency
	yield, PFU/ml		no. of large plaques		
	L-type	S-type	without IDU	with IDU	
SI+G	2.4×10 ⁷	5.0×10 ⁷	4475	490	10.9
G	7.0×10 ⁷	—	6425	0	0

DISCUSSION

Wildy⁶⁾ studied the recombination with herpes simplex virus using a variety of strains which differed in the type of lesions on chorioallantois and virulence on mice or cornea of the rabbit. He found some recombinants in the progeny viruses obtained by mixed

infection which possessed degree of virulence intermediate between those of parents. Subak-Sharpe⁵⁾ reported that the recombination was possible between some temperature-sensitive mutants of herpes simplex virus.

IDU-resistant viruses described in the present report were genetically stable in their characters, and showed

the same antigenic behavior and thermostability as the original viruses from which they derived. Large plaque formers resistant to IDU, designated LI, formed almost equal number of plaques in the presence and in the absence of IDU. Therefore, the recombination frequencies could be estimated by comparing the infectivities obtained in the presence and absence of IDU in agar overlay medium if an experiment was designed to produce LI as a recombinant.

When the mixed infection was done with SI and L or SI and G, one recombinant should be LI or GI. The results from crossing of SI and L described above demonstrate that a recombinant LI was produced at high frequency in the progeny. Relatively low recombination frequencies have been reported generally with animal viruses even if the recombination occurred, except with influenza and reoviruses. The high frequencies of recombination of the two viruses have been ascribed to the segmented genomes which were also proved by chemical analysis of the nucleic acids ³⁾. Although such a situation was not obvious with herpes simplex virus, Frenkel and Roizman pointed that the herpes virions contain only one species of double-stranded DNA molecules and the value determined by renaturation kinetics was in agreement with the molecular weight of viral DNA, $(99 \pm 5) \times 10^6$ daltons ²⁾. At present, therefore, it is not likely to consider that the high recombination frequency obtained in our experiment is associated with the segmented genomes in the virion. It might be possibly due to the high molecular weight of viral DNA.

In the present experiment of mixed infection with SI and L, LI which is only one of the two possible recombinants could be detected. The reciprocal recombinant or S was probably present

in the progeny viruses. It is thought that the demonstration of the reciprocal recombinant and estimation of the frequency of its production is important in a viewpoint of accounting for the mechanism included in the recombination process. The reciprocal recombinant remains to be looked for and it will be found by isolating many clones from the small plaques formed by the progeny viruses and test separately the IDU-resistance of them. Furthermore, it may be necessary to account for whether the two possible recombinant could be produced in a single cell mixedly infected with SI and L, since Slade and Pringle⁴⁾ found only one of the two possible recombinants in the case of recombination with foot-and-mouth disease virus in a single cell.

It should be noted that the unexpected recombinants such as L and LI were found in the progeny viruses from mixed infection with SI and G. Since the accurate frequencies of each types of viruses were not determined the genetical significance is not clear, but it may be speculated that phenotypic appearance of G character (polykaryocytosis) is exhibited by at least two cistrons which determine the appearance of L character when they function separately. Accordingly, G mutant comprises at least two different L cistrons in the genome which is phenotypically identical. If this is the case, the genetic analysis will afford the informations on the sequential steps included in polykaryocytosis or cell fusion by herpes simplex virus, and furthermore give the biochemical methods by which clarify the mechanisms of polykaryocyte formation.

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