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SELECTION OF HERPES SIMPLEX VIRUS MUTANTS SENSITIVE TO SULPHATED AGAR POLYSACCHARIDES

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Summary. — Twenty-eight mutants sensitive to agar polysaccharides were isolated from microplaques produced by herpes simplex virus in chick embryo cell cultures under agar overlay. Nineteen of them were induced by hydroxylamine. The frequency of spontaneous and hydroxylamine-induced mutations was studied.

Introduction

Natural and synthetic sulphated polysaccharides are known to inhibit the adsorption and reproduction of many RNA- and DNA-containing viruses such as Newcastle disease (Thiry, 1964), encephalomyocarditis (Takemoto and Liebhaber, 1961*a*, *b*), polio (Agol and Chumakova, 1963; Pagano, 1965), ECHO (Takemoto and Liebhaber, 1961*b*), Venezuelan equine encephalomyelitis (Kaverin, 1965) and herpes simplex (Takemoto and Spicer, 1965; Takemoto and Fabish, 1964; Vaheri and Cantell, 1963; Vaheri, 1964) viruses.

Data in the literature on the sensitivity of herpes simplex virus to agar polysaccharides are contradictory. According to Tytell and Neuman (1963) the addition to agar overlay of protamine sulphate or substitution of agar with methyl-cellulose led to increase in plaque numbers. Contradictory results were obtained by Taniguchi and Ioshino (1964) who showed that substitution of agar with methyl-cellulose resulted in increased plaque size but did not increase plaque numbers. According to De Mayer and Schonne (1964) the agar overlay influenced both the size and the number of plaques produced. These contradictory data probably could be attributed to differences among strains of herpes simplex virus with respect to sensitivity to sulphated agar polysaccharides. Strains of herpes simplex virus that had completely lost the resistance to agar polysaccharides have not been reported.

The resistance to sulphated polysaccharides is a stable genetic character and mutants with a different degree of sensitivity were obtained in some viruses (Thiry, 1964; Takemoto and Liebhaber, 1961*a*, *b*, 1962).

So far, data on the selection of herpes simplex virus mutants with changed level of resistance to sulphated polysaccharides have not been reported. We studied, therefore, the possibility of obtaining such mutants by selection or induction by mutagens.

Materials and Methods

Virus. The L2k5 strain of herpes simplex virus producing plaques in chick embryo cell (CEC) monolayers under agar overlay (Porterfield, and Allison, 1960) was used. The strain was purified by three times repeated cloning from single plaques.

Selection of mutants. Mutants sensitive to agar polysaccharides were selected from microplaques (approx. 0.1 mm in diameter) that occurred rarely in agar-overlaid CEC monolayers infected with 10-fold dilutions of original virus. The plaques were examined under a dissecting microscope (magnification × 7) on the 4th – 5th day of incubation at 37° C. To obtain sensitive mutants, diethylaminoethyl- (DEAE-) dextran was added to the agar overlay in limiting concentration (0.1 mg/ml).

To *select spontaneous reversions*, 1 ml samples of undiluted suspension of a mutant were inoculated in CEC monolayers under agar overlay without DEAE-dextran and after 4—5 days of incubation at 37° C the plaques were scored and isolated. The infectious titre of mutant suspension was determined by inoculating 10-fold dilutions on CEC monolayers under agar overlay containing 0.5 mg/ml DEAE-dextran and expressed in plaque forming units (PFU) per ml.

The reversion frequency was determined as the ratio of average number of plaques per culture flask to average number of virus particles (PFU) in the inoculum. The rate of mutations leading to sensitivity to sulphated agar polysaccharides was calculated as the ratio of absolute number of microplaques to total number of plaques determined visually. Only mutants that turned out stable upon further investigation were taken into account in calculations of mutation frequency.

Hydroxylamine treatment. A 4 M $NH_2OH.HCl$ solution in 0.5 M phosphate buffer was prepared before use and its pH adjusted to 7.5 with NaOH. Then 2.25 ml of this solution was mixed with 2.25 ml of a 2.6 M NaCl solution and 0.5 ml of undiluted virus suspension. The mixture was incubated at 37° C. After 0, 1, 2, 3 and 4 hours 0.2 ml samples were withdrawn and diluted 1 : 50 in Hanks' solution containing 2% acetone to inactivate hydroxylamine. To obtain mutants sensitive to sulphated agar polysaccharides, thus treated virus suspension was diluted in 10-fold steps in Hanks' solution and inoculated on CEC monolayers (1 ml of the appropriate dilution per culture flask). Agar overlay containing 0.1 mg/ml DEAE-dextran was used. The microplaques and "normal" plaques were scored after 5 days and the mutation frequency and survival rate were determined.

In selecting reversions, the hydroxylamine-treated suspension of the mutant, after inactivation of hydroxylamine, was inoculated in 1 ml volumes per flask on CEC monolayers under agar overlay without DEAE-dextran. The titre and survival rate of the mutant were determined by inoculating 10-fold dilutions on CEC monolayers under agar overlay containing 0.5 mg/ml DEAE-dextran.

Results

The original L2k5 strain used in our experiments produced plaques 1—2 mm in diameter after 4—5 days of incubation at 37° C in CEC embryo monolayers under agar overlay. The addition to the overlay of DEAE-dextran in a concentration of 0.5 mg/ml led to a 30—40% increase in plaque numbers but did not influence the plaque size. Sometimes plaques of approx. 0.5 mm diameter were observed. However, attempts to select a homogeneous small plaque variant were not successful. After infection of monolayers with suspensions of such small plaques we observed the same variation in plaque size as in the original strain.

When examining infected CEC monolayers after 4—5 days of incubation at 37° C at low magnification (× 7) we rarely observed microplaques that were hardly discernible with the naked eye. After inoculation of the suspension from single microplaques on CEC monolayers under agar overlay, no microplaques were not observed. But in CEC cultures with fluid medium (medium 199) the same suspensions produced a cytopathic effect. The virus propagated in this way produced no plaques when agar overlay was used, while the titre of virus in the suspension, determined by the limiting dilution method, was $10^{4.75}$ ID_{50}/ml.

The variants obtained were characterized as plaqueless and designated

with the symbol fpp (failing to produce plaques). The alternative allele of the original strain was designated as app (able to produce plaques).

We found that fpp variants produced plaques 0.5—1 mm in diameter after 4—5 days of incubation at 37° C under agar overlay containing 0.5 mg/ml DEAE-dextran. At a concentration of 0.1 mg/ml of DEAE-dextran in the overlay, the microplaques were produced. The addition to a liquid overlay of the water extract of agar polysaccharides obtained by Takemoto and Libhaber's (1961*a*) method inhibited the reproduction of fpp variants. The results obtained allow us to consider the fpp variants as mutants sensitive to sulphated agar polysaccharides.

Table 1. Frequency of spontaneous fpp mutations in the $L2_{k5}$ strain of herpes simplex virus

Exp. No.	Concentration of DEAE-dextran in overlay (mg/ml)	Number of plaques studied	Number of microplaques (fpp mutants)	Frequency of fpp mutations
1	0	1333	1	7.5×10^{-4}
2	0	3842	1	2.6×10^{-4}
3	0	8531	0	$< 1.17 \times 10^{-4}$
4	0	8068	0	$< 1.23 \times 10^{-4}$
5	0	14430	2	1.38×10^{-4}
6	0	8284	0	$< 1.2 \times 10^{-4}$
7	0	8030	0	$< 1.24 \times 10^{-4}$
8	0	14289	2	1.39×10^{-4}
9	0	8170	0	$< 1.22 \times 10^{-4}$
10	0	7410	0	$< 1.34 \times 10^{-4}$
11	0	10038	1	9.96×10^{-5}
12	0.1	8277	1	1.2×10^{-4}
13	0.1	8195	1	1.22×10^{-4}
Total		108897	9	8.26×10^{65}

The results of 13 experiments on the determination of the frequency of fpp mutations are presented in Table 1. These data demonstrate that frequency of fpp mutations was rather high and ranged from 9.9×10^{-5} to 7.5×10^{-4} in indidividual experiments. The mutation frequency calculated from all 13 experiments was 8.26×10^{-5}. There was no considerable difference in the frequency of fpp mutations in experiments with and without the addition of a limited concentration of DEAE-dextran. However, when overlay containing 0.1 mg/ml DEAE-dextran was used, the microplaques were more marked which made their isolation more easy.

The exposure of original virus to 2 M hydroxylamine hydrochloride led to increased frequency of fpp mutations, depending on the dose used. Nineteen fpp mutants were thus obtained with frequencies of 3.2×10^{-3} and 2.2×10^{-2} at survival rates of 3.2 and 0.18%, respectively. At survival rates of $\geqq 36\%$ and 0.018% fpp mutants were not detected (Table 2).

The next step in our investigations was the determination of the frequency of reversions to app in some fpp mutants. We found that the mutants studied (fpp_1, fpp_2, fpp_3, fpp_{10} and fpp_{14}) reverted with a frequency of 2.1×10^{-8}, 2.7×10^{-8}, 5.6×10^{-8}, 4.4×10^{-8} and 5.3×10^{-8}, respectively. Hydroxylamine failed to induce reversions both in fpp_{10} and fpp_{14} mutants that had been obtained by means of this mutagen and in spontaneous mutants fpp_1

Table 2. The dependence of survival rate and frequency of fpp mutations on the time of treatment of herpes simplex virus with 2 M NH_2.HCl, pH 7.5

Time of treatment (hours) at 37° C	Titre (PFU/ml)	Survival (%)	Number of plaques studied	Number of micro-plaques (fpp mutants)	Frequency of fpp mutations
0	5×10^5	100	4271	0	$< 2.3 \times 10^{-4}$
1	1.8×10^5	36	1255	0	$< 9.9 \times 10^{-4}$
2	1.6×10^4	3.2	1225	4	3.2×10^{-3}
3	9.2×10^2	0.18	660	15	2.2×10^{-2}
4	9×10^1	0.018	99	0	$< 1 \times 10^{-2}$

and fpp_3. It induced reversions in the spontaneous fpp_2 mutant with frequencies of 4.3×10^{-4} and 2.8×10^{-3} at survival rates of 9.5 and 0.8%, respectively.

Thus the frequency of spontaneous and hydroxylamine-induced mutations to fpp exceeded considerably the frequency of both spontaneous and hydroxylamine-induced reversions from fpp to app.

Discussion

In experiments on herpes simplex virus (strain L2k5), mutants sensitive to sulphated agar polysaccharides were obtained. Nine spontaneous fpp mutants were selected; the frequency of these mutations in the cloned population of the original virus was 8.26×10^{-5}. When the original virus suspension was treated with hydroxylamine, a considerable dose-dependent increase in frequency of fpp mutations was detected. A delay in phenotypic expresion of fpp mutants was observed; it was manifested by primary production of microplaques and absence of their production upon subsequent passaging in CEC monolayers under agar overlay without DEAE-dextran. The reasons of this delay have not yet been elucidated.

The spontaneous reversion to app type was observed in the population of fpp mutants, but their frequency was much lower than the frequency of mutations from app to fpp. We failed to induce the reversion by means of hydroxylamine in some spontaneous and hydroxylamine-induced fpp mutants. These findings are in agreement with data by Freese *et al.* (1961) on hydroxylamine mutagenesis in T4 phage.

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