JOURNAL OF VIROLOGY, Mar. 1969, p. 326-330

Vol. 3, No. 3
Printed in U.S.A

Recombination Between Guanidine-resistant and Dextran Sulfate-resistant Mutants of Type 1 Poliovirus

DINA SERGIESCU, ANDREI AUBERT-COMBIESCU, AND RADU CRAINIC
Cantacuzino Institute, Bucharest, Rumania

Received for publication 30 October 1968

Mixed infection of monkey kidney cells with two mutants of the LSc2ab strain of poliovirus, one resistant to guanidine and the other resistant to both dextran sulfate and 2-(α-hydroxybenzyl)-benzimidazole (HBB), yielded progeny in which the number of *gua*^{r} *dex*^{r} particles exceeded by a factor of 7 to 10 the expected number of similar particles occurring through spontaneous mutation; recombination would explain the fairly high excess of doubly mutant particles that was obtained. Scoring of HBB resistance in 50 *gua*^{r} *dex*^{r} clones suggested that, during recombination, resistance to dextran sulfate is not associated with HBB resistance.

After the pioneer work of Hirst (7) and Ledinko (11), no similar investigations of recombination in poliovirus were published for a rather long time. A different approach to the genetic mapping of poliovirus, i.e., complementation between thermosensitive mutants, was used in Cooper's experiments (3-5). In a previous paper (15), we reported that, when a guanidine-resistant type 2 poliovirus strain was crossed with a guanidine-sensitive type 1 strain, a significant increase in the frequency of guanidine-resistant type 1 particles was found.

This paper describes attempts to use dextran sulfate resistance and 2-(α-hydroxybenzyl)-benzimidazole (HBB) resistance as additional markers in mixed-infection experiments yielding recombinants of the *gua*^{r} *dex*^{r} phenotype, in which the fate of the unselected *hbb*^{r} character was investigated.

MATERIALS AND METHODS

Virus. The parental strains used in mixed-infection experiments were two plaque-purified mutants of the attenuated LSc2ab type 1 poliovirus strain, which was kindly supplied by M. Chumakov (Institute of Poliomyelitis, Moscow).

The first, a guanidine-resistant, dextran sulfate- and HBB-sensitive (*gua*^{r} *dex*^{s} *hbb*^{s}) mutant, labeled LG, was selected by three exposures to 10^{-3} M guanidine (g), alternating with several passages performed in the absence of the inhibitor. It was chosen from a great number of *gua*^{r} candidate clones because of its stability of resistance to high concentrations of the drug (10^{-3} M).

The second, a *gua*^{s} *dex*^{r} *hbb*^{r} mutant, labeled LDH, was selected by a first exposure to 100 μg of dextran sulfate per ml followed by simultaneous exposure to dextran sulfate (100 μg/ml) and HBB (4×10^{-4} M), alternating with passages performed in the absence of these inhibitors. Since stable *dex*^{r} mutants were readily obtained, the main difficulty in choosing between candidate clones was in finding the clone which was most resistant to HBB while being fully sensitive to guanidine.

Cells and media. Rhesus monkey kidney cell cultures (MK) were used throughout. The maintenance and plaque assay media have been described in previous papers (9, 15).

Virus cloning technique. The plug of agar collected over the center of a plaque was transferred into a rubber-capped vial containing 4 ml of Hanks' basal salt solution; it was then subjected to freezing and thawing and was homogenized by means of a mechanical rotatory rocking device.

Mixed-infection experiments. These experiments were devised so as to ensure equivalent, high-input multiplicities of the two partners; input multiplicities were computed by taking into account the actual titer [total number of plaque-forming units (PFU)] of *gua*^{r} or *dex*^{r} particles in the two parental stock suspensions, respectively (Table 1). The two suspensions were mixed and inoculated into MK cultures (each of a series of 10 cultures received the same inoculum) at an input multiplicity of 10 PFU/cell for each parent in one of the experiments and at an input multiplicity of 15 PFU/cell in another experiment. The cultures were washed, inoculated, and incubated for 30 min at 37 C. The inocula were removed, and the monolayers were washed and further incubated at 34 C in roller drums. Eight hours after inoculation, the cultures were subjected to three cycles of freezing and thawing followed by centrifugation at 3,000 rev/min for 30 min; the supernatant fluid was distributed in small volumes and kept at

−20 C for virus assay. For self-cross controls, similarly handled parallel cultures were inoculated with double concentrations of either one or the other parental virus.

Viral inhibitors. In these experiments, we used 10^{-3} M guanidine hydrochloride (Nutritional Biochemicals Co., Cleveland, Ohio; recrystallized in our laboratory), 4×10^{-4} M HBB (synthesized by V. Blazsek, Tg. Mures, Rumania), and 100 μg of dextran sulfate (Pharmacia Fine Chemicals, Uppsala) per ml. These rather high concentrations of the drugs had been found previously to reduce leakiness to a minimum and to reduce the influence of host fluctuations and antiguanidinic factors (8).

Virus and marker assay. Titrations were performed as described in a previous paper (15), and titers were computed according to Lorenz (12). The sensitivity to inhibitors was tested either by the conventional method of comparing the number of plaques formed in the absence and in the presence of the drug, or by the drug gradient plaque method (1), which affords a more economical rough estimate of the sensitivity of a clonal population to inhibitors.

Statistical processing of the data. The statistical significance of the increase in the frequency of double mutants was tested by analysis of variance in a two-way experiment, with the arcsin transformation for proportions (16).

RESULTS

Yield of mixed infection and quantitative evidence for recombination. The sensitivity to guanidine, dextran sulfate, and HBB displayed by the stock suspensions of the LG and LDH viruses used as parental strains is shown in Table 1. The viral populations resulting from mixed infection and from the two self-crosses (Table 2) had equivalent total virus titers, and the mixed yields contained the two parental types (*gua*^{r} and *dex*^{r}) in similar proportions.

Scoring of recombinants was performed by selecting only for *gua*^{r} *dex*^{r} particles; therefore, the presence of a drug-sensitive fraction in the parental strains, as well as in their progeny, did not confuse the final analysis of the results.

The frequency of particles displaying a *gua*^{r} *dex*^{r} phenotype (Table 2) was 0.88% (mean of three successive determinations for the experiment performed at the lower multiplicity) and 1.6% (for the higher multiplicity) in the mixed progeny, whereas the self-cross of the *gua*^{r} parent yielded only 0.25 and 0.23%, respectively, and the self-cross of the *dex*^{r} partner yielded 0.041 and 0.026%, respectively. The statistical significance of the differences recorded in three successive determinations, between the frequency of *gua*^{r} *dex*^{r} particles in the mixed yield (input multiplicity, 10 + 10) and the corrected frequencies recorded in self-cross controls (Table 2), was assessed. The analysis of variance showed that the differences between the found and expected frequencies of *gua*^{r} *dex*^{r} mutants were significant ($P < 0.025$), whereas the variations between the successive determinations were not.

The fact that the excess over the expected number of *gua*^{r} *dex*^{r} particles in this experiment (5.7 times higher than the frequency of the spontaneous double mutants) was statistically significant might be attributed to recombination. In the experiment in which the higher input multiplicity was used, this excess was even higher (9.0). Therefore, the results were considered as quantitative evidence of recombination between *gua*^{r} and *dex*^{r} mutants.

Scoring of an unselected marker (resistance to HBB) in recombinants. The fate of another determinant present on the genome of the LDH strain, i.e., that of the *hbb* character, was investigated.

The simplest experimental approach, consisting of the direct exposure of mixed yields to the simultaneous presence of guanidine, dextran sulfate, and HBB, could not be utilized because the cultures did not resist contact with guanidine and HBB in the concentrations required for the selection of highly resistant particles. Therefore, we resorted to an indirect procedure, i.e., analysis of the sensitivity to HBB of *gua*^{r} *dex*^{r} clones isolated from the progeny of mixed infection. Since clones were isolated by picking up plaques formed at terminal dilutions and since the number

TABLE 1. *Distinctive markers of the parental strains used in mixed infection with gua*^{r} *dex*^{s} *hbb*^{s} *and gua*^{s} *dex*^{r} *hbb*^{r} *mutants of the same type 1 poliovirus strain*

Parental strain	Titer of control (PFU/ml)	Plaque-forming fraction in the presence of						Genotype
		Guanidine hydrochloride (10^{-3} M)		Dextran sulfate (100 μg/ml)		HBB (4×10^{-4} M)		
		PFU/ml	*gua*^{r} Fraction	PFU/ml	*dex*^{r} Fraction	PFU/ml	*hbb*^{r} Fraction	
			%		%		%	
LG.....	1.35×10^7	9.25×10^6	68.6	2.5×10^3	0.018	6.0×10^2	0.0044	*gua*^{r} *dex*^{s} *hbb*^{s}
LDH.....	2.45×10^7	8.00×10^2	0.0033	3.0×10^7	120.0	5.2×10^6	21.0	*gua*^{s} *dex*^{r} *hbb*^{r}

TABLE 2. *Frequency of double mutants in the progeny of crosses between gua^r and dex^r mutants of a type 1 poliovirus strain*[a]

Type of cross	Cross	Input multiplicity	Determination no.	Titer (PFU/ml) in the presence of				Frequency of double mutants (%)			Expected[b] frequency of *gua^r dex^r* particles (%)	Excess over expected/expected
				No inhibitor	Guanidine	Dextran sulfate	Guanidine + dextran sulfate	*gua^r dex^r*/*gua^r*	*gua^r dex^r*/*dex^r*	*gua^r dex^r*/total virus		
Self-crosses	*gua^r* × *gua^r*	20	1	2.95×10^6	3.63×10^6		6.30×10^3	0.18				
			2	1.33×10^6	1.45×10^6		5.00×10^3	0.34				
			3	1.80×10^6	1.20×10^6		4.30×10^3	0.36				
			Mean	2.03×10^6	2.03×10^6		5.20×10^3	0.25				
		30	1	2.20×10^6	1.55×10^6		3.50×10^3	0.23				
	dex^r hbb^r × *dex^r hbb^r*	20	1	3.25×10^6		3.41×10^6	9.80×10^2		0.027			
			2	2.26×10^6		2.10×10^6	1.20×10^3		0.058			
			3	9.00×10^5		8.60×10^5	4.60×10^2		0.056			
			Mean	2.14×10^6		2.12×10^6	8.80×10^2		0.041			
		30	1	3.30×10^6		3.80×10^6	9.00×10^2		0.026			
Mixed infection	*gua^r* × *dex^r hbb^r*	10 + 10	1	3.00×10^6	1.50×10^6	1.63×10^6	2.40×10^4			0.80	0.098	(0.88 − 0.13)/ 0.13 = 5.7
			2	1.50×10^6	6.00×10^5	7.80×10^5	1.87×10^4			1.2	0.16	
			3	1.20×10^6	4.20×10^5	6.70×10^5	7.40×10^3			0.62	0.15	
			Mean	1.90×10^6	8.40×10^5	1.03×10^6	1.67×10^4			0.88	0.13	
		15 + 15	1	6.00×10^6	3.90×10^6	2.05×10^6	9.50×10^4			1.60	0.16	(1.6 − 0.16)/ 0.16 = 9.0

[a]The inhibitors were used at the same concentrations shown in Table 1.

[b]Computed from the frequencies of double mutants recorded in self-crosses and the titers of *gua^r* and *dex^r* particles found in the mixed yield, in parallel determinations; the two figures were added together and related to the total virus titers of the mixed yield.

of *gua*^{r} *dex*^{r} particles was known to exceed that of phenotypically similar spontaneous double mutants by a factor of 7 to 10, it was very likely that the majority of these clones were recombinants.

Fifty plaques formed by the mixed progeny in the presence of both guanidine and dextran sulfate and 10 plaques formed under similar conditions by the progeny of each of the two self-crosses were isolated and tested for their *hbb* marker by means of the drug gradient technique.

Most of the recombinants (as for example the four clones shown in the lower part of Fig. 1) behaved like the *gua*^{r} *dex*^{r} clones isolated from the self-cross of the LG (*hbb*^{s}) parent. However,

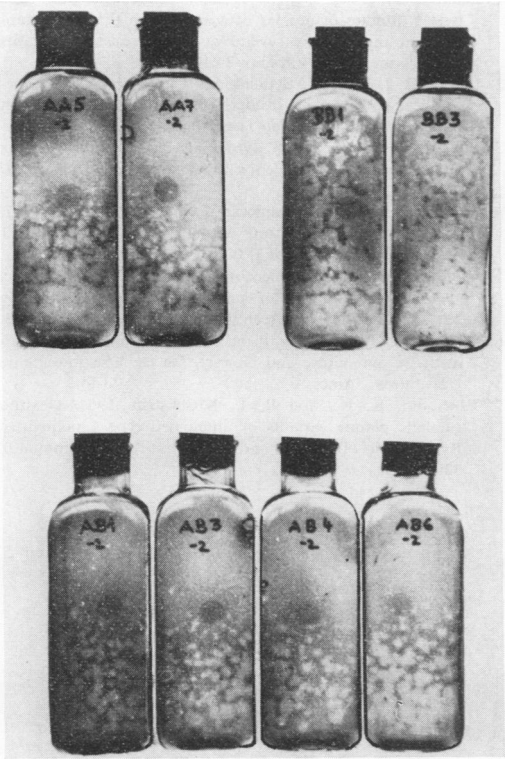

FIG. 1. *Sensitivity to a gradient of HBB [ranging from zero (bottom) to 4 × 10*$^{-4}$ *M] of the gua*^{r} *dex*^{r} *clonal populations derived from the progeny of crosses between gua*^{r} *dex*^{s} *hbb*^{s} *and gua*^{s} *dex*^{r} *hbb*^{r} *mutants and of the self-crosses of the mutants. AA = gua*^{r} *dex*^{r} *clones isolated from the self-cross progeny of the gua*^{r} *dex*^{s} *hbb*^{s} *parent; BB = gua*^{r} *dex*^{r} *clones isolated from the self-cross progeny of the gua*^{s} *dex*^{r} *hbb*^{r} *parent; AB = gua*^{r} *dex*^{r} *recombinant clones isolated from the progeny of mixed infection. All cultures were infected with an inoculum containing 10*$^{3.0}$ *to 10*$^{3.5}$ *PFU. Distance from bottom to top of each gradient bottle, 12 cm.*

in 17 of the 50 recombinant clones, the zone of the gradient reached by the plaque front suggested the existence of an *hbb*^{r} fraction comprising more than 0.01% of the population. Therefore, these 17 clones were further subjected to quantitative determinations of their *hbb*^{r} particle content; however, in only 3 of them was the *hbb*^{r} fraction found to exceed 1% of the population. Thus, it seems that the *dex*^{r} and *hbb*^{r} characters were not distributed together during recombination.

DISCUSSION

The experiments reported above showed that, when *gua*^{r} and *dex*^{r} mutants were crossed, an increase of doubly mutant particles was observed that significantly exceeded the background of spontaneous double mutants.

Sensitivity to HBB was chosen as the unselected character, because it offered the additional possibility of investigating the redistribution, during recombination, of the *gua*^{r} and *hbb*^{r} characters. However, since this possibility could not be investigated by simultaneously exposing the cells to both guanidine and HBB, a method more indirect and cumbersome had to be applied, i.e., analysis of the sensitivity of a significant number of doubly mutant *gua*^{r} *dex*^{r} clones to HBB. The fact that not all of the *dex*^{r} parental particles formed plaques in the presence of HBB (Table 1) did not affect the assay of HBB resistance in *gua*^{r} *dex*^{r} progeny clones; the comparison was made between *gua*^{r} *dex*^{r} clones isolated by plating an inoculum amounting to about 10^3 PFU (10^{-3} of the viral progeny yielded either by the mixed infection or by the self-crosses). Under these conditions, all of the *gua*^{r} *dex*^{r} clones isolated from the self-cross of the *dex*^{r} parent proved to be *hbb*^{r}.

Scoring of 50 *gua*^{r} *dex*^{r} recombinant clones for the unselected *hbb* marker revealed in only 3 cases a proportion of *hbb*^{r} comparable to that of the *dex*^{r} *hbb*^{r} parent, suggesting an independent fate of the *dex* and *hbb* characters during recombination.

The available data concerning the mechanism of action of dextran sulfate (2), as well as some genetic aspects of the *dex* character (10, 14, 17), support the hypothesis that this character is related to properties of the viral coat protein. The *gua* and *hbb* characters are believed to be connected in some way with the synthesis of the viral replicase system (6, 13) and, hence, might be under the control of a segment of the genome distinct from the one governing the synthesis of the coat protein(s). Our findings, that recombination between *dex* and *gua* occurred at a similar rate as between *ho* and *gua* (11) and that *dex*

and *hbb* behave independently, do not disprove the above-mentioned views; moreover, these findings could add some potentially useful data for the genetic analysis of poliovirus with the aid of selective inhibitors.

ACKNOWLEDGMENTS

We thank J. L. Melnick for helpful advice and encouragement. The excellent technical assistance of Ileana Brucher is gratefully acknowledged.

ADDENDUM

After a previous version of this paper had been submitted for publication, the authors were informed about the comprehensive results just published by Cooper (Virology **35**:584, 1968) and Bengtsson (Acta Pathol. Microbiol. Scand. **73**:592, 1968) on genetic mapping of poliovirus by recombination. Nevertheless, since our data also bear on another potentially useful marker (*hbb*), they might be of use for further work in this line.

LITERATURE CITED

1. Aubert-Combiescu, A., D. Sergiescu, and F. Horodniceanu. 1966. A drug gradient plaque test for the study of the behaviour of animal viruses towards synthetic inhibitors. Arch. Ges. Virusforsch. **18**:111–115.
2. Bengtsson, S. 1965. Mechanism of dextran sulfate inhibition of attenuated poliovirus. Proc. Soc. Exptl. Biol. Med. **118**: 47–53.
3. Cooper, P. D. 1965. Rescue of one phenotype in mixed infections with heat defective mutants of type 1 poliovirus. Virology **25**:431–438.
4. Cooper, P. D. 1967. Genetic analysis of animal viruses: new approaches and new vistas. Brit. Med. Bull. **23**:155–160.
5. Cooper, P. D., R. T. Johnson, and D. J. Garwes. 1966. Physiological characterization of heat-defective (temperature sensitive) poliovirus mutants; preliminary classification. Virology **30**:638–649.
6. Eggers, H. J., N. Ikegami, and I. Tamm. 1959. Comparative studies with selective inhibitors of picornavirus reproduction. Ann. N.Y. Acad. Sci. **130**:267–281.
7. Hirst, G. K. 1962. Genetic recombination with Newcastle disease virus, polioviruses and influenza. Cold Spring Harbor Symp. Quant. Biol. **28**:303–308.
8. Horodniceanu, F., D. Sergiescu, A. Aubert-Combiescu, and R. Crainic. 1968. Distinctive features and comparative value of the genetic markers defining the behaviour of poliovirus towards different selective inhibitors. Proc. Congr. Permanent Sect. Microbiol. Standard Intern. Assoc. Microbiol. Soc., 10th; Prague, 1967. Progr. Immunobiol. Standard, vol. 3. Karger, Basel.
9. Horodniceanu, F., D. Sergiescu, R. Klein, M. Zamfirescu, and A. Aubert-Combiescu. 1964. Characters of guanidine resistant mutants of type 1 poliovirus. Arch. Ges. Virusforsch. **14**:238–252.
10. Klein, R., D. Sergiescu, and M. Teodorescu. 1966. Induction with nitrous acid of dextran sulfate resistance in type 1 poliovirus. Virology **30**:145–147.
11. Ledinko, N. 1963. Genetic recombination with poliovirus type 1. Studies of crosses between a normal horse serum-resistant mutant and several guanidine-resistant mutants of the same strain. Virology **20**:107–119.
12. Lorenz, R. J. 1964. Die Statistik des Plaque-Testes. *In* Biometrisches Seminar für Virologen. Bundesforschungs Anstalt für Viruskrankheiten der Tiere, Tübingen.
13. Ménard, D., and A. Lwoff. 1967. Existence probable de deux RNA réplicases chez le poliovirus. Compt. Rend. **264**: 2350–2352.
14. Sergiescu, D., F. Horodniceanu, and R. Crainic. 1967. Mutation towards dextran sulphate resistance in type 1 poliovirus. Nature **215**:313–315.
15. Sergiescu, D., F. Horodniceanu, R. Klein, and R. Crainic. 1966. Genetic transfer of guanidine resistance from type 2 to type 1 poliovirus. Arch. Ges. Virusforsch. **18**:231–243.
16. Snedecor, G. W. 1962. Statistical methods applied to experiments in agriculture and biology, 5th ed. The Iowa State Univ. Press, Ames.
17. Takemoto, K. K., and R. L. Kirschstein. 1964. Dextran sulphate plaque variants of attenuated type 1 poliovirus. Relationship of in vitro and in vivo markers. J. Immunol. **92**:329–333.