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ISOLATION OF ANTIGENIC MUTANTS OF TYPE 1 POLIOVIRUS: GROWING THE VIRUS IN THE PRESENCE OF HOMOLOGOUS ANTISERUM

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The antigenic mutants, which were significantly different intra-typically from the parental Mahoney-1709 type 1 poliovirus, were repeatedly isolated after a number of serial virus passages in the presence of homologous anti-Mahoney sera.

The levels of the antigenic variation of those mutant viruses varied, depending on the individual antiserum employed for mutant selection and the passage numbers with same antiserum.

The susceptibility to certain serum inhibitors of the antigenic mutants varied according to the antisera AMS₃ and AMS₄ employed for the mutant selection, but was not dependent on the passage numbers using the same antiserum.

There is evidence that the antisera contained antibodies indistinguishable from the serum inhibitors in the specific antiviral activity, and that the production of these antibodies varied with the results of individual variations in the animals receiving the antigenic stimulus.

INTRODUCTION

Since antigenic differences between intra-typic strains of poliovirus were demonstrated by WENNER et al.¹²⁾, detailed antigenic analyses among those strains have been done by many workers^{3,5,11,13)} using various serological methods. However, the mechanism which caused the differentiation in the antigenicity among strains within a type is still not clear. In the previous paper⁴⁾, it was reported that the antigenicity of the inhibitor resistant mutant (M-H11) derived from a type 1 poliovirus in the presence of an inhibitory equine serum (H-11) differed definitely from the parent virus.

Attempts have been made to isolate the antigenic mutants from the Mahoney strain of type 1 poliovirus by growing the virus in the presence of an homologous antibody. The grades of antigenic variation and the alteration of susceptibility against serum inhibitors of the antigenic mutants obtained in this way were also analyzed.

MATERIALS AND METHODS

All materials and methods employed herein parallel those in the previous paper⁴.

Cell cultures A stable line of monkey kidney cells (MS) was subcultivated with YLE medium containing 10% bovine serum. The monolayer cultures were prepared in 70 mm Petri-dishes for plaque assay and in 2 ounce prescription bottles for virus passages.

Solution and medium Diluent for virus and serum contained 0.1% gelatin in DULBECCO'S PBS. Cell growth medium (CGM) consisted of YLE, 0.075% NaHCO₃, and 10% inhibitor free bovine serum. Virus growth medium (VGM) consisted of YLE, 0.15% NaHCO₃, and 0.4% gelatin. Regular overlay medium consisted of YLE, 0.15% NaHCO₃, and 1% Bacto-Agar (Difco, Detroit, Mich.). Second overlay medium contained 0.01% neutral red in regular overlay medium. All media contained antibiotic concentrations of 100 units of penicillin, 100 μ g streptomycin, and 2.5 μ g fungizon (Squibb, Pharmaceutical, New York) per ml.

Virus The clone 1709 of Mahoney strain of type 1 poliovirus, its antigenic mutants, and its equine serum inhibitor resistant mutant (M-H11) were used. All of the viruses employed were purified by three successive plaque passages on MS monolayers with a regular overlay medium, unless otherwise indicated. Stocks were prepared by infecting MS monolayers in VGM. After incubation at 37°C for 16 hr, the virus was extracted from cells by two cycles of freezing and thawing. The viral fluid was centrifuged at 2,000 rpm for 10 min, and the supernatant was stored in small aliquats at -20°C.

Antisera Pre-sera were collected from all of the Japanese white rabbits employed. Antisera to Mahoney-1709 and antigenic mutants were prepared by immunizing the inhibitors-free rabbits with a virus grown in MS cells and purified by two cycles of extraction with fluorocarbon. The early immune serum containing exclusively the IgM antibody was collected 2 days after a single i. v. injection with 10⁶ plaque forming units (PFU) of virus, as described by SVEHAG & MANDEL⁸. Thereafter, the animals were boosted 6 times with intravenous injections spaced 5 days apart, containing approximately 10^{8.3} PFU of the same virus. The hyperimmune serum, consisting mostly of IgG antibody, was collected 35 days after the first injection. All sera were stored at -20°C and heated at 56°C for 30 minutes before use.

Plaque assay The duplicate culture plates with MS monolayers were washed once with PBS, and the inoculum of 0.4 ml per plate was added at room temperature (RT) for 1 hr. The infected plates were overlaid with 8 ml of a regular overlay medium and incubated at 37°C in a 5% CO₂ incubator. The second overlay medium containing neutral red was added 2 days later. The plaques were counted on the following day.

Plaque neutralization test A virus suspension containing approximately 500 PFU per ml of virus was mixed with an equal volume of serum diluted in 2-fold steps. Then the mixture was kept at 37°C for 1 hr and over-night at 4°C. The neutralizing titer

was expressed as the reciprocal of the serum dilution at which the number of plaques was reduced to 50% of that in the control plates.

Kinetic neutralization test A suspension containing approximately 10^6 PFU per ml of virus and correctly diluted antiserum was kept in a water bath at 37°C. These were mixed together with equal volumes at time zero. At time intervals 0.3 ml of the mixture was transferred into 29.7 ml of cold diluent and further diluted in 10-fold steps. A control mixture containing virus and diluent was diluted in the same manner. The differences in log residual plaque counts between the virus-serum mixture and the virus-diluent control were plotted against the time of incubation. The K value—the neutralization rate constant—was calculated from the slope of the first order kinetic by equation described by DULBECCO et al.²⁾ In order to compare the K value of one virus in different sera as well as that for one serum with different viruses, the normalized K value (NK) was calculated as presented by MCBRIDE³⁾.

$$NK = \frac{\text{K of each virus}}{\text{K of homologous virus}} \times 100$$

Plaque isolation and plaque passages Plaques were picked up by capillary pipettes and suspended with agar debris into 1 ml of diluent. The virus suspension was diluted in 10-fold steps and assayed for plaque formation using a regular overlay medium. The virus suspension from the 3rd plaque passage was inoculated into an MS cell bottle culture to obtain the stock virus.

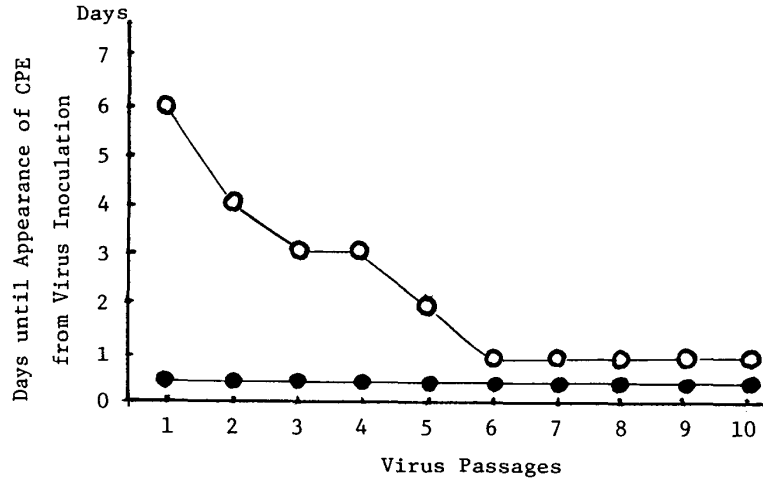
Serial passages of virus in presence of homologous antiserum Approximately $10^{7.0}$ PFU per ml of Mahoney-1709 virus was mixed with an equal volume of diluted antiserum. After incubation at 37°C for 2 hr, 2 ml of the mixture was inoculated into an MS bottle culture containing about 10^6 cells with 3 ml of VGM and then incubated at 37°C. When cellular destruction was completed, the viral fluid was centrifuged at 2,000 rpm for 10 min and the supernatant collected. This supernatant was again mixed with the same diluted antiserum for virus neutralization and incubated at 37°C for 2 hr, and then inoculated onto the monolayer cells in the bottle. The process was repeated for desired number of serial passages using the undiluted virus fluid with the same antiserum. The viral fluid at final passage was diluted $10^{-4.0}$ to eliminate the antibody and inoculated onto an MS cell monolayer. The virus grown in the absence of antiserum was used to test its antigenicity.

RESULTS

Effect of antiserum on virus passages

Using antiserum as a selective pressure, a virus less sensitive to the homologous serum was propagated in due course of the virus passages. An anti-Mahoney-1709 hyperimmune serum produced in rabbit number 4 (AMS₄) was employed at a 1:50 dilution containing 800 units per ml of neutralizing antibody calculated by means of

FIGURE 1 *Relationship between subculture of Mahoney-1709 strain of type 1 poliovirus in the presence of anti-Mahoney-1709 hyperimmune rabbit serum (AMS₄) and intervals until the appearance of CPE on MS monolayers after virus inoculation*



Viral fluid was mixed with an equal volume of 1:50 dilution of AMS₄ containing 800 units/ml of neutralizing antibody, and 2 ml of the mixture were inoculated in the MS culture bottle with 3 ml of VGM.

○—○, 1:50 diluted AMS₄; ●—●, 1:20 diluted normal rabbit serum (NRS₄)

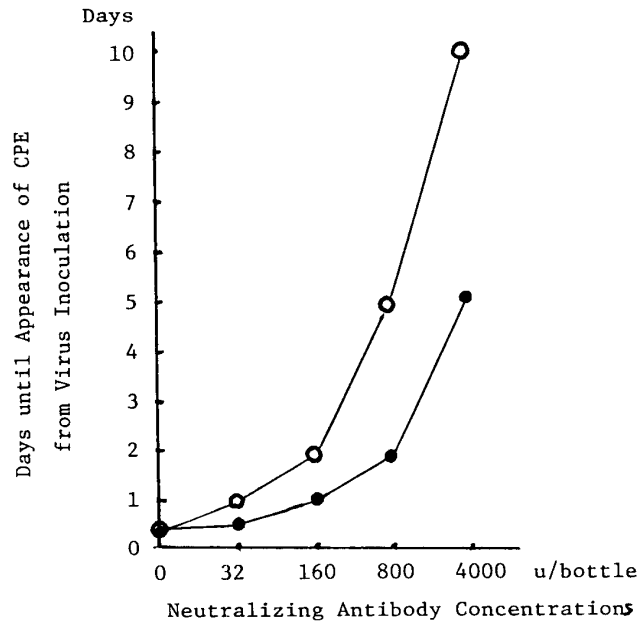
the plaque neutralization test using the homologous Mahoney-1709 virus.

A virus suspension containing approximately $10^{7.0}$ PFU per ml of Mahoney-1709 was mixed with an equal volume of the 1:50 diluted AMS₄. The control passages were carried out using 1:20 dilution of normal serum (NRS₄) bled from the rabbit before preparation of AMS₄. The results are shown in figure 1.

Complete cellular destruction of the first passage appeared 6 days after inoculation with the virus-antiserum mixture. However, with the increased number of successive virus passages, the appearance of a cytopathic effect (CPE) became noticeable at gradually shorter time intervals. CPE from the 6th to 10th passages appeared consistently at 24 hr after viral fluid inoculation. In contrast to this, CPE on all control series with 1:20 diluted NRS₄ appeared almost 12 hr after virus inoculation. The virus from the 10th passage with AMS₄ grown once by infecting MS cells in the absence of the antibody was named AMS₄ p-10 and employed without cloning. Using the same virus concentration and varied antiserum concentrations, Mahoney-1709 and AMS₄ p-10 viruses were inoculated onto MS monolayers, and then at intervals until the appearance of CPE from the virus inoculation was comparable. The results are shown in figure 2.

AMS₄ was used at 4 different antibody concentrations ranging from 32 to 4,000 units per ml. A virus suspension containing $10^{7.7}$ PFU/ml was mixed with an equal volume of the diluted antiserum. After incubation at 37°C for 2 hr, 2 ml of the mixture

FIGURE 2 *Effect of antibody concentrations on the appearance of CPE of Mahoney-1709 and AMS₄p-10 viruses on MS cells*



A virus suspension containing approximately $10^{7.7}$ PFU/ml was mixed with an equal volume of diluted anti-Mahoney-1709 hyperimmune rabbit serum (AMS₄) and 2 ml of the mixture were inoculated onto the MS cells.

○—○, Mahoney-1709 virus; ●—●, AMS₄ p-10 virus

were inoculated into an MS bottle containing 3 ml of VGM.

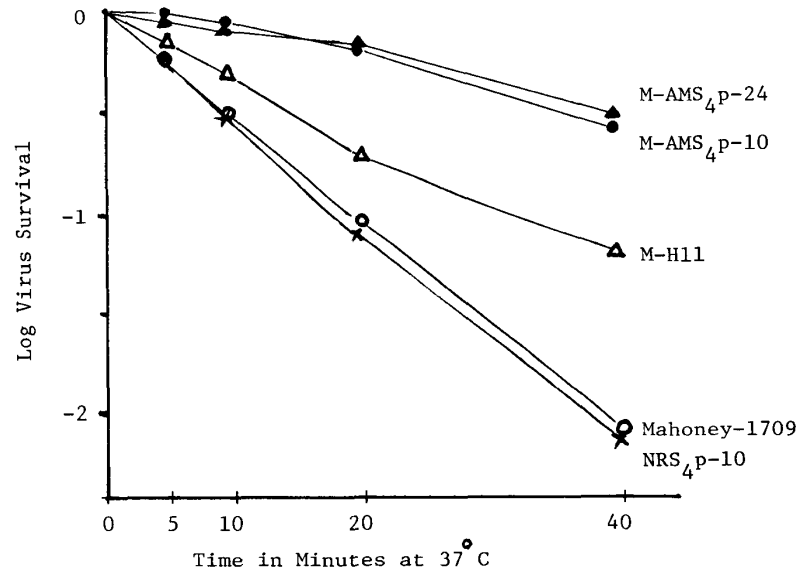
CPE appeared much earlier in the AMS₄ p-10 virus infection than in the Mahoney-1709 virus in the presence of AMS₄; this tendency intensified with the increased antibody concentration. Another serial passage using the same concentration of AMS₄ was then started in order to examine the reproducibility of isolation of the antigenic mutant until the 24th passage.

Characteristics of antigenic mutants selected in the presence of AMS₄

To determine the antigenic differences between Mahoney-1709 and its derivatives, the kinetic neutralization test using homologous AMS₄ was carried out. The viruses from the 10th and 24th passages obtained in the separated series with AMS₄ were purified by 3 serial plaque passages and labelled as M-AMS₄ p-10 and M-AMS₄ p-24, respectively. The results, including an equine serum inhibitor resistant mutant (M-H11), which was distinct in antigenicity intra-typically from the Mahoney-1709 virus, and a control virus from the 10th passage with NRS₄ named NRS₄ p-10, are shown in figure 3.

No difference was seen in the antigenicity between the parental Mahoney-1709 and the NRS₄ p-10 viruses. However, the two viruses, M-AMS₄ p-10 and M-AMS₄ p-24,

FIGURE 3 *Kinetic curves of neutralization of Mahoney-1709 and its mutant viruses with anti-Mahoney-1709 hyperimmune rabbit serum (AMS₄)*



A virus suspension containing 10^6 PFU/ml was mixed with an equal volume of a 1:400 dilution of anti-Mahoney-1709 hyperimmune rabbit serum (AMS₄)

were more heterologous to the Mahoney-1709 virus than to M-H11. Although there was in the different series and a great difference in the passage number with the AMS₄, no dissimilarity was observed between these two antigenic mutants.

The AMS₄ used for the mutant selection had been, unfortunately, exhausted. Therefore, another series of virus passages was started to isolate the antigenic mutants using an anti-Mahoney-1709 hyperimmune serum (AMS₃) prepared in rabbit number 3. The procedure for the selection of highly heterologous antigenic mutants was as follow.

First passage was carried out using the Mahoney-1709 virus suspension containing 32 units of neutralizing antibody per ml. When cellular destruction was completed, the viral fluid was mixed with an equal volume of the diluted AMS₃. After incubation at 37°C for 2 hr, 2 ml of the mixture were inoculated into an MS bottle culture containing 3 ml of VGM. At every 5th successive passage, the antiserum concentration was raised in 5-fold steps; after the 16th passage AMS₃ was used constantly at a dilution of 1:32 containing 4,000 units per ml of neutralizing antibody.

In total 50 serial passages were carried out in which the diluted AMS₃ was mixed with undiluted viral fluid prior to each passage; control passages were done until the 30th passage using the 1:32 dilution of normal serum (NRS₃) collected from the rabbit before preparation of AMS₃.

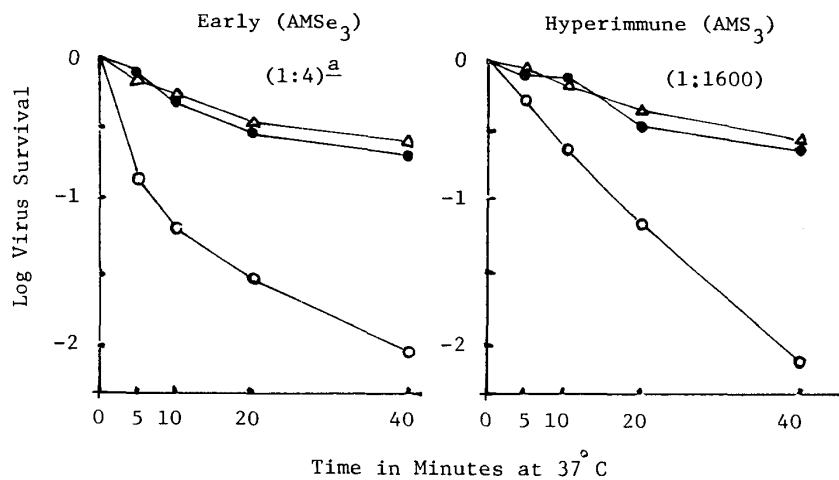
Cellular destruction occurred within a maximum of 7 days until the serial 10 pas-

sages were completed; however, until the appearance of CPE from the virus inoculation on the cells, the intervals were slightly delayed when compared with the lower antiserum concentration found in the earlier passages. In the presence of a high concentration of antibody (4,000 u), complete cellular destruction was barely obtainable from the 16th to the 25th passage, even 10 days after virus inoculation. However, after the 26th passage with the same antibody concentration, the intervals for the appearance of CPE gradually became shorter. These results indicate that the antigenic pattern of the virus may have been undergoing alteration as the passage continued.

Determination of antigenic mutants selected in the presence of AMS₃

The viruses with AMS₃ in passages 30th and 50th were purified by 3 serial plaque passages, and labelled M-AMS₃ p-30 and M-AMS₃ p-50, respectively. Kinetic neutralization tests were performed again to determine the antigenic changes of these 2 derivatives using both anti-Mahoney-1709 early (AMS₃e) and hyperimmune (AMS₃) sera prepared from the same rabbit. The results are shown in figure 4.

FIGURE 4 *Kinetic neutralization of antigenic mutants with anti-Mahoney-1709 rabbit early and hyperimmune sera (R-3)*



A virus suspension containing $10^{6.0}$ PFU/ml was mixed with an equal volume of diluted anti-Mahoney-1709 rabbit serum.

^a Antiserum dilution; ○—○, Mahoney-1709 virus; ●—●, M-AMS₃ p-30 virus; △—△, M-AMS₃ p-50 virus

These two viruses were distinguished clearly from Mahoney-1709 using both the anti-Mahoney sera containing either IgM or IgG antibodies. No difference, however, was observed between these two mutant viruses.

Reciprocal kinetic neutralization test of Mahoney and its antigenic mutants

The cross-neutralization test of Mahoney-1709 and its antigenic mutants was carried out using hyperimmune sera in the kinetic neutralization test. The hyperimmune sera

TABLE 1 *NK-values of the antigenic mutants*

VIRUSES	ANTISEA					
	AMS		M-AMS ₃ p-30		M-AMS ₃ p-50	
	R-3	R-4	R-15	R-16	R31	R-35
Mahoney-1709	100.0 ○	100.0 ○	121.9 ○	82.3 ○	40.5 ●	35.5 ●
M-AMS ₃ p-30	35.4 ●	72.6 △	100.0 ○	100.0 ○	37.5 ●	16.3 ●
M-AMS ₃ p-50	32.1 ●	57.8 △	67.5 △	86.4 ○	100.0 ○	100.0 ○
M-AMS ₄ p-24	56.3 △	40.0 ●	ND	ND	ND	ND

○ : homologous, $NK \geq 81$; △ : intermediate, $NK 51-80$; ● : heterologous, $NK \leq 50$; ND : not done

were prepared separately using 2 rabbits for each virus employed.

The results are summarized in table 1. The normalized K values were classified into 3 groups: homologous, NK over 81; intermediate, NK 51 to 80; and heterologous, NK under 50. Using AMS₄ the antigenicity of the corresponding M-AMS₄ p-24 virus was classified in the heterologous group against the Mahoney-1709 virus but, using the same antiserum, those of M-AMS₃ p-30 and M-AMS₃ p-50 belonged to the intermediate group against the Mahoney-1709 virus. Similarly, there were results when the NK values were compared with the viruses using AMS₃. The antigenicity of both viruses, M-AMS₃ p-30 and M-AMS₃ p-50, was identified as heterologous intra-typically against the Mahoney-1709 virus, but the M-AMS₄ p-24 virus was identified as intermediate to it. No significant difference in NK values, however, was observed between M-AMS₃ p-30 and M-AMS₃ p-50 viruses when tested with either AMS₃ or AMS₄. Using both anti-M-AMS₃ p-30 sera, R-15 and R-16, parental Mahoney-1709 showed high NK values which were classified as homologous against the M-AMS₃ p-30 virus. Using R-15 serum, the M-AMS₃ p-50 virus was somewhat low, and it was classified as intermediate and as homologous using R-16. It was noted that the anti-M-AMS₃ p-30 sera were indistinguishable for those differences between the parental Mahoney-1709 and the M-AMS₃ line viruses, despite the corresponding AMS₃ which could differentiate them. Using both anti-M-AMS₃ p-50 sera, R-31 and R-35, however, the Mahoney-1709 and M-AMS₃ p-30 viruses showed markedly low NK values classified as heterologous against the M-AMS₃ p-50 virus. These results indicate that the antigenicity of the M-AMS₃ p-50 virus was markedly distinct intra-typically in a reciprocal way from that of both the Mahoney-1709 and the M-AMS₃ p-30 viruses. On the other hand, the antigenicity of the M-AMS₃ p-30 virus was intermediate between the Mahoney-1709 and M-AMS₃ p-50 viruses owing to the one way cross reaction.

Susceptibility of antigenic mutants to serum inhibitors

In the previous paper⁴⁾ it was demonstrated that the antigenicity of an inhibitor resistant mutant (M-H11) derived from the Mahoney-1709 virus in the presence of an inhibitory equine serum (H-11) was fairly different from the parental Mahoney virus. Reciprocal experiments were then performed to see whether or not the antigenic mutants selected in the presence of homologous antiserum were due to any alteration in their susceptibility to serum inhibitors.

The two derivatives from the Mahoney-1709 virus, NRS₄ p-24 (24th passage with 1:20 dilution of NRS₄) and NRS₃ p-30 (30th passage with 1:32 dilution of NRS₃), which did not alter the antigenicity, were included in this experiment. A total of 7 viruses—parental Mahoney-1709, its 2 control derivatives (NRS₄ p-24 and NRS₃ p-30), and its 4 antigenic mutants (M-AMS₄ p-10, M-AMS₄ p-24, M-AMS₃ p-30 and M-AMS₃ p-50) were employed.

TABLE 2 *Susceptibility of the antigenic mutants to serum inhibitors*

INHIBITORY SERUM	PARENT	CONTROL		ANTIGENIC MUTANTS			
	Mahoney 1709	NRS ₄ p-24	NRS ₃ p-30	M-AMS ₄ p-10	M-AMS ₄ p-24	M-AMS ₃ p-30	M-AMS ₃ p-50
Bovine							
B-20	256 ^a	256	256	256	256	256	256
B-35	64	64	64	64	64	<4	<4
B-55	<4	<4	<4	<4	<4	64	64
Equine							
H-11	512	512	512	<4	<4	512	512

^a: Neutralizing titer by plaque neutralization test. Box indicates the alteration of susceptibility to serum inhibitors of the antigenic mutants selected from Mahoney-1709 virus in the presence of anti-Mahoney-1709 rabbit serum.

The characteristics of the bovine serum inhibitors, B-30, B-35 and B-55 and the equine serum inhibitor H-11 employed herein, were described in the previous paper⁴⁾. Cross neutralization reaction with the 7 viruses and the 4 inhibitory sera were performed by means of the plaque neutralization test. The results are shown in table 2.

No difference in the susceptibility to those inhibitors was observed between the Mahoney-1709 and the control derivatives which did not associate with the antigenic variations. When the M-AMS₄ p-10 and M-AMS₄ p-24 viruses, which were isolated in the presence of AMS₄, were tested, however, both were definitely resistant to the equine serum inhibitor H-11. On the other hand, the two antigenic mutants, the M-AMS₃ p-30 and the M-AMS₃ p-50 viruses, which were isolated in the presence of AMS₃, continued to be resistant to the bovine serum inhibitor B-35, and acquired susceptibility

to bovine serum B-55, which did not inactivate the parental Mahoney virus.

In the 2 lines of antigenic mutants, M-AMS₄p-10 and M-AMS₄p-24, and M-AMS₃p-30 and M-AMS₃p-50, the susceptibility to serum inhibitors varied according to the antisera AMS₄ and AMS₃, which were used for mutant selection, but was not dependent on the passage numbers.

DISCUSSION

The preliminary attempts to demonstrate the antigenic change in a poliovirus strain were done by WENNER et al.¹³, McBRIDE⁶ and VONKA et al.⁹, who were unable to obtain an antigenic variant even several passages of the virus in tissue culture in high concentration of homotypic antibody, and concluded that the antigenicity of poliovirus might be highly stable in vitro system. However, WASSERMAN & FOX¹⁰, VONKA et al.⁹, GARD³ and NAKANO et al.⁷ indicated that antigenic variants were readily produced when the vaccine strain of poliovirus was allowed to multiply in the human intestinal tract. Subsequently, McBRIDE⁶ isolated several antigenic variants from 10 lines of LSc2ab when they were subcultivated serially for 30 passages in tissue culture in the presence of an homologous antiserum.

The results of this paper show that the intervals until the appearance of CPE in the monolayer cells from the virus inoculation were gradually accelerated with an increased number of virus passages in the presence of the homologous antiserum. The viruses M-AMS₄p-24 and M-AMS₃p-50 from the latest passage were less sensitive to their corresponding antiserum AMS₄ or AMS₃ than to the parental virus. On the other hand, no demonstrable alteration in the antigenic pattern of the control virus passages using normal rabbit serum was observed. The process of the antigenic mutant selection of poliovirus in the presence of antiserum seems to be similar to that of ARCHETTI & HORSFALL¹¹, who presumed that when a large number of influenza A virus were mixed with an homologous antibody, only those mutant viruses which were less neutralized by antibodies retained their ability of multiplication. Accordingly antigenic mutants, which were contained in a very low frequency in the stock virus, may increase gradually in the course of virus passages with the antiserum, and finally take the place of the original virus in the fluid.

The antigenicity of M-AMS₃p-30 and M-AMS₃p-50 viruses using AMS₃ was determined as heterologous against the parental Mahoney virus; however, the M-AMS₃p-50 virus could not be differentiated from the M-AMS₃p-30 virus even by using the anti-M-AMS₃p-30 sera, R-15 and R-16. In the contrast, the M-AMS₃p-30 virus could be differentiated from the M-AMS₃p-50 virus using the anti-M-AMS₃p-50 sera. Therefore, it was suggested that the M-AMS₃p-30 virus underwent only minor antigenic variation, which could not be recognized in vivo as an antigenic stimulus, while the M-AMS₃p-50 virus apparently underwent major antigenic variations, which could be

recognized in both in vivo and in vitro. These facts indicate that the antigenicity of the mutant (M-AMS₃p-50) isolated at later passage in the M-AMS₃ line was more heterologous against the original virus than its mutant (M-AMS₃p-30). It is suggested that the intra-typic antigenic variation of poliovirus may be due to the sequential changes of diverse antigenic determinant sites on the virus surface as a result of the number of point mutations of the virus.

In the previous paper⁴, it was found that the equine serum inhibitor resistant mutant (M-H11) derived from the Mahoney-1709 virus in the presence of an inhibitory equine serum (H-11) was associated with an alteration of antigenicity. The data presented in Table 2 shows the reciprocal result. The antigenic mutants, the M-AMS₄p-10 and M-AMS₄p-24 viruses, which were selected in the presence of AMS₄, were resistant to the H-11 inhibitor affecting the parental virus. When the M-AMS₃ line mutants isolated in the presence of AMS₃ were tested against the serum inhibitors, however, they were resistant to the bovine serum inhibitor B-35, but not to the H-11. The above findings suggest that some antibodies produced in rabbits, R₃ and R₄, after poliovirus immunization, were identical in specific antiviral activity to the serum inhibitor. Further, there was evidence that the antigenic alteration and serum inhibitor susceptibility of these antigenic mutants varied according to the antibodies contained in the antiserum, AMS₃ or AMS₄, used for mutant selection. It may be presumed that the recognition of each antigenic determinant site and/or sites on the viral surface in vivo could vary with the individual variations of receiving the same antigenic stimulus in the same manner.

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