Characterization of Genetic Changes Occurring in Attenuated Poliovirus 2 During Persistent Infection in Mouse Central Nervous Systems

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Genomic changes occurring in the attenuated W-2 strain of poliovirus 2 during persistent infection of the central nervous system of immunosuppressed mice were analyzed. The RNase T1 oligonucleotide fingerprints of 34 different viruses, isolated from the brains and spinal cords of paralyzed and nonparalyzed mice during a 105-day period, were used to quantitate and compare the mutations occurring in each isolate. Although mice were inoculated with plaque-purified virus, genetically distinct viruses were recovered from the central nervous system. The number of oligonucleotide changes occurring in isolates from paralyzed mice generally was greater than that observed in isolates from nonparalyzed mice. However, differences in the extent of mutation in isolates from the two groups of mice did not appear to be related to the level of virus replication. In paralyzed mice, the number of oligonucleotide changes on average was greater in viruses isolated during the first 60 days of the infection than in the last 45 days. The number of oligonucleotide changes was essentially constant throughout the infection, however, in viruses isolated from the brains of nonparalyzed mice. In addition, several specific oligonucleotide changes were found only in viruses isolated from paralyzed animals.

A high frequency of mutation has been shown to be common for several RNA viruses (for review see reference 10). This ability to rapidly mutate has been proposed as being important to the mechanism by which some RNA viruses escape the immune response of the host and persist (9, 16). In an effort to understand the mechanisms underlying RNA virus persistence, RNase T1 oligonucleotide fingerprinting has been used to quantitate mutation and characterize oligonucleotide changes occurring in the genomes of persisting viruses. This technique was used to study several RNA viruses persisting in cell culture (Semliki Forest virus [15]; Theiler's murine encephalomyelitis virus [18]; and vesicular stomatitis virus [21]), but there are only a few examples of oligonucleotide fingerprinting used to study RNA viruses persisting in human or animal hosts.

There have been reports of persistent enterovirus infections in humans by vaccine strains of poliovirus (8, 23, 24) and by wild-type echovirus (22). Only persistent polioviruses, however, have been subjected to genetic analysis. Polioviruses, isolated from persistent intestinal infections of immunodeficient and immunocompetent patients, have been shown to have oligonucleotide maps that differ by as much as 3% from that of the parental vaccine virus (13, 24). Although a few polioviruses with genetic changes were recovered from the central nervous system (CNS) (13), it is not clear whether those viruses actually persisted there. RNase T1 oligonucleotide fingerprinting was used to show the extent of mutation in different strains of Theiler's virus (Theiler's murine encephalomyelitis virus, a mouse enterovirus) during persistent infection of the mouse CNS (17, 19). In one of these studies, mutation occurred throughout the infection, but the largest number of mutations was found late in the infection (19). Another RNA virus, visna virus, a member of the retrovirus family, which has been isolated from leukocytes of persistently infected sheep, contained

oligonucleotide changes that were mapped to the region of the genome believed to code for the virus glycoprotein (5). Changes in this protein suggest that the virus may persist by evading neutralizing antibodies. In other persisting RNA viruses, however, the relationship between mutation and persistence or the ability to cause disease is unclear.

This study is part of a larger investigation of the mechanisms of poliovirus persistence in the CNS of immunosuppressed mice (B. Jubelt and J. B. Meagher, *Neurology*, in press). We have used RNase T1 mapping of poliovirus 2 to examine the genomic changes occurring during the persistent infection. In addition, we have quantitated the mutations and determined whether any pattern of oligonucleotide changes could be identified in separate brain and spinal cord isolates. Finally, oligonucleotide changes of viruses obtained from paralyzed and nonparalyzed mice were compared to see if any changes could be associated with either condition.

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MATERIALS AND METHODS

Cell culture and virus. Human RD (rhabdomyosarcoma) cells, obtained from the American Type Culture Collection, Rockville, Md., were cultured at 37°C in Dulbecco modified Eagle medium (DMEM) containing 10% fetal calf serum (KC Biologicals, Lenexa, Kans.), 100 µg of streptomycin sulfate per ml, and 100 U of penicillin per ml. The attenuated W-2 strain of human poliovirus 2 was obtained from the American Type Culture Collection and was plaque purified twice before it was grown into a stock. The titer of this stock was 3.8×10^8 PFU/ml or 2.1×10^8 50% tissue culture doses (TCD₅₀) per 0.2 ml.

Virus titrations. The plaque-purified stock was quantitated by plaque assay on monolayers of RD cells as described

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previously (11). Viruses which were isolated from the CNS were quantitated by a TCD₅₀ assay in microtiter plates (12).

Animal inoculations and virus isolations. Six-week-old male BALB/c mice (barrier colony; Cumberland View Farms, Clinton, Tenn.) were inoculated in the left cerebral hemisphere with 0.02 ml of the plaque-purified stock of W-2 virus (about 8×10^5 PFU or 2×10^6 TCD₅₀). Mice were given cyclophosphamide intraperitoneally: 150 mg/kg on day 1 postinfection (PI) and 50 mg/kg weekly thereafter. At intervals PI, paralyzed and nonparalyzed mice were anesthetized; the brains (cerebrum and diencephalon) and spinal cords (excluding the brain stem) were removed, and each was prepared as a clarified 10% homogenate in DMEM. The virus content of the homogenates was determined by a TCD₅₀ assay before passing virus twice in RD cells at 37°C to amplify the virus titer.

³²P-radiolabeling and virus purification. RD cells in five T-150 flasks were infected at a multiplicity of 10 to 100 and incubated with DMEM at 37°C. After 2 h, DMEM was removed and replaced with phosphate-free medium containing 1 mCi of ³²Pi. The infection was stopped when cytopathic effect was total, usually about 22 h PI. The infected cells were pooled with the culture fluid, sonicated, clarified, and virus was precipitated by 8% polyethylene glycol (20). The concentrated virus lysate was treated with 1% sodium dodecyl sulfate for 10 min and then centrifuged in a 15 to 45% sucrose gradient at $96,000 \times g$ in a Beckman SW28 rotor.

Oligonucleotide fingerprinting. The visible virus band was diluted twofold in a buffer containing 0.15 M NaCl and 0.015 M Tris-hydrochloride (pH 7.4), and sodium dodecyl sulfate and carrier yeast RNA (BDH Chemicals Ltd., Poole, England) were added to final concentrations of 1% and 200 µg, respectively. Virus RNA was extracted twice with a mixture of phenol and *m*-cresol as described previously (20). The RNA was precipitated in absolute ethanol, suspended in 10 µl of a buffer containing 0.002 M EDTA and 0.02 M Tris-hydrochloride (pH 7.4), and digested with RNase T1 (Calbiochem-Behring, La Jolla, Calif.; 2 U/10⁶ cpm of virus RNA) for 30 min at 37°C. Oligonucleotides were separated by two-dimensional polyacrylamide gel electrophoresis with a 10% acid urea gel in the first dimension and a 22% gel (pH 8.3) in the second dimension. The apparatus and the conditions employed for two-dimensional electrophoresis have been described previously (7, 20). Gels were fluorographed for 48 to 72 h with Kodak XS-1 film and Dupont Cronex Lightning-Plus intensifier screens.

The fingerprint of W-2 virus after centrifugation in sucrose was identical to that after an additional equilibrium density centrifugation of virus in Cs₂SO₄. Thus, purification of virus isolates by centrifugation in sucrose was concluded to satisfactorily prevent contamination by cellular RNAs.

RESULTS

Isolation of viruses from persistently infected mice. Since it was demonstrated that W-2 poliovirus persists only in immunosuppressed mice, cyclophosphamide was administered weekly to maintain immunosuppression (Jubelt and Meagher, in press). The first mouse became paralyzed at day 10 PI, and by day 90 PI ca. 55% of the mice were paralyzed. Virus always was recovered from both the brains and spinal cords of paralyzed mice, whereas virus generally was recovered from brains but not spinal cords of nonparalyzed animals (Jubelt and Meagher, in press). In instances in which virus was not recovered, virus may have been cleared from the CNS or was less than the level of detection ($0.5 \log_{10}$

TCD₅₀ per 0.02 g of tissue). To prevent selecting a single virus as representative of several genetically distinct viruses that may have been present in the CNS, viruses obtained from mice were not plaque purified.

Viruses isolated from 12 paralyzed and 10 nonparalyzed mice were selected for oligonucleotide fingerprint analysis at ca. 30-day intervals starting at day 10. Isolates from the CNS of nonparalyzed mice with higher virus levels were chosen for RNase T1 mapping so that the number of passages required to amplify their titers could be limited to two. CNS isolates from paralyzed mice were chosen randomly since they all had high titers. The virus content in the brains of paralyzed mice was slightly greater (about $0.5 \log_{10}$ TCD₅₀) than the titers from nonparalyzed mice (Fig. 1). In paralyzed mice, the virus titers in the spinal cords were 10- to 100-fold greater than the titers in the brain (Fig. 1A).

Analysis of oligonucleotide changes in persistent virus isolates. There were a total of 24 viruses fingerprinted from paralyzed mice (12 each from brains and spinal cords) and 10 viruses fingerprinted from nonparalyzed mice (brains only). A total of 55 large RNase T1-resistant oligonucleotides, resolved in the second-dimension gel, were used to make comparisons between fingerprint patterns (Fig. 2a and b).

(i) Estimation of the frequency of oligonucleotide change. Fingerprint changes in virion RNAs, which consisted of loss of oligonucleotides and appearance of new oligonucleotides compared with the original virus, were found in all paralyzed mice (Table 1). However, many more new oligonucleotides appeared in viruses than were lost. In nonparalyzed mice, oligonucleotide changes, which consisted only of new oligonucleotides, were found in 7 of 10 mice (Table 1 and Fig. 1B). The number of oligonucleotide changes generally was

FIG. 1. Virus titers in the CNS of BALB/c mice inoculated with W-2 poliovirus. (A) Isolates from paralyzed mice and (B) isolates from nonparalyzed mice. Symbols: ○ and ●, isolates from brains; ■, isolates from spinal cords. Virus was not detected in the spinal cords of nonparalyzed animals. Solid symbols show isolates with altered fingerprints compared with the W-2 plaque-purified virus, and open symbols indicate isolates with no fingerprint changes.

FIG. 2. RNase T1 oligonucleotide fingerprints of plaque-purified W-2 poliovirus and selected isolates from the CNS of paralyzed mice. Each virus was passed twice in RD cells and labeled with ^{32}P . Virus was purified by rate zonal centrifugation, and the virion RNA was digested with RNase T1 and subjected to two-dimensional electrophoresis (left to right in the first dimension and bottom to top in the second dimension). Gels then were fluorographed as described in the text. The position of each new oligonucleotide is indicated by an arrow ($\uparrow$), and the position of each lost oligonucleotide is indicated by an asterisk (*). (a) Fingerprint of W-2 poliovirus and (b) diagrammatic representation of W-2. (c, d, e, and f) Isolates from paralyzed mice: (c) day 30 brain isolate, P-2; (d) day 30 spinal cord isolate, P-2; (e) day 105 brain isolate, P-1; and (f) day 105 spinal cord isolate, P-1. Some new oligonucleotides, which appeared in submolar concentrations (see text), were faint and did not reproduce well during normal exposure times; therefore, two overexposed fingerprints showing these faint oligonucleotide spots appear in Fig. 4.

TABLE 1. RNase T1 oligonucleotide changes of poliovirus during persistence in the CNS of mice

Day ^a	Isolate	Paralyzed (P) ^b				Nonparalyzed (N) ^b		
		Brain		Spinal cord		Isolate	Brain	
		New	Lost	New	Lost		New	Lost
10	P-1	1, 6, 9, 16, 23		1, 6, 7, 9, 15, 19, 28		N-1	11	
	P-2	1, 6		1, 6, 9, 14, 17, 23, 29		N-2 ^c N-3	14	
30	P-1	1, 6, 17		10	2	N-1	17, 21	
	P-2	17, 33		1, 5, 6, 7, 9, 17, 33		N-2	14	
60	P-1	1, 6, 9, 17, 33		1, 6, 9, 17, 33	31	N-1	8, 11	
	P-2	1, 3, 4, 6, 9, 11, 22, 27	26	1, 4, 6, 9, 17, 27, 33	26	N-2 ^c		
90	P-1 ^c			6		N-1	12	
	P-2	1, 6, 11, 13, 28		6, 11		N-2	9, 17	
	P-3	6		5, 6				
105	P-1	17		6, 16, 30	18, 25	N-1 ^c		
	P-2	1, 6, 9, 17, 33		24	18			
	P-3		32	6				

^a Virus isolates are listed according to the day PI; each isolate was obtained from a different mouse, except in paralyzed mice in which isolates were obtained from both brains and spinal cords.

^b Each new oligonucleotide or lost oligonucleotide in the RNase T1 fingerprints of the isolates was assigned a number. Refer to Fig. 5 for the location of each oligonucleotide change in the T1 maps.

^c In these instances, the fingerprints were identical to poliovirus W-2.

substantially greater in viruses from paralyzed mice than in viruses from nonparalyzed animals.

Assuming that ca. 10% of the genome is analyzed by RNase T1 fingerprinting and that the large oligonucleotides are representative of the entire genome, it is possible to approximate the total number of nucleotide changes occurring in each isolate (19). For example, in the day 10, P-1 brain isolate, there were a total of five observed oligonucleotide changes in the fingerprint (Table 1), reflecting a total of

50 base changes. Since the virion RNA of poliovirus is about 7,500 nucleotides in size (14), 50 nucleotide changes represent a 0.7% change in the genome. The overall estimated frequency of change in the genomes of viruses isolated from paralyzed mice ranged from 0 to 1.2% for isolates from brains and from 0.1 to 1.0% for isolates from spinal cords (Table 2). For isolates from nonparalyzed mice, the range was 0 to 0.3%. As was demonstrated, more than a single virus genotype is present in each CNS homogenate [see

TABLE 2. Estimated percent change in genomes of persisting W-2 virus isolates^a

Day	Paralyzed (P)					Nonparalyzed (N)		
	Isolate	Brain	Mean	Spinal cord	Mean	Isolate	Brain	Mean
10	P-1	0.7		0.9		N-1	0.1	
	P-2	0.3		0.9		N-2 N-3	0 0.1	
30	P-1	0.4	0.6 ± 0.4	0.3	0.8 ± 0.3	N-1	0.3	0.13 ± 0.1
	P-2	0.3		0.9		N-2	0.1	
60	P-1	0.7		0.8		N-1	0.3	
	P-2	1.2		1.0		N-2	0	
90	P-1	0	0.3 ± 0.3	0.1	0.3 ± 0.2	N-1	0.1	
	P-2	0.7		0.3		N-2	0.3	
	P-3	0.1		0.3				
105	P-1	0.1		0.7		N-1	0	
	P-2	0.7		0.3				
	P-3	0.1		0.1				

^a Percent change = $\left(\frac{\text{number of oligonucleotide changes} \times 10}{7,500 \text{ nucleotides}} \right) \times 100\%$. See text for explanation. Since more than a single polio-

virus genotype was present in each CNS homogenate, these frequencies of genetic change represent the overall estimate of genetic change for all viruses in each CNS isolate, rather than for a single virus.

section (iii) below]. Therefore, these frequencies of genetic change represent an estimate of genetic change for all viruses present in each CNS isolate, rather than for a single virus.

On the average, there was a larger number of oligonucleotide changes in brain and spinal cord isolates obtained from paralyzed animals during the first 60 days than in those obtained in the final 45 days of the infection. The mean change during the first 60 days was 0.6% for isolates from brains and 0.8% for those from spinal cords, in contrast to a 0.3% mean change for isolates obtained from either brains or spinal cords after day 60 PI (Table 2). This difference was not observed in viruses isolated from the brains of nonparalyzed mice in which the mean change for the entire infection was only 0.13% (Table 2). These frequencies of genetic change fall within the expected range of values based on the computer-simulated model of Aaronson et al. (1) in which T1 map changes were correlated with sequence changes. They showed that extrapolation from the number of oligonucleotide changes to sequence changes should be accurate when the sequence homology between the two RNAs being compared is at least 90%. Although we have no sequence information, it is likely that the CNS isolates have sequences very close to that of W-2, since W-2 was the parent of each isolate. In addition, based on the results of the oligonucleotide maps, the genomes clearly did not undergo massive mutation during persistence.

(ii) Specific oligonucleotide changes. Brain and spinal cord isolates from the same paralyzed mouse did not have identical oligonucleotide maps, but many isolates from the same mouse were similar, with some new oligonucleotide spots shared (Table 1). For example, oligonucleotides 1, 6, and 9 were present in the brain and spinal cord isolates of the day 10, P-1 mouse, but there were two additional spots in the brain isolate and four additional spots in the spinal cord isolate which were not shared. These findings were similar for viruses from other paralyzed mice (e.g., Fig. 2c and d), especially those recovered during the first 2 months. (See Fig. 5 for the location of oligonucleotide changes.)

Oligonucleotide changes in isolates did not accumulate with time; the changes seen in isolates recovered at later times did not consist of all changes found in earlier isolates. This suggests that subpopulations of virus, which are present in the CNS [section (iii) below], may fluctuate with time. Some oligonucleotide changes, however, occurred frequently in different isolates obtained throughout the infection. Oligonucleotides 1, 6, and 9 appeared as new oligonucleotides individually or in some combination in 17 of the 24 oligonucleotide fingerprints of isolates from paralyzed mice (e.g., Fig. 3a and c). In contrast, other oligonucleotides such as 15, 22, and 23, appeared much less frequently. Oligonucleotide 26 was the only oligonucleotide which was lost from both the brain and spinal cord isolates of the same mouse; otherwise, there was no pattern to the loss of oligonucleotides. Also, no pattern of specific oligonucleotide changes was seen in nonparalyzed mice.

In the 24 RNase T1 maps of isolates from paralyzed mice, two patterns were identical in more than one isolate. The maps of the day 90, P-1 (spinal cord); day 90, P-3 (brain); and day 105, P-3 (spinal cord) isolates were identical and had only one change compared with the W-2 virus. The maps from brain isolates from paralyzed mice at day 60, P-1 and day 105, P-2 also were identical. For isolates with altered maps from the nonparalyzed group, two were identical: day 10, N-3 and day 30, N-2 (Table 1). In addition, since spinal cord isolates were derived from viruses originally replicating

in the brain, it seems likely that viruses isolated from brains and spinal cords of the same animal would be genetically similar, that is, share many of the same oligonucleotides. This was found to be the case for many brain and spinal cord isolates, and in one instance, the RNase T1 maps for the brain and spinal cord isolates had only a single oligonucleotide difference (the day 60, P-1 mouse; Table 1). It also would be expected that since viruses in the brain and spinal cord of the same mouse replicate independently of one another at some point during the infection, some oligonucleotides might not be shared.

RNase T1 fingerprints of W-2 poliovirus and several representative isolates from paralyzed and nonparalyzed mice are shown in Fig. 2 and 3. The oligonucleotide maps of paired brain and spinal cord isolates from two paralyzed mice sacrificed at days 30 and 105 are shown in Fig. 2c and d and Fig. 2e and f, respectively. In Fig. 3, brain isolates from paralyzed and nonparalyzed mice sacrificed at days 10 and 90 are shown. Since many fainter oligonucleotide spots reproduced poorly in photographs during the regular exposure periods of the fingerprints shown in these figures, Fig. 4 is provided as an intentional overexposure of two representative fingerprints to demonstrate the presence of the lighter oligonucleotide spots. Finally, the positions of all oligonucleotide changes that occurred in each isolate from both groups of mice are summarized in the diagram in Fig. 5.

(iii) Mixed virus populations. Many of the new oligonucleotide spots (numbers 1, 5, 6, 7, 8, and 9, for example) were less intense than the majority of spots in the fingerprints and therefore appeared to be submolar (Fig. 4). Similar light spots in the fingerprints of Semliki Forest virus isolates from persistently infected mouse L929 cells (15) and Theiler's virus isolates from persistently infected mice (19) were concluded to be submolar and to originate from mutant viruses. These results suggest that the submolar spots in the fingerprints of the W-2 isolates also originate from subpopulations of viruses derived by mutation of the W-2 virus during replication in the CNS.

The fact that not all new oligonucleotides, submolar or molar, always appeared simultaneously in the same RNase T1 map suggests that genetically distinct viruses are present in each CNS homogenate. For example, oligonucleotides 1, 6, and 9 occasionally appeared together in the same fingerprint, but more often, they appeared individually or in pairs. Thus, the RNA sequences giving rise to these three oligonucleotides must be carried in viruses of different genotypes.

(iv) Control experiment. It was important to determine whether the oligonucleotide changes identified by RNase T1 fingerprinting could have occurred during the process of virus isolation and amplification. Successive high multiplicity lytic passage of vesicular stomatitis virus in cell culture has been shown to cause more virus mutation than low multiplicity passage (21). Therefore, W-2 poliovirus was passed 10 times in RD cells at high multiplicity, and the 10th passage fingerprint was compared with that of the original virus. The two fingerprints were identical (data not shown), thus indicating that substantial mutation was not likely to occur during the *in vitro* amplification of the CNS isolates. This is in agreement with other studies in which no mutations were detected during *in vitro* passages of another poliovirus 2 (24) and two strains of Theiler's murine encephalomyelitis virus (17, 19).

DISCUSSION

RNase T1 mapping of persistent W-2 polioviruses demonstrated that substantially more oligonucleotide changes oc-

FIG. 3. RNase T1 oligonucleotide fingerprints of early and late isolates from the brains of paralyzed and nonparalyzed mice. See legend to Fig. 2 for experimental details. (a and b) Day 10 isolates from paralyzed, P-1, and nonparalyzed, N-1, mice, respectively; (c and d) day 90 isolates from paralyzed, P-2, and nonparalyzed, N-1, mice, respectively.

curred in viruses isolated from the CNS of paralyzed mice compared with those isolated from nonparalyzed mice. Since all animals were treated with cyclophosphamide to induce immunosuppression, it seems unlikely that immunological pressures were present to select for specific virus mutants and hence account for these T1 map differences. In fact, the neutralizing antibody response to W-2 virus was not detected in both animal groups, and general assays for both humoral and cell-mediated immune responses also were suppressed (Jubelt and Meagher, in press). In addition, since virus titers from brains of paralyzed and nonparalyzed mice were similar, no relationship can be established between virus titer and the extent of mutation.

Paralysis caused by poliovirus is known to be associated with virus replication in anterior horn cells in the spinal cord (2, 11). Since W-2 virus was inoculated into mice intracerebrally, the virus must spread to the spinal cord if it is to cause paralysis. It is possible, therefore, that the greater number of oligonucleotide changes seen in isolates from paralyzed mice might be the result of mutations that facilitate the spread of virus to the spinal cord or allow infection of anterior horn cells. After intracerebral inoculation, both

W-2 virus and the virulent Lansing strain of poliovirus replicate to the same level in the brain by day 3 PI (12; Jubelt and Meagher, in press). However, it takes much longer for W-2 to cause paralysis than it does for the Lansing strain (11; Jubelt and Meagher, in press). This longer time for W-2 virus to cause paralysis appears to occur because it takes W-2 virus longer to spread from the brain to the spinal cord. In fact, a characteristic of attenuated polioviruses is the inability to disseminate rapidly in the nervous system as compared with wild-type viruses (3, 4). If W-2 virus is inoculated directly into the spinal cord, however, the time to paralysis is markedly reduced and is similar to that of the Lansing strain (12; Jubelt and Meagher, in press). Thus, the prolonged period before paralysis after intracerebral inoculation does not appear to be due to an inability of W-2 virus to infect anterior horn cells. This does not preclude mutation as being a necessary step for infection of the anterior horn cells by either wild or attenuated viruses, but it does lend support to the idea that if mutation of W-2 virus is a prerequisite for paralysis, it more likely is related to the ability of the virus to spread to the spinal cord.

Another finding was the decrease in oligonucleotide

FIG. 4. Overexposure of two oligonucleotide fingerprints showing submolar oligonucleotides. These same fingerprints, normally exposed, appear in Fig. 2d and 3c, respectively. (a) Day 30 spinal cord, P-2; (b) day 90 brain, P-2. See legends to Fig. 2 and 3 for details.

changes in viruses isolated from brains and spinal cords of paralyzed mice after day 60. This result contrasts with the finding from nonparalyzed mice in which oligonucleotide changes were reasonably constant (although less frequent) throughout the infection. This decrease in the number of oligonucleotide changes in isolates from paralyzed animals did not appear to be related to virus titer, since the virus concentration in brains and spinal cords remained at about the same level over the course of the infection. This was unexpected in light of other persistent virus infections in which the frequency of mutation increased with increasing time after infection. For example, only one or two oligonucleotide changes were found in Theiler's viruses persisting in the mouse CNS during the first 3 months PI, but by 6 months, as many as nine oligonucleotide changes were found (19). In visna viruses isolated from a persistently infected sheep, nine oligonucleotide changes at 1.5 years and four oligonucleotide changes at 3 years PI were found (6), but the number of viruses fingerprinted was small and possibly not a representative sample. The frequencies of mutation in polioviruses isolated from humans appears to be variable, with no particular pattern relating to the number of oligonucleotide changes (13, 24). It is possible that the immunosuppressed condition, in which host antibody does not play a selective role, permits replication of W-2 virus mutants ordinarily eliminated. However, it remains unexplained why mutation declines after day 60.

A major difficulty is being able to separate mutations which may be important to the pathogenesis of virus disease and persistence from background mutations. In spite of this problem, the appearance of new oligonucleotides 1, 6, and 9 in most of the 24 fingerprints of isolates from paralyzed animals suggested that they were important in the paralytic process. However, these oligonucleotides were not detected in all isolates from paralyzed mice. This fact detracts from the role the mutations causing these oligonucleotide changes may play in the paralytic process. Since we are not certain that each virus present in the CNS was amplified to high titer during in vitro passage, it is possible that viruses from which these oligonucleotides originated were in too low a concentration in some CNS homogenates to be detected by finger-

printing. Also, other virus mutants could have been involved in the paralytic process but were in numbers too small to be detected. Therefore, because of these possibilities, a large number of virus isolates was fingerprinted in an effort to obtain a representative sample of oligonucleotide changes.

FIG. 5. Summary of oligonucleotide changes occurring in RNase T1 oligonucleotide fingerprints of the persistent virus isolates. This diagram represents a composite oligonucleotide pattern of the 30 isolates with fingerprints different from the original poliovirus W-2. Symbols: ●, oligonucleotides lost; ○, new oligonucleotides present in molar or near molar quantities; ○, new oligonucleotides present in submolar quantities.

Several conclusions can be drawn from this study. First, the extent of mutation in viruses from paralyzed and nonparalyzed mice was markedly different as exemplified by many more oligonucleotide changes in viruses from paralyzed animals. The levels of virus replication in the brains of the two animal groups were comparable and did not explain this difference. In addition, since the selective pressures of virus-neutralizing antibodies were not present, other host factors probably are involved in promoting the high rate of W-2 virus mutation during replication in animal hosts. Second, specific oligonucleotide changes were detected exclusively in viruses from paralyzed mice. Work is in progress to examine the relationship these oligonucleotide changes have to the paralyzed state. In addition, we are mapping specific oligonucleotide changes found in viruses from paralyzed mice on the poliovirus genome to determine in which gene(s) the mutations giving rise to these changes occurred.

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