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Biochemical Analysis of DA Strain of Theiler's Murine Encephalomyelitis Virus Obtained Directly from Acutely Infected Mouse Brain

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Growth and neurovirulence of a number of neurotropic viruses show pronounced differences after passage in cell culture compared with continued in vivo passage in the central nervous system. The DA strain of Theiler's murine encephalomyelitis virus provides a model for studying these issues since DA virus grown in mouse brain produces acute neuronal disease in weanling mice, but tissue culture-passed DA virus does not. In addition, DA virus grown in mouse brain has a greater 50% mouse lethal dose/50% tissue culture infective dose ratio than tissue culture-passed DA virus. Comparison of these viruses required the analysis of virus purified directly from infected mouse brain, without tissue culture passage. Capsid proteins from DA virus grown in mouse brain were resolved on sodium dodecyl sulfate-polyacrylamide gels and shown to have the same profile as tissue culture-passed DA virus. Viral RNAs were the same size, with no evidence of defective interfering particle production. Two-dimensional gels of in vitro-labeled RNase T_1-digested RNA showed that virus variants were more apparent during acute in vivo passage. These genomic differences may be critical in determining the biological behavior of the virus.

Why particular viruses or strains of viruses are neurotropic and how viruses change their biological activity during passage in vitro or in vivo are complex, largely unanswered questions. For example, despite the effective use of poliovirus vaccination for 20 years, it is still not known why the poliovirus vaccine strains lack neurovirulence. The DA strain of Theiler's murine encephalomyelitis virus provides a model system in which to study these topics of viral neurotropism and in vitro adaptation.

The passage history of DA strain has a prominent effect on its biological activity (11–13, 23). Although DA passed both in suckling mouse brain (DAMB) and in tissue culture cells (DATC) produces a late, persistent, white matter-demyelinating infection when inoculated into weanling mice, only DAMB produces clinical and histopathological evidence of an early, acute gray matter neuronal encephalomyelitis. In addition, DAMB has a higher 50% suckling mouse lethal dose/50% tissue culture infective dose ratio compared with DATC.

A biochemical analysis of DAMB requires virus to be studied directly from the central nervous system, without tissue culture passage, for several reasons. DAMB does not grow well in tissue culture and usually requires a number of passages to build up sufficient titer for analysis. This in vitro passage may favor selection and amplification of rapidly growing species of DA virus, with less infectious viral mutants present in the central nervous system becoming obscured. Tissue culture passage of DAMB may also eliminate biochemical differences that exist between DAMB, which has not been passed in tissue culture, and DATC. Therefore, to perform a comparison of DAMB and DATC, we have developed methods involving purification of DAMB directly from infected and pooled, as well as single, mouse brains.

The analyses of DAMB and DATC described in this report show that mouse brain-derived virus is composed of a collection of virus subpopulations, whereas DATC is considerably more homogeneous.

MATERIALS AND METHODS

Growth of cells and virus. Monolayer cultures of BHK-21 cells (MBHK), used for preparing virus stocks, and L929 cells, used for assaying virus infectivity, were grown on Dulbecco modified Eagle medium and minimum essential medium, respectively, containing 10% calf serum, 2 mM L-glutamine, and 100 μg of streptomycin and 100 U of penicillin per ml. Suspension cultures of BHK-21 cells were grown in Joklik modified minimum essential medium of Eagle containing 10% calf serum, 10% tryptose phosphate, nonessential amino acids, 2 mM L-glutamine, and 100 μg of streptomycin and 100 U of penicillin per ml.

A mouse brain-passed stock of DA (obtained from J. Lehrich, Harvard Medical School, Boston, Mass.) that had been passed at least 5 times in suckling mouse brain was designated DAMB1. DAMB2 was passed an additional time in suckling mouse brain. For these passages, 1- to 2-day-old C3H/HeJ mice (Jackson Laboratories) were inoculated intracerebrally with 0.02 ml of a 10^{-1} dilution of virus. Brains from mice were harvested when the animals were lethargic and no longer suckling. DAMB stocks were 10% clarified homogenates of single or pooled brain in Hanks buffered saline solution.

DATC stocks prepared from the original mouse brain stock of DA were passed 9 times in MBHK cells and further processed as follows: one was passed 3 more times in MBHK cells (DATC1); one was passed 7 more times in MBHK cells (DATC2); and one was passed twice in BHK-21 suspension culture cells (DATC3).

Virus purification. DATC was purified as described previously (20). In brief, an infected BHK cytoplasmic lysate was clarified, made 1% (vol/vol) sodium dodecyl sulfate (SDS), pelleted, and banded in CsCl. A visible band of virus was collected.

In the case of DAMB, brain homogenates from infected

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suckling mice were extracted with 1,1,2-trichloro-1,2,2-trifluoroethane (Freon). As a control, uninfected mouse brains were similarly and separately processed. The extractions consisted of adding an equal volume of the Freon to the clarified homogenates, vortexing for 10 min, and centrifuging at 1,500 rpm for 10 min at 4°C. The upper (aqueous) phase was collected and reextracted. After three extractions, the aqueous phase was diluted in 20 mM Tris-hydrochloride (pH 7.5) and pelleted through 3 ml of 35% sucrose in an SW27 rotor at 24,000 rpm at 4°C for 17 h. The pellet was diluted in 20 mM Tris-hydrochloride (pH 7.5) and centrifuged on a 15 to 30% continuous sucrose gradient in an SW28.1 rotor at 27,000 rpm for 4 h at 4°C or in an SW40 rotor at 38,000 rpm for 2.75 h at 4°C. Fractions were collected by pumping from the bottom; in some cases, optical density at 260 nm was continuously recorded. Fractions with an absorbance peak, or fractions corresponding in location to a peak of radioactivity from [^{35}S]methionine-labeled purified 150S marker DATC2, prepared as previously described (20) and run in a companion gradient, were collected.

Isolation of viral RNA and proteins. DATC or DAMB was extracted twice with phenol saturated with TNE (0.1 M NaCl, 0.001 M EDTA, 0.005 M Tris-hydrochloride [pH 8.3]) at room temperature. The aqueous phase was removed and adjusted to 0.2 M with sodium acetate (pH 5.5), and the RNA was precipitated with 2.5 volumes of 95% ethanol at −20°C overnight. The precipitated RNA was collected by centrifugation at 16,000 × *g* for 30 min at 4°C. The phenol phase was made 10% (vol/vol) with acetone, left overnight at 4°C, and centrifuged at 10,000 rpm for 10 min at 4°C in a Sorvall HB-4 rotor. The precipitated proteins were dissolved in 20 mM Tris-hydrochloride (pH 7.5) and the appropriate sample buffer for SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE. Electrophoresis was performed in 10 or 12.5% polyacrylamide slab gels according to Laemmli (10). Sample buffers used were: (i) 10% glycerol–5% 2-mercaptoethanol–3% SDS–63 mM Tris-hydrochloride (pH 6.8)–0.002% bromphenol blue; (ii) 2 M urea–3% SDS–0.002% bromphenol blue; (iii) 10% glycerol–3% SDS–63 mM Tris-hydrochloride (pH 6.8)–0.002% bromphenol blue. Gels were stained with Coomassie blue or silver (15).

Oligonucleotide fingerprints. The alcohol-precipitated RNA was labeled in vitro by the technique of Pedersen and Haseltine (18). In brief, less than 750 ng of RNA (when measured) in 5 μl was digested with 10 U of RNase T_1 (Calbiochem-Behring) and 10^{-2} U of intestinal alkaline phosphatase (Boehringer-Mannheim Biochemicals) in 20 mM Tris-hydrochloride (pH 8.0)–2 mM EDTA. The digest was then mixed with a 45-μl reaction mixture containing 10 mM potassium phosphate (pH 9.5), 10 mM magnesium acetate, 5 mM dithiothreitol, 25 μCi of [γ-^{32}P]ATP (Amersham Corp.), and 40 U of polynucleotide kinase (P-L Biochemicals, Inc.). The mixture was incubated at 37°C for 4 h until the labeling was stopped with an equal volume of 0.6 M ammonium acetate. Yeast carrier RNA (30 μg) was added, and the oligonucleotides were phenol extracted, alcohol precipitated, and separated on two-dimensional gels as previously described (7). In vivo-labeled DATC3 oligonucleotide fingerprints, prepared as previously decribed (20), were used for comparison.

Northern blots. RNAs from clarified crude homogenates of pooled, DA-infected suckling mouse brains and from purified DATC3 were phenol extracted, alcohol precipitated, electrophoresed on methyl mercury hydroxide-agarose gels (2), and transferred to activated diazobenzyloxymethylcellulose paper. The Northern blot procedure and [^{32}P]cDNA probe prepared from DATC were as previously described (1, 20).

RESULTS

The DA mouse brain stocks (DAMB1 and 2) had been passed at least 5 and 6 times in suckling mouse brain since the original DA virus isolation, whereas the three DA tissue culture stocks (DATC1, 2, and 3) had been obtained by passing DAMB various numbers of times (12, 16, and 11 times, respectively) in BHK-21 cells (See above). During the initial passages in BHK-21, DA virus grew poorly but eventually grew to high titer in tissue culture with a relative decline in encephalitogenicity compared with DAMB, as previously described (11–13, 23). Table 1 gives the 50% suckling mouse lethal dose/PFU ratio of DAMB2 compared with DATC1. The DAMB2 ratio was 9.6×10^{1}, compared with the DATC1 ratio of 5.6×10^{-2}. Although both DAMB and DATC produced a late demyelinating disease in weanling mice, only DAMB produced clinicopathological evidence of anterior horn cell disease (data not shown).

Comparison of DAMB with DATC required the direct isolation of DAMB from mouse brain, without tissue culture passage, to prepare virus for analysis. We therefore developed methods that would yield virus preparations directly from infected mouse brain that were suitable for biochemical study. DAMB1 and DAMB2 virus from single and pooled brain were purified as described above. In one experiment with DAMB1, for example, approximately 2 g of brain tissue from 14 infected suckling mice was homogenized, Freon extracted, pelleted through sucrose, and applied to a continuous sucrose gradient. Figure 1 shows a peak of absorbance at 260 nm, which corresponded in location to fractions that exhibited a radioactivity peak from a companion gradient on which labeled 150S marker DATC2 had been applied, indicating that DAMB and DATC had the same sedimentation properties. The number of virus particles in DAMB1 was estimated at 1.5×10^{13}, assuming that the recorded absorbance represented pure virus and that an optical density at 260 nm of 1 represents 10^{13} virus particles per ml (21). The same purification scheme was used for infected single brains and control (uninfected) mouse brains, but since no absorbance peak was seen in fractions from the sucrose gradient, fractions of DAMB and control mouse brains corresponding in location to the labeled peaks of the companion tube were collected blindly.

The DAMB preparations described above were extracted with phenol for analysis of both proteins (phenol phase) and RNA (aqueous phase). Figure 2a shows a Coomassie blue-stained SDS-PAGE gel of the proteins precipitated with acetone from the phenol phase in a preliminary study of a DAMB1 pooled brain preparation. Three major polypeptides, detected by Coomassie blue stain, comigrated with VP1, VP2, and VP3 (molecular weights between 25,000 and 35,000). A few minor bands were present. To increase sensitivity and to detect contaminating bands, SDS-PAGE gels were silver stained in subsequent studies. An identical

TABLE 1. Comparison of DAMB and DATC

Virus	$SMLD_{50}$/ml[a]	No. of PFU/ml	$SMLD_{50}$/PFU ratio
DAMB2	5×10^{7}	5.2×10^{5}	96
DATC1	5×10^{5}	8.8×10^{6}	0.056

[a] $SMLD_{50}$, 50% Suckling mouse lethal dose.

pattern, occasionally with visualization of a polypeptide of 36,000 daltons (VPO) and of less than 10,000 daltons (VP4), was seen in other preparations of DAMB1 from pooled mouse brains (Fig. 2b) and DATC (Fig. 2c). Despite intentional overdevelopment, DAMB exhibited the polypeptide pattern of previously characterized DA wild-type virus grown in MBHK (14), whereas the DATC preparation displayed some additional bands that represented contaminating proteins, since they were not present in other, more pure DATC preparations (Fig. 2e). In some gels, VP2 appeared broadened (Fig. 2a) or as a doublet (Fig. 2d and e) for reasons that are not clear. Of note was the appearance of several high-molecular-weight bands that represented artifacts of silver staining in all lanes of the gel, even empty lanes containing sample buffer alone (24; Fig. 2, "x" marks). These bands were less evident when sample buffer containing urea was used (Fig. 2d and e). Proteins from DAMB1 and DAMB2 from a single mouse brain were also detectable with the characteristic DA polypeptide pattern by silver staining gels (data not shown).

Because it had been reported that the WW strain of Theiler's murine encephalomyelitis virus which has biological properties similar to those of the DA strain, had a 58,000-molecular-weight protein when purified from acutely infected mouse brain and analyzed by SDS-PAGE under nonreducing conditions (22), DAMB2 proteins were also run in the absence of 2-mercaptoethanol (Fig. 2f). Identical polypeptide patterns were seen with and without nonreducing conditions. No high-molecular-weight bands, aside from silver stain artifactual bands, were observed that were unique to DAMB (Fig. 2f and g).

The relatively low infectivity in tissue culture of DAMB compared with DATC raised the possibility that defective interfering particles were present in the infected brains. Therefore, RNAs from alcohol-precipitated, phenol-extracted homogenates of acutely infected brains or purified DATC3 were electrophoresed on methyl mercury hydroxide-agarose gels, transferred to activated diazobenzyloxymethylcellulose paper, and hybridized with [^{32}P]cDNA. The size of the hybridized RNA extracted from purified DATC3 was identical to that extracted from the brain homogenates

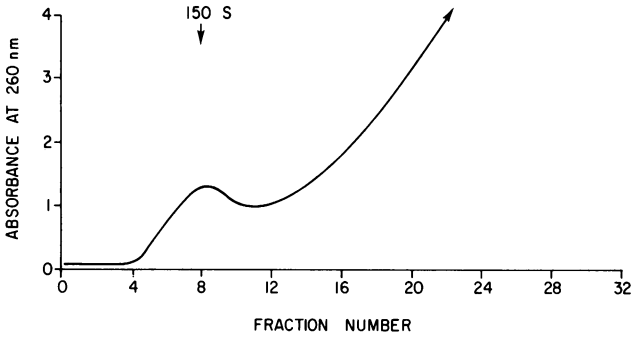

FIG. 1. Analysis of DAMB1 by rate zonal centrifugation on a continuous sucrose gradient. Freon-extracted, DA-infected mouse brain homogenates were pelleted and then analyzed by centrifugation in gradients of 15 to 30% sucrose (wt/wt) for 2.75 h at 38,000 rpm in a Beckman SW40 rotor at 4°C. Fractions were collected by pumping from the bottom, and the optical density at 260 nm was continuously recorded. The position of a fraction corresponding in location to a peak of radioactivity from [^{35}S]methionine-labeled purified 150S marker DATC2 run in a companion gradient is shown at the arrow.

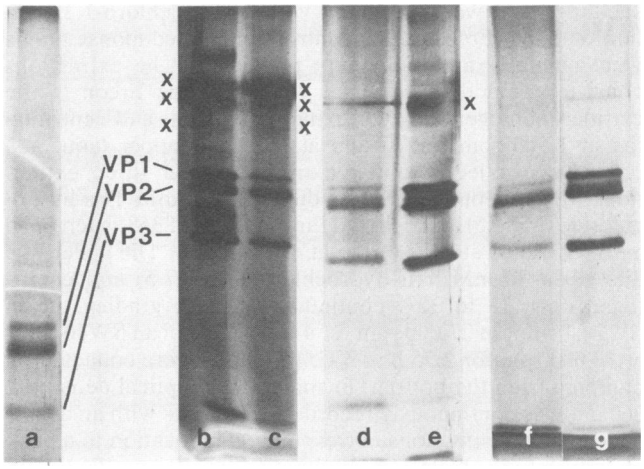

FIG. 2. SDS-PAGE analysis of DA virus capsid polypeptides. Virus was purified as described in the text, run on 12.5% polyacrylamide slab gels, and stained with silver, except for lane a, which was run on 10% polyacrylamide slab gels and stained with Coomassie blue. Samples for lanes a to c were applied in sample buffer 1 (see the text); lanes d and e in sample buffer 2; and lanes f and g in sample buffer 3. (a) DAMB1 from pooled mouse brains; (b) DATC; (c) DAMB1 from pooled mouse brains; (d) DAMB2 from pooled mouse brains; (e) DATC; (f) DAMB2 from pooled mouse brains; (g) DATC3. The positions of VP1, VP2, and VP3 are indicated. "x" is marked next to prominent bands that were artifacts of silver staining.

(Fig. 3) and corresponded to approximately 7,400 nucleotides, as estimated by the relative mobilities of 28S and 18S ribosomal RNA markers. No other homogeneous RNA species indicative of defective interfering particles were detectable.

The biological differences that are known to exist between DAMB and DATC suggested that passage in mouse brain might select genetic variants of the virus. Such genetic changes are generally monitored by RNase T_1 oligonucleotide fingerprints. Virus is usually grown in tissue culture in medium containing ^{32}P, which is incorporated into the genome; two-dimensional gel analysis after digestion with RNase T_1 results in an oligonucleotide map. We have previously analyzed the DA virus genome after multiple lytic passages in tissue culture and found the T_1 fingerprint to be stable. In contrast, isolates from a persistent DA virus in vitro infection and isolates (passed in tissue culture) from chronically DA virus-infected mice showed evidence of genomic mutation (19, 20). These methods were not applicable in the present study, since DAMB grows relatively poorly in tissue culture and since its growth in tissue culture may lead to selection against relatively noninfectious viral mutants. Production of ^{32}P-labeled virus directly from mouse brain has not been shown to yield viral RNA of sufficient specific activity to analyze by fingerprinting methods. We therefore sought to determine whether fingerprints could be obtained from viral RNA isolated and purified directly from mouse brain by labeling RNase T_1 oligonucleotide digests in vitro with polynucleotide kinase and [γ-^{32}P]ATP. Previous reports concerning other viruses (grown in tissue culture) showed that in vitro fingerprints could be obtained that were similar to in vivo-labeled fingerprints (18).

Therefore, to characterize the genome of mouse brain-derived DA virus, RNase T_1 oligonucleotide maps of the

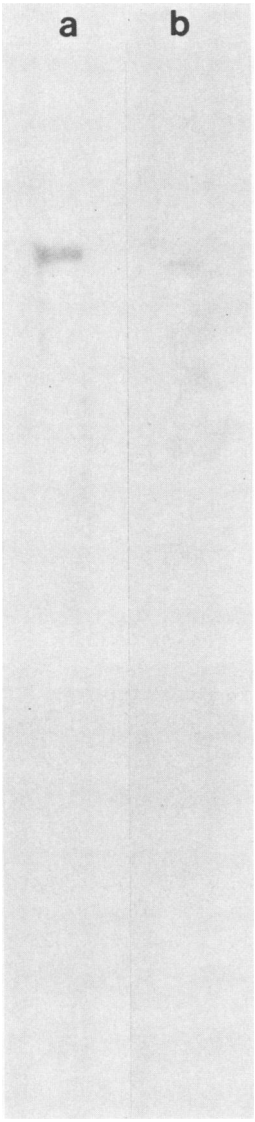

FIG. 3. Northern blot of DA RNA. Purified viral RNA was extracted, electrophoresed in methyl mercury hydroxide-agarose gels, transferred to activated diazobenzyloxymethylcellulose paper, and hybridized with a [^{32}P]cDNA probe from DATC as described in the text. The sources of the RNA were (a) DAMB1; (b) DATC3. Migration of RNA species was from top to bottom.

genome were prepared by using in vitro labeling with [γ-^{32}P]ATP and polynucleotide kinase. The fingerprints of in vitro-labeled RNA from DATC1, 2, and 3 (Fig. 4c, d, and e) were very similar to those of in vivo-labeled DATC3 RNA (Fig. 4b), except for some loss in resolution and slight differences in the prominence and intensity of individual oligonucleotides; the loss of resolution of the in vitro-labeled fingerprints may have been due partly to the small amount of RNA being manipulated and our reluctance to further purify or extensively wash the alcohol-precipitated RNA before labeling. DATC2 (Fig. 4d) seemed to have one oligonucleotide (no. 18, as indexed in Fig. 4a) that was not as prominent, or was missing, when compared with the other DATC fingerprints. Submolar oligonucleotides were seen between no. 5 and 2 in DATC1 and DATC2. Oligonucleotide 23 was artifactually deleted in DATC1 and DATC2. Our previous studies with in vivo-labeled RNase T_1 oligonucleotide maps demonstrated stability of the DA genome with lytic tissue culture passage (20).

RNase T_1 oligonucleotide maps were obtained from in vitro-labeled RNA from purified DAMB1 and DAMB2 from single and pooled brain (Fig. 5). Even more so than with in vitro-labeled DATC, there were differences in the prominence of individual oligonucleotides compared with in vivo-labeled DATC (Fig. 4b), which may have resulted from preferential labeling of some oligonucleotides; this variation did not change with longer labeling times with polynucleotide kinase. Two exposures of a fingerprint of DAMB2 RNA from pooled brain (Fig. 5c and d) were clearly identified as DA fingerprints but demonstrated remarkable differences from the DATC fingerprints. Although certain spots in Fig. 5c had an intensity equivalent to that seen in the corresponding oligonucleotides in other fingerprints, other spots were barely visible if at all. A longer exposure (Fig. 5d) permitted visualization of a complete DA fingerprint, but extra oligonucleotides (some marked with an arrow) became apparent. Although the resolution of spots was not optimal in fingerprints from DAMB1 and DAMB2 RNA from single brains (Fig. 5b and e), perhaps due to the small amounts of RNA present, the fingerprints were all easily recognizable as DA fingerprints. Since DAMB2 single brain was purified, in vitro labeled, and electrophoresed at a different time from DAMB2 pooled brain, the similarity in their fingerprints (Fig. 5e and d) suggests that the extra oligonucleotides seen are not artifactual contaminants; in addition, these large "extra oligonucleotides" were not seen when normal uninfected brain was processed and its RNA was labeled and run in an identical fashion to DAMB RNA (data not shown). The similarity in their fingerprints also suggests that the heterogeneity seen in the pooled brain was not due to different isolates in different brains but that the heterogeneity existed in each single brain. In both DAMB1 (Fig. 5a) and DAMB2 (Fig. 5d) from pooled brain, spots 3, 4, and 6, in comparison with DATC fingerprints, were more vertically aligned, demonstrated an extra associated oligonucleotide, and had a prominence of spot 3. An extra oligonucleotide of lesser intensity was present between spots 1 and 2 in Fig. 5a and d. In all of the DAMB fingerprints, spots 21 to 23 were artifactually deleted.

DISCUSSION

Little is known concerning biochemical determinants of viral neurotropism and the biochemical changes responsible for tissue culture adaptation. As previously mentioned, it is still unclear how the vaccine strain of poliovirus became attentuated and lost neurovirulence. Both the wild Mahoney strain and the Sabin vaccine strain of type 1 poliovirus have been cloned and sequenced, revealing base differences in 57 nucleotides (16); however, the mutations critical for the change in tropism and the mechanism for this selection in tissue culture passage remain uncertain. In many respects, Theiler's murine encephalomyelitis virus and its easily studied animal host, the mouse, provide a unique opportunity to answer many of the questions that have remained unsolved with poliovirus.

The DA strain of Theiler's murine encephalomyelitis virus varies in biological behavior depending on passage history. The increased encephalitogenicity, decreased tissue culture infectivity, and the tropism for motor neurons of DAMB have been previously documented (11–13, 23) and were substantiated in this study. In situ hybridization studies have

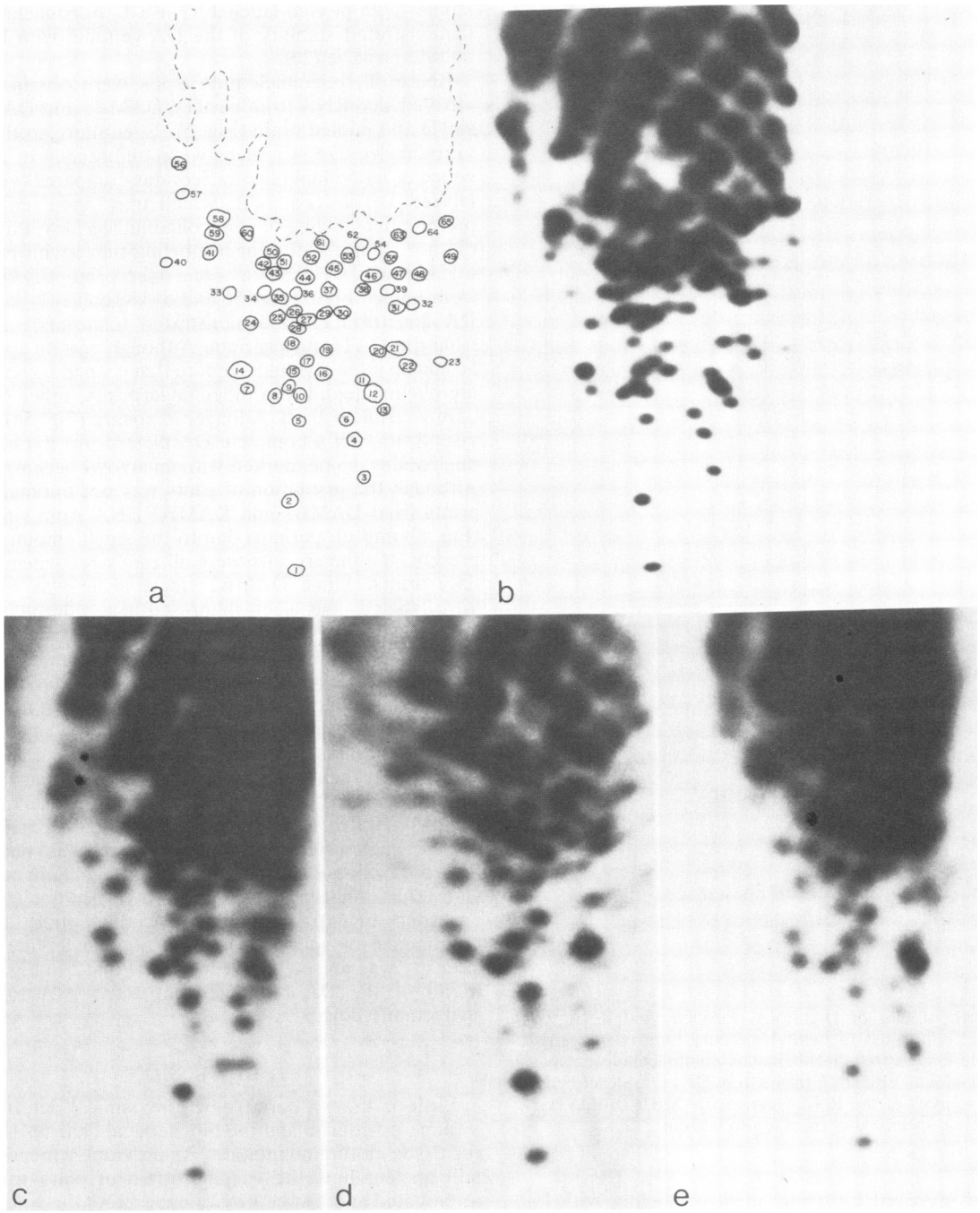

FIG. 4. Two-dimensional oligonucleotide maps of RNase T_1-digested RNA from DATC. For comparative purposes, (a) and (b) are in vivo labeled, whereas all other maps are in vitro labeled. (a) Tracing of in vivo-labeled DATC3 with oligonucleotide spots increasing in number arbitrarily from the bottom; (b) in vivo-labeled DATC3; (c) DATC1; (d) DATC2; (e) DATC3. Conditions are described in the text. The first dimension is from left to right; the second dimension is from bottom to top.

shown viral RNA located in motor neurons and glia in mouse central nervous system tissue after inoculation of weanling mice with mouse brain-passed WW strain (a strain similar to DA), but viral RNA is located only in glia after inoculation of tissue culture-passed WW (4, 23). The use of special methodology enabled us to analyze DAMB and compare it biochemically with DATC.

DAMB and DATC viruses showed identical sedimentation properties in sucrose gradients. We found no difference in viral capsid polypeptides of DAMB compared with DATC when analyzed by SDS-PAGE. This finding is in contrast to a previous report of a 58,000-dalton polypeptide, especially evident under nonreducing conditions, in purified iodinated virus obtained from brain tissue infected with WW strain. Perhaps DA virus does not contain the 58,000-dalton polypeptide.

Northern blot analysis demonstrated an identically sized RNA in DAMB and DATC, with no homogeneous small

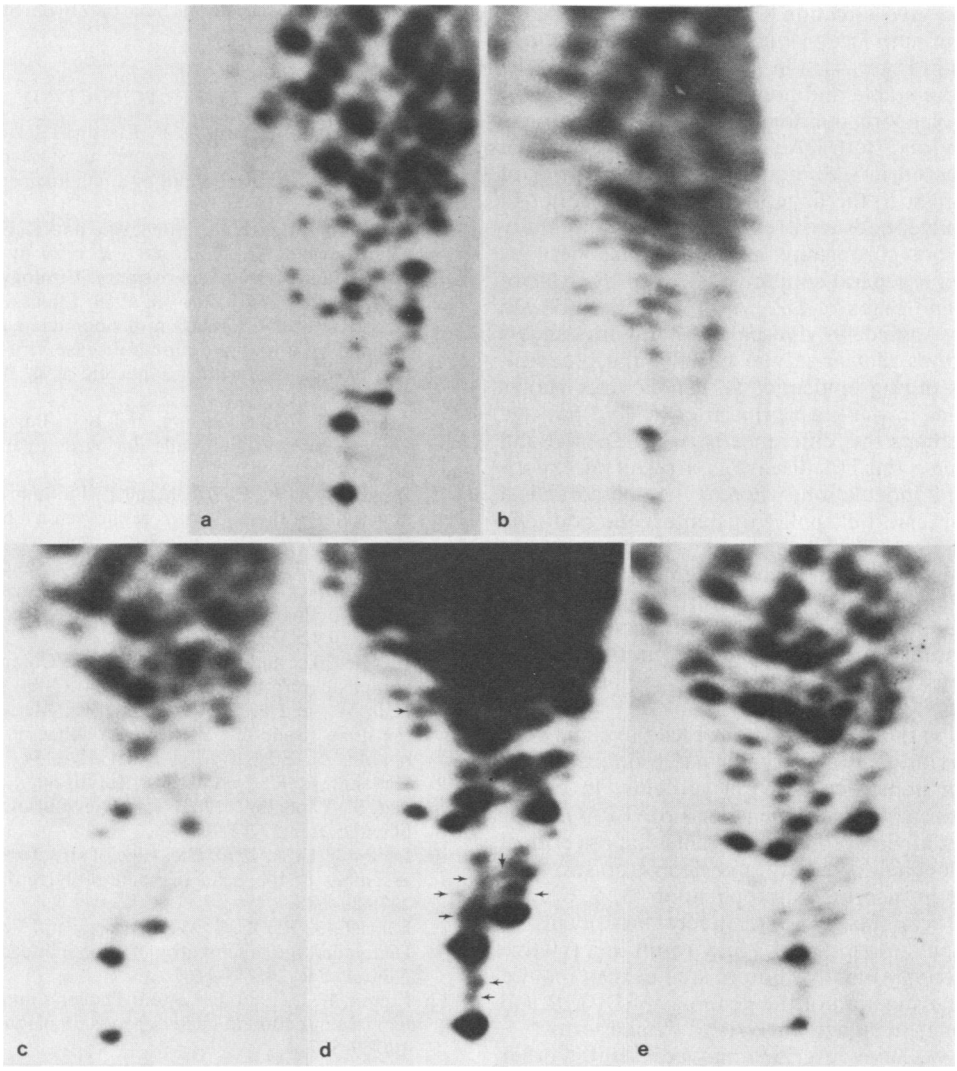

FIG. 5. Two-dimensional oligonucleotide maps of RNase T_1-digested RNA from DA. (a) DAMB1 from pooled mouse brains; (b) DAMB1 from single mouse brain; (c) DAMB2 from pooled mouse brains, first exposure; (d) DAMB2 from pooled mouse brains, second exposure; (e) DAMB2 from single mouse brain. Conditions are described in the text. Arrows indicate the positions of oligonucleotides in (d) that were not present in in vivo-labeled DATC3 (Fig. 4b). The first dimension is from left to right; the second dimension is from bottom to top.

species representing defective interfering particles. Two-dimensional oligonucleotide maps demonstrated a great similarity of DATC fingerprints to one another, as has been seen in in vivo-labeled DATC after lytic tissue culture passage (19). In contrast, the pooled brain virus contained a number of extra oligonucleotides, some of varying intensity, in addition to the oligonucleotides of DATC fingerprints. Since the large, readable oligonucleotides of these gels represent only ca. 10% of the complete viral genome, these changes may be more remarkable. The finding that DAMB fingerprints are basically similar to DATC fingerprints, but differ primarily in the variation in intensities of individual oligonucleotides as well as the appearance of extra oligonucleotides of relatively low intensity, may reflect a heterogeneity of the DAMB virus pool and the presence of virus variants.

A potential criticism of the study is related to the suboptimal resolution of the fingerprints. Perhaps the fingerprints would have been "cleaner" had we washed the alcohol-precipitated RNA more extensively. The analysis of virus taken directly from tissue clearly needs further refinement, but one can still cautiously accept and interpret information derived from such new techniques. We have never previously seen the "extra oligonucleotides" visible in Fig. 5d in other DA fingerprints. Therefore, the data suggest that there is a greater heterogeneity of the DAMB fingerprint compared with DATC. The possibility that contamination by cellular RNA occurred cannot be definitely excluded; however, the similarity of the fingerprints of the pooled and single DAMB2 despite their separate processing makes this less likely, as does the absence of the extra oligonucleotides in similarly processed uninfected mouse brains.

Holland et al. (9) recently suggested that RNA viruses contain a mixture of variants that arise as a result of a high mutation frequency. The mutation frequency is not apparent in lytic infections because selective pressures allow the faster-growing wild type to outgrow the variants. These selective pressures are probably more active in the rapid, acutely lytic tissue culture passage of DA than over the

longer course of in vivo infection. For this reason, variants may be more apparent in DA produced during acute, lytic in vivo passage than in DA passed in tissue culture, where the genome seems more stable and homogeneous.

It is useful to compare our findings for DA with those reported for poliovirus. Both DA and poliovirus have stable genomes when passed in tissue culture. The fingerprint of DAMB is very similar to the fingerprint of DATC, although some extra oligonucleotides are present. Our previous study of DA isolates from chronically infected mouse nervous tissue also showed a general similarity between their oligonucleotide maps and maps of DA wild-type RNA (19). In contrast, naturally spread, in vivo-passed poliovirus shows continuous mutations that arise and become the predominant viral species during epidemics (17); poliovirus shows many more changes in the fingerprint after in vivo passage than does DA. Perhaps this difference between DAMB and poliovirus is because the DA disease is a result of experimental intracerebral inoculation, whereas the poliovirus is a result of natural spread, i.e., poliovirus enters the body via the gastrointestinal tract, replicates locally, produces a viremia, and then spreads to the nervous system. The spread of poliovirus during in vivo passage in nature may allow time for the emergence and establishment of variants. It is also possible in these studies (17) that the tissue culture passage of poliovirus isolates artifactually led to the amplification of one of the variants.

The presence of variants of DAMB may lead to changes in its biological behavior. Certain variants may produce important alterations in nonstructural viral proteins, in capsid proteins (not apparent in one-dimensional SDS-PAGE), or enzymes important in viral replication events that are critical in determining encephalitogenicity and neurotropism; host factors, important in picornavirus replication (3, 5, 6) may interact with the variants. The relatively less available DATC-like subpopulation in DAMB may result in a relative diminution of infectivity in vitro. Future studies may require a more detailed investigation of the genomes of DAMB and DATC, for example, sequence analysis of cloned virus.

The techniques we have described may be useful in other biological studies. The methodology we used avoids problems that may occur when virus isolated from the brain is grown in tissue culture. For example, there is a potential danger, in analyzing virus isolates that have undergone in vitro passage, of unavoidably selecting populations of rapidly growing lytic virus, even though the central nervous system contains a predominantly noninfectious form. Such a situation occurs, for example, in subacute sclerosing panencephalitis, in which cultured isolates usually demonstrate all viral proteins, although M protein can be shown to be absent when viruses are examined directly from the brain (8). These powerful techniques may be valuable in the study of central nervous tissue from subacute sclerosing panencephalitis, from DA chronically infected mice, and from other diseases in which there may be relatively noninfectious viral material. As we have demonstrated (for the first time), RNA fingerprints can be obtained directly from brains, even from a single mouse brain.

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