JOURNAL OF VIROLOGY, Mar. 1980, p. 1165-1172
0022-538X/80/03-1165/08$02.00/0

Vol. 33, No. 3

Purification of Theiler's Murine Encephalomyelitis Virus and Analysis of the Structural Virion Polypeptides: Correlation of the Polypeptide Profile with Virulence

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Theiler's murine encephalomyelitis viruses (TMEV) are separable into two groups based on their biological behavior: those highly virulent isolates which are unable to cause persistent infection and the less virulent isolates which regularly produce persistent central nervous system infection in mice. Two highly virulent and five less virulent TMEV were found to have the same buoyant density (1.34 g/ml) on isopycnic centrifugation and virion structure by electron microscopy. Negatively stained virus particles purified in Cs_2SO_4 gradients appeared to have icosahedral symmetry and measured 28 nm in diameter. Mature virions were found to possess three major structural polypeptides, VP1, VP2 and VP3, in the range of 25,000 to 35,000 daltons, and a smaller fourth major polypeptide, VP4, of 6,000 daltons on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The precursor of VP2 and VP4, VP0, which is a minor polypeptide of mature picornavirus particles, was also identified. However, a slight but consistent difference in several of the capsid polypeptides between the highly virulent and less virulent TMEV was found. VP1 was slightly larger (34,000 versus 33,500 daltons) and VP2 was slightly smaller (31,000 versus 32,000 daltons) for the highly virulent strains compared to the same polypeptide species in the less virulent viruses. VP0 was also slightly smaller (35,500 versus 36,000 daltons) for the highly virulent isolates compared to their less virulent counterparts. Finally, trypsin which was used initially in our purification procedure resulted in preferential cleavage of a 2,000-molecular-weight fragment or fragments from VP1 of only the less virulent isolates.

Theiler's murine encephalomyelitis viruses (TMEV) are naturally occurring enteric pathogens of mice. A number of different isolates have been recovered from paralyzed as well as asymptomatic mice, and all appear to share a close serological relationship (9). However, TMEV are separable into two groups based on their biological behavior in vivo and in vitro (10). GDVII and FA viruses are highly virulent agents which produce a rapidly fatal encephalitis in mice and large plaques in cell culture. These viruses do not appear to be able to establish persistent central nervous system infections in mice. Other TMEV, which resemble Theiler's original (TO) isolates, including DA, WW, TO4, Yale, and BeAn8386 viruses, are at least 1,000-fold less virulent and form small plaques when grown in cell culture. The TO isolates are capable of producing a poliomyelitis-like disease in mice, and surviving animals regularly develop a persistent central nervous system infection which results in a chronic, inflammatory demyelinating disease process (5). Persistent TMEV infection in mice is one of the few available experimental animal models of virus-induced demyelination and, as such, it provides a useful system for studying the pathogenesis of diseases like multiple sclerosis.

A better characterization of these viruses is necessary to begin to understand the basis for the observed differences in their biological behavior. This paper presents an initial investigation of some of the physical properties as well as the structural polypeptide composition of seven isolates representative of the two groups of TMEV. From this, it is now clear that TMEV which have been previously shown to contain RNA (6), fulfill the criteria necessary for their inclusion in the genus *Enterovirus* of the family *Picornaviridae* (4).

MATERIALS AND METHODS

Cells. Baby hamster kidney cells (BHK21) and RD cells, derived from a human rhabdomyosarcoma (12) were maintained in Dulbecco-modified Eagle medium (DMEM) containing 0.1 mM L-glutamine, 100 μg of streptomycin, and 100 units of penicillin per ml, 25 mM $MgCl_2$, and 2% fetal calf serum.

Viruses. The GDVII, DA, TO4, WW, and Yale isolates of TMEV were adapted to grow and produce cytopathic effect in cell culture as described (9). FA virus was purchased from the American Type Culture Collection, and BeAn8386 virus was obtained from Robert Shope (New Haven, Conn.). The mouse brain pools of FA and BeAn8386 isolates were passed twice in BHK21 cells, at which time cytopathic effect developed. The TMEV isolates were plaque purified one to three times, and stocks were prepared in BHK21 cells after several more passages to amplify virus titers. A wild-type (virulent) poliovirus type 1, isolated from a patient that died of polio in 1977, was obtained from William Whetsell (Mt. Sinai School of Medicine, New York, N.Y.).

Radiolabeling of virus for purification. Monolayer cultures in 75-mm plastic flasks were infected with a multiplicity of 10 to 15 PFU/cell. After virus adsorption for 1 h, maintenance medium was added. At 4 to 5 h, the infected cells were washed once with phosphate-buffered saline (pH 7.2), 100 μCi of L-[^{35}S]methionine (810 Ci/mmol; Amersham Corp.) per ml in methionine-free medium, or 100 μCi of [^{3}H]-uridine (51.5 Ci/mmol; Amersham Corp.) per ml of DMEM was added, and the infection was allowed to proceed until there was extensive cytopathic effect. A 4-μg amount of actinomycin D per ml was present in all the media after adsorption.

Virus purification. Virus was purified from infected cells by a modification of the procedure described by Ziola and Scraba (16). Lysates from infected monolayer cultures were disrupted by three cycles of freezing and thawing and clarified at 10,000 × *g* at 4°C for 30 min in an RC2-B Sorvall centrifuge. Trypsin (Sigma Chemical Co.) was added to the supernatant to give a final enzyme concentration of 0.5 mg/ml, and the mixture was incubated at 37°C for 10 min. Sodium dodecyl sulfate (SDS; Sigma Chemical Co.) was added to give a final concentration of 1%, and the mixture was incubated at 24°C for 30 min. The virus was sedimented at 85,000 × *g* at 24°C for 2 h in a type 35 Spinco rotor. This pellet was suspended in 0.1 M sodium phosphate buffer (pH 7.4) and sedimented through 10 ml of 15% sucrose layered over 10 ml of 30% sucrose by centrifugation at 80,000 × *g* at 24°C for 20 h in an SW25.1 rotor. The pellet was resuspended in the sodium phosphate buffer, mixed with an aqueous solution of Cs_2SO_4 to give a final density of 1.33 g/ml, and centrifuged at 180,000 × *g* at 4°C for 20 h in an SW50.1 rotor. Either the visible band below the center of the tube was aspirated by puncturing the tube from the side or the entire gradient was collected in 100-μl fractions by puncturing the tube from the bottom. When appropriate, the density of every fifth fraction was determined by using a Zeiss refractometer, and radioactivity of trichloroacetic acid-precipitable counts in 5-μl samples from collected fractions was determined in a Packard liquid scintillation counter. Purified virus was suspended in the sodium phosphate buffer and centrifuged at 180,000 × *g* at 4°C for 2 h in an SW50.1 rotor.

Labeling and preparation of cytoplasmic extracts. Petri dishes (35-mm size) containing BHK21 cell monolayers were either mock infected or infected with TMEV isolates at a multiplicity of 15 PFU/cell. The cells were pulse-labeled with 50 μCi of L-[^{35}S]-methionine per ml in methionine-free medium from 6 to 7 h postinfection. At the end of the pulse, the cells were washed twice with cold phosphate-buffered saline, 0.5% Nonidet P-40 (Bethesda Research Laboratories, Inc.) was added, and the dishes were placed on ice for 10 min until the cells were disrupted. The extract was centrifuged at 1,200 rpm at 4°C for 10 min in an International PR-2 centrifuge to remove nuclei, and the supernatant was saved.

Polyacrylamide gel electrophoresis and autoradiography. Electrophoresis of radioactive polypeptides or cytoplasmic extracts was performed in polyacrylamide gel slabs containing SDS and using the discontinuous buffer system of Laemmli (8). The resolving gel was prepared to a final concentration of 12.5% acrylamide, 0.33% bisacrylamide, 0.03% *N,N,N',N'*-tetramethylethylenediamine, and 0.1% SDS in a buffer containing 0.375 M Tris-hydrochloride (pH 8.8). Polymerization was catalyzed with 0.03% ammonium persulfate. The stacking gel contained 3% acrylamide, 0.08% bisacrylamide, and 0.1% SDS in 0.125 M Tris-hydrochloride buffer (pH 6.8). The electrophoresis buffer consisted of 0.025 M Tris, 0.192 M glycine (pH 8.3), and 0.1% SDS. Samples were diluted 1:2 in sample buffer (0.06 M Tris-hydrochloride (pH 6.8), 2% SDS, 5% 2-mercaptoethanol, 0.001 bromophenol blue, and 10% glycerol) and boiled for 2 min before use. Electrophoresis was carried out for 3 h at a current of 25 mA. The slabs were fixed and stained for 12 h with a solution of 50% methanol–7% acetic acid containing 0.05% Coomassie brilliant blue, destained with a solution of 10% methanol–7% acetic acid, and soaked for 1 h in 10% methanol–2% glycerine. The gels were dried and then exposed to Kodak X-Omat R XR-1 film.

Electron microscopy. Negative staining of purified virus was performed by placing 50 μl of virus samples on 400-mesh carbon-coated grids. The droplets were allowed to stand for 10 min under a petri dish cover at 24°C. Grids were washed with 3 drops of 1% (wt/vol) sodium phosphotungstate (pH 7), stained in the same solution for 40 s, and dried by blotting with Whatman no. 1 filter paper. A JEM 100 CX electron microscope with 80 kV of accelerating voltage was used to obtain electron micrographs.

Hemagglutination. Fifty microliters of human type O erythrocytes was mixed with an equal volume of twofold dilutions of dialyzed gradient fractions in phosphate-buffered saline containing 0.5% bovine plasma (Armour Pharmaceutical Co.; fraction V). After incubation for 2 h at 4°C, the endpoint was read as the highest dilution at which hemagglutination occurred.

RESULTS

Physical characterization of TMEV. The buoyant density of a highly virulent (GDVII virus) and a less virulent (DA virus) TMEV were determined by centrifuging the radiolabeled viruses to equilibrium on Cs_2SO_4 gradients. Cs_2SO_4 was used since, in initial experiments, these viruses were found to be partially disrupted by CsCl, as appears to be the case for

some other picornaviruses (2). Symmetrical peaks of radioactivity at a density of 1.34 g/ml were obtained for ^{35}S-labeled DA virus and ^{3}H-labeled GDVII virus when they were centrifuged separately (Fig. 1A and B) and when run together (Fig. 1C). For both viruses, infectivity determined by hemagglutination coincided with the radioactive peaks (Fig. 1A and B).

A sample of the peak fractions from the gradients of each virus was negatively stained and examined by electron microscopy. Large numbers of virions which appeared to possess icosahedral symmetry were seen (Fig. 2A and B). Both DA and GDVII virus particles had an average diameter of 28 nm. These results demonstrate that GDVII and DA virions have the same density in Cs_2SO_4 and that they are identical in appearance by electron microscopy. The other five TMEV isolates (see above) were found to have the same buoyant density and electron microscopic appearance.

When larger amounts of virus lysates (more than four 75-cm^3 flasks) were purified by isopycnic banding in Cs_2SO_4, two narrowly separated bands were usually seen. The upper band contained predominantly empty capsids, whereas the lower band contained full particles by electron microscopy. In the experiments that follow, no attempt was made to separate empty from full virions.

Structural polypeptides of TMEV. The polypeptide composition of purified preparations of GDVII and DA viruses was examined by SDS-polyacrylamide gel electrophoresis. A 12.5% gel was found to be the optimum concentration of acrylamide for maximal resolution of all of the TMEV polypeptides, particularly VP1 and VP2, which are similar in size (see below). Purified wild-type poliovirus type 1 was also electrophoresed in the same slabs to provide a comparison with a picornavirus in which the polypeptides have been well characterized. GDVII virus had three major polypeptides, VP1, VP2 and VP3, in the range of 25,000 to 35,000 daltons and a smaller virion polypeptide, VP4, of less than 10,000 daltons (Fig. 3A). VP0 was also present, indicating that the purified virus preparations also contained empty capsids. The polypeptides of DA virus were similar to GDVII virus except that DA virus appeared to have only two major polypeptides, VP2 and VP3, as well as the smaller VP4. VP0 was present, but cannot be seen in this photograph of the autoradiogram (Fig. 3A). Thus, DA virus appeared to lack VP1 in this gel, and VP2 migrated slightly behind VP2 of GDVII virus. VP1 also appeared to be missing when other preparations of purified DA virus were electrophoresed on slab gels.

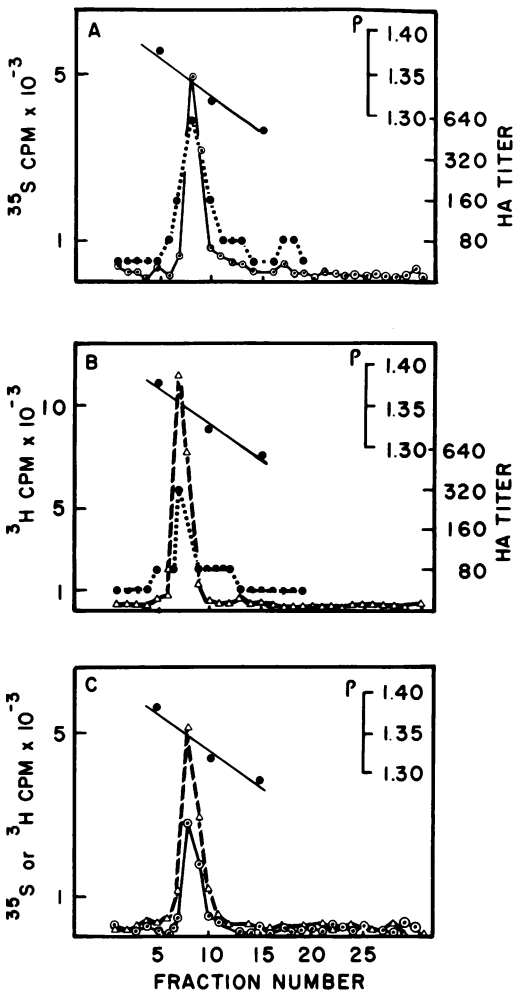

FIG. 1. *Sedimentation of two TMEV which differ in neurovirulence in Cs_2SO_4 gradients either separately (A, B) or together (C) to show the distribution of radioactivity and infectivity.* ⊙, *^{35}S-labeled DA virus;* △, *^{3}H-labeled GDVII virus;* ●, *infectivity determined by hemagglutination.*

It was possible that DA virion VP1 was in fact not missing, but that it comigrated with VP2. This was suggested by the denser VP2 band seen in this autoradiogram (Fig. 3A). However, in most of the gels, the increased density of VP2 was not apparent. To determine if DA virion VP1 comigrated with VP2, a transverse 0 to 8 M urea gradient was incorporated into the polyacrylamide gel. Figure 4A shows an autoradiogram from a 12.5% gel containing the urea gradient in which DA virion polypeptides were electrophoresed. Separation of VP2 into two bands is seen in the middle two-thirds of this gradient, demonstrating that DA virus does con-

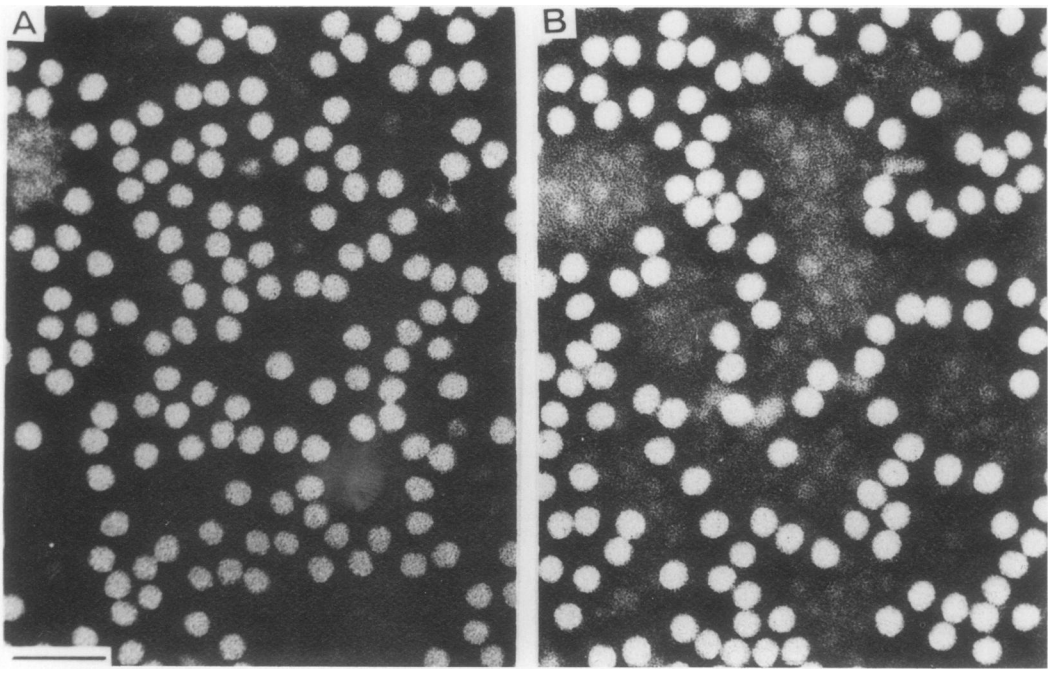

FIG. 2. *The appearance of negatively stained virions of (A) DA virus and (B) GDVII virus from the peaks of Cs_2SO_4 gradients similar to the ones shown in Fig. 1.*

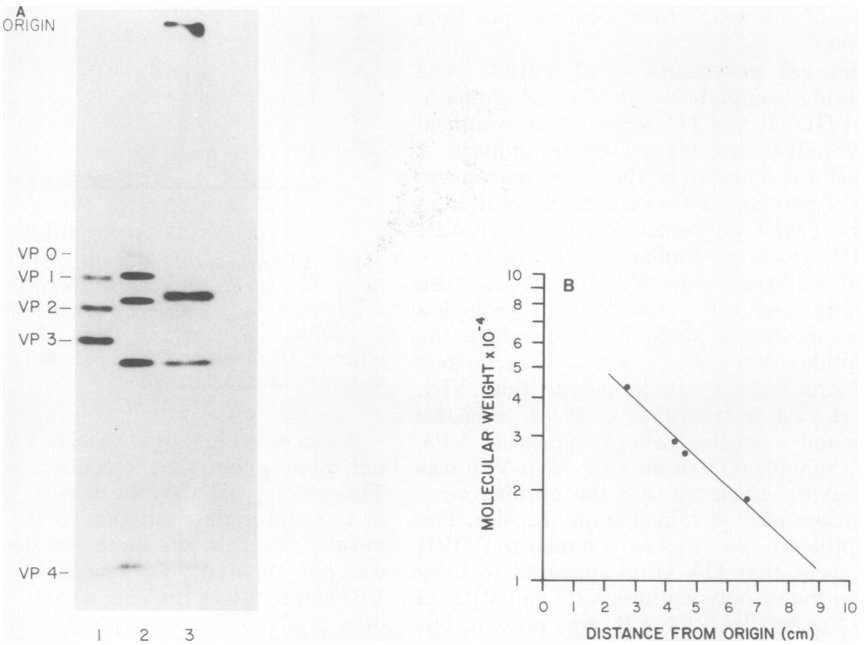

FIG. 3. *(A) Autoradiogram of a 12.5% SDS-polyacrylamide slab gel showing the separated capsid polypeptides of ^{35}S-labeled purified virions. Lanes: (1) wild-type poliovirus type 1; (2) GDVII virus; (3) DA virus. (B) In this and subsequent gels, internal standards were electrophoresed and their molecular weights were determined by staining as described in the text. The standards used were bovine serum albumin, molecular weight, 69,000 (not shown here); ovalbumin, molecular weight, 43,000; carbonic anhydrase, molecular weight, 30,000; alpha chymotrypsinogen, molecular weight, 25,000; myoglobin, molecular weight, 17,000; and ribonuclease A, molecular weight, 12,000 (not shown here).*

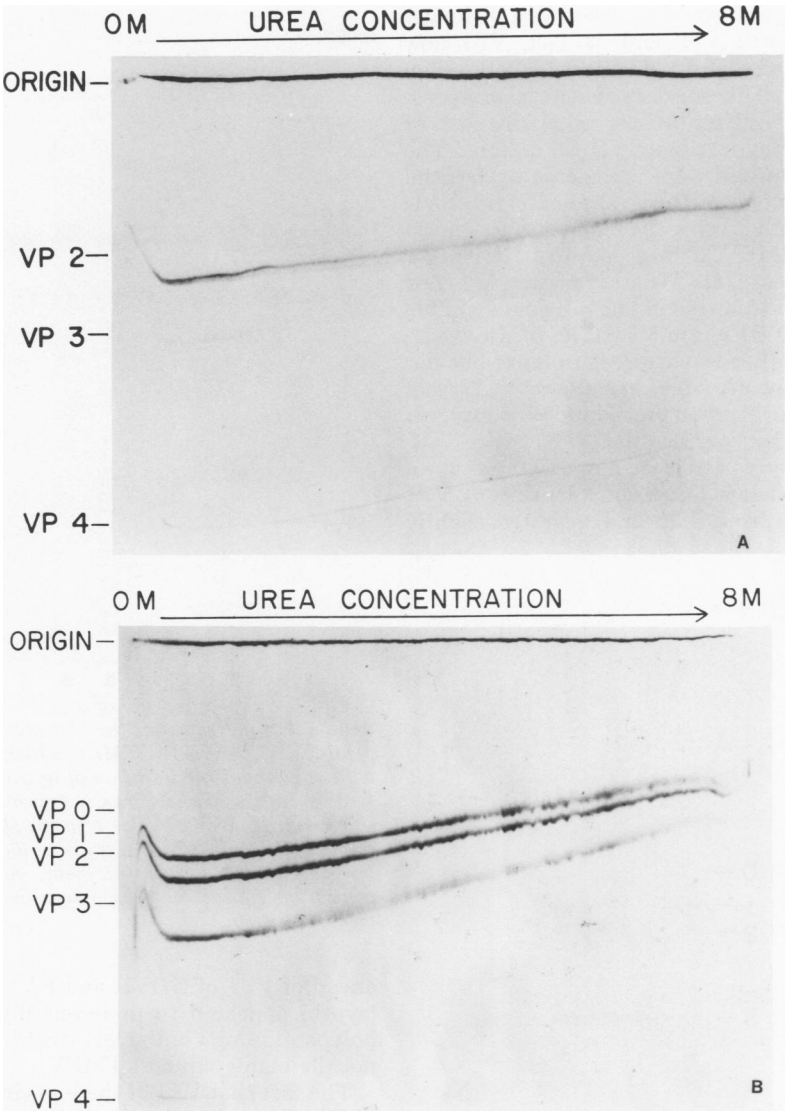

FIG. 4. *Autoradiogram of 12.5% SDS-polyacrylamide slab gels with a transverse 0 to 8 M urea gradient in which the capsid polypeptides of ^{35}S-labeled and purified (A) DA and (B) GDVII viruses were separated.*

tain both polypeptides VP1 and VP2. Therefore, VP1 had comigrated with VP2 in the regular SDS-polyacrylamide gel system. For comparison, GDVII virion polypeptides were also electrophoresed in a slab gel containing a 0 to 8 M gradient, and only the polypeptides identified in the SDS-polyacrylamide gel without urea were observed (Fig. 4B).

Another highly virulent TMEV, FA virus, was found to possess virion polypeptides identical in number and rate of migration to those demonstrated for GDVII virus, whereas four other less virulent TMEV (WW, Yale, TO4, and BeAn8386 viruses) were found to have a polypeptide profile similar to DA virus (see below). For all of the less virulent strains of TMEV, VP1 comigrated with VP2. In addition, VP2 migrated slightly behind VP2 of the two highly virulent TMEV, GDVII and FA viruses. Therefore, all of the less virulent viruses appeared to differ from their highly virulent counterparts in that VP1 tended to comigrate with VP2, and VP2 was slightly larger in molecular weight.

Trypsin sensitivity of the less virulent strains of TMEV. Subsequently, we found that when trypsin was omitted from the purification

procedure (see above), DA virion VP1 no longer comigrated with VP2 and, in fact, VP1 now migrated only slightly ahead of GDVII virion VP1 (Fig. 5). This suggests that trypsin hydrolyzed VP1, resulting in loss of a fragment or fragments of approximately 2,000 daltons. The other polypeptides were unaffected by trypsin. The fact that only VP1 was altered by trypsin is consistent with it being the major surface capsid protein of TMEV, as it seems to be for other picornaviruses (3, 11). Whether trypsin was used in virus purification did not have a demonstrable effect on GDVII virion VP1 (Fig. 5). However, VP1 of the other less virulent isolates, but not highly virulent FA virus, was altered by trypsin used in the purification procedure. When trypsin was not used in purification, WW, Yale, TO4, and BeAn8386 virion VP1 now migrated in an identical fashion to DA virion VP1 (Fig. 6). VP1 was separate from VP2 and migrated slightly ahead of VP1 of GDVII and FA viruses. Thus, trypsin appeared to preferentially alter virion polypeptide VP1 of the less virulent TMEV, but not the highly virulent TMEV.

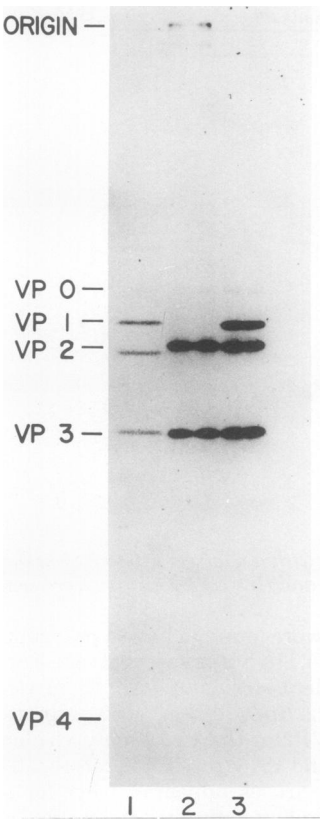

FIG. 5. *Autoradiogram of a 12.5% SDS-polyacrylamide slab gel showing the separated capsid polypeptides of ^{35}S-labeled TMEV which were purified with (+) or without (−) the addition of trypsin to the initial lysates. Lanes: (1) GDVII virus, − trypsin; (2) DA virus, + trypsin; (3) DA virus, − trypsin.*

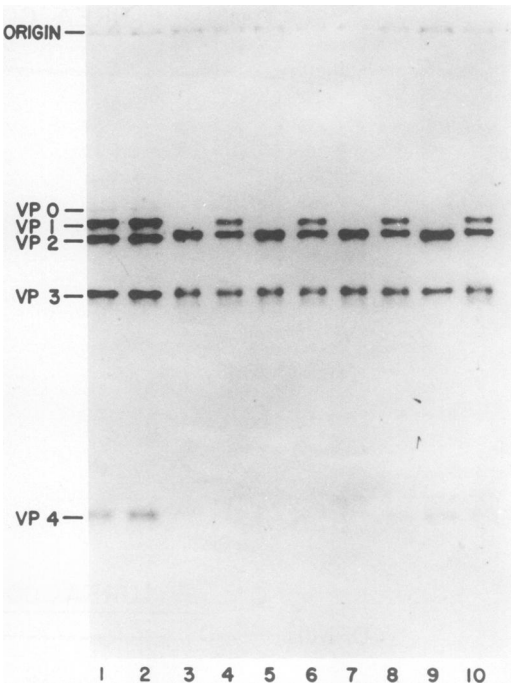

FIG. 6. *Autoradiogram of a 12.5% SDS-polyacrylamide slab gel showing the separated capsid polypeptides of ^{35}S-labeled TMEV which were purified with or without the addition of trypsin to the initial lysates. Lanes: (1) FA virus, + trypsin; (2) FA virus, − trypsin; (3) WW virus, + trypsin; (4) WW virus, − trypsin; (5) Yale virus, + trypsin; (6) Yale virus, − trypsin; (7) TO4 virus, + trypsin; (8) TO4 virus, − trypsin; (9) BeAn8386 virus, + trypsin; (10) BeAn8386 virus, − trypsin.*

The fact that VP1 of the less virulent isolates of TMEV migrated only slightly ahead of VP1 of the highly virulent isolates, and not in the position of VP2, was also demonstrated by radiolabeling these viruses and running the cytoplasmic extracts on a 12.5% polyacrylamide gel (data not shown).

Molecular weights of virion polypeptides. The molecular weights of the structural polypeptides were determined by their electrophoretic migration relative to internal standards of known molecular weight (Fig. 3B). The apparent molecular weights of the polypeptides of the highly virulent versus the less virulent TMEV in comparison with wild-type poliovirus type 1 are summarized in Table 1. The values given are for TMEV purified in the absence of trypsin. The molecular weights of the polypep-

TABLE 1. *Molecular weights of the structural polypeptides of Theiler's murine encephalomyelitis viruses and wild-type poliovirus type 1*

Polypeptide	Mol wt		
	GDVII virus[a]	DA virus[b]	Poliovirus type 1
VP0	35,500	36,000	—[c]
VP1	34,000	33,500	33,500
VP2	31,000	32,000	30,000
VP3	25,000	25,000	27,000
VP4	6,000	6,000	5,000

[a] Representative of the highly virulent TMEV.
[b] Representative of the less or relatively avirulent TMEV.
[c] —, Not observed.

tides of poliovirus type 1 are in good agreement with published results (15). The sum of the molecular weights of the TMEV cleavage products VP2 and VP4 are within 5% of the molecular weight of their precursor, VP0.

DISCUSSION

Two earlier reports in the literature contained information on polioviruses suggesting that the virion polypeptides might differ between wild and attenuated strains (1, 14). Recently, Milstien et al. (13) were able to correlate a change in the polypeptide gel pattern with a decrease in neurovirulence of poliovirus type 1. They found that VP1 comigrated with VP2 for attenuated polioviruses derived from the Mahoney strain, but not for the virulent parent Mahoney strain. In contrast, Roland Rueckert and co-workers (personal communication) have been unable to find a correlation between the polypeptide pattern and virulence amoung 14 virulent and attenuated human poliovirus type 1, 2, and 3 stocks that they examined. Therefore, it was of particular interest that there was a slight but consistent difference in several structural polypeptides between the two highly virulent and five less virulent TMEV by SDS-polyacrylamide gel electrophoresis. VP1 was slightly larger (34,000 versus 33,500 daltons) and VP2 was slightly smaller (31,000 versus 32,000 daltons) for the highly virulent strains compared to the same polypeptide species in the less virulent viruses.

The significance of the differences in the capsid polypeptides between the highly virulent and less virulent TMEV in terms of the properties of virulence or viral persistence is as yet unknown. We have found that the precursor polypeptides 1a and 3a of the highly virulent GDVII virus migrated slightly behind 1a and 3a of the less virulent TMEV DA virus (unpublished data). Therefore, the differences in the virion structural polypeptides between the different TMEV may reflect differences in RNA composition. However, it is also possible that the observed differences in the structural polypeptides are due to modifications in the post-translational cleavage of the polyprotein (1). These possibilities are the subjects of current study.

The finding that trypsin which was used initially in our virus purification procedure resulted in preferential cleavage of a 2,000-molecular-weight fragment of VP1 of the less virulent isolates was totally unexpected. The use of trypsin to treat virus lysates was based on the use of neutral proteases to obtain highly purified Mengo (16) and EMC viruses (7). VP1 is probably the major capsid polypeptide of the TMEV as it appears to be for most other picornaviruses (3, 11) and, therefore, would be more likely to be affected by trypsin than the other polypeptides. The selective effect of trypsin on the less virulent TMEV isolates probably reflects either presence or exposure of susceptible peptide bonds in VP1 in these viruses, but not in the virulent TMEV. This result is difficult to understand in light of our recent electron microscopic observations on the replication of GDVII and DA viruses in BHK21 cells (A. Friedmann and H. L. Lipton, Virology, in press). We found that the intracellular development of DA virus was closely associated with unique membranous structures consisting of two double-membrane units enclosing a single layer of virions. Upon cell lysis, DA virions were not freely released but were found to be still trapped within these membranes in the extracellular space. In contrast, GDVII virus replication did not result in the formation of membranes; instead, well-developed crystalline arrays of virions were observed in the cytoplasm of infected cells, and virus was readily released upon cell lysis. Therefore, one might have anticipated that GDVII virus would be more susceptible to trypsin since DA virus would appear to be inaccessible and even protected by the membranous structures. Other possible explanations for the preferential effect of trypsin on the less virulent TMEV include trypsin activating a cellular protease, perhaps associated with the membranes, which in turn cleaves VP1 or even a greater susceptibility of membrane-associated virions to trypsin. In any event, these results indicate another difference between the highly virulent and less virulent TMEV and draw attention to the potential danger of using agents, such as neutral proteases, in the purification of picornaviruses.

ACKNOWLEDGMENTS

This study was supported by Public Health Service grant AI 141139. H.L.L. is the recipient of Public Health Service Career Development Award 1KO4-AI 00228. A.F. was the J.D.

and Iva Leiper visiting fellow at Northwestern University during the conduction of these experiments.

We thank Kimiko K. Matsutani, Barbara Klement, and Kathy Ley for excellent technical assistance and Kathleen Rundell for her helpful advice and discussions. We thank Roland R. Rueckert for suggesting incorporating a transverse urea gradient in the SDS-polyacrylamide gel electrophoresis system to separate the comigration of TMEV polypeptides.

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