Structural and Physiological Properties of Mengovirus: Avirulent, Hemagglutination-defective Mutants Express Altered Alpha (1 D) Proteins and Are Adsorption-defective

By

KEVIN ANDERSON* and CLIFFORD W. BOND

Department of Microbiology, Montana State University, Bozeman, Mont., U.S.A.

With 6 Figures

Summary

Structural and physiological properties of two mutants of mengovirus, 205 and 280, were compared to those of wild-type virus to understand the molecular basis of changes exhibited in their biological function. Two dimensional gel electrophoresis of wild-type and mutant structural proteins revealed alterations in the isoelectric character of the alpha (1 D) protein of both mutant 205 and 280. These data suggest that alterations in the alpha (1 D) protein may be responsible for the phenotypic changes by the mutants. A delay in detectable virus-specified protein synthesis was exhibited in mutant-infected cells in comparison to wild-type. The amount of RNA synthesized in mutant- and revertant-infected cells was less than that synthesized in wild-type infected cells. Changes in virus-specified macromolecular synthesis in mutant and revertant-infected cells reflected a decrease in the ability of the viruses to attach to cells.

Introduction

Biological properties of two mengovirus mutants, 205 and 280, were compared with those of wild-type virus (2). These mutants were defective in their ability to agglutinate erythrocytes, produced smaller plaques in cell culture, were avirulent in mice, but were not temperature-sensitive. These data suggested that changes in the structure of the mutant viruses may be responsible for the expression of altered phenotypic traits.

---

* Present address: Department of Microbiology, School of Medicine, University of Alabama at Birmingham, University Station, Birmingham, AL 35294, U.S.A.

In this communication, the structural proteins of the wild-type and mutant viruses were compared by SDS-PAGE, peptide mapping, and two-dimensional gel electrophoresis to determine which of the mutant structural proteins differed from that of wild-type and the extent of homology among analogous proteins. The structural proteins of two revertants of mutant 205 were also examined by two-dimensional gel electrophoresis to relate changes in biological function to structural differences in the analogous proteins of the mutants and wild-type mengovirus. In addition, the progression of events associated with wild-type, mutant, and revertant virus infection were examined to identify factors responsible for physiological changes associated with mutant and revertant virus-specified macromolecular synthesis.

Materials and Methods

Viruses and Cells

The growth and assay of wild-type, mutant and revertant viruses using BHK-21 cells have been described previously (2). Mutant and revertant virus stocks used in the experiments described below were from the second passage of virus isolated by two successive rounds of plaque purification.

Radiolabeling of Virus-specified Proteins

Purified virions were radiolabeled in vivo with [^{14}C(U)]-L-amino acid mixture (6 µCi/ml) [New England Nuclear (NEN), NEC-445] were prepared and analyzed by SDS-polyacrylamide gel electrophoresis as described previously (2).

Surface tyrosine residues of purified virions were labeled with [^{125}I]-sodium iodide [(NEN), NEZ-033 H] using a modification of the method described by Millar and Smith (15). Pellets containing purified virions were resuspended in TN buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl] and quantitated by absorbance at 260 nm as described (19). Suspensions of purified virus in TN buffer containing 0.3 absorbance units (260 nm), 200 µCi of ^{125}I, and 100 µg of Iodogen (Pierce) were mixed in a total volume of 0.3 ml and agitated for 10 minutes. An equal volume of 0.4 µM NaI, 5.0 µM 2-mercaptoethanol was added to the mixture and layered onto Sephadex G-25 columns (60 × 7 mm) equilibrated with TN buffer. The excluded volumes were collected and centrifuged in an SW 41 rotor at 37,000 rpm for 90 minutes at 6° C. Pellets were prepared for preparative SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Virus-specified intracellular proteins were labeled with L-[^{35}S]-methionine, immunoprecipitated and analyzed by SDS-PAGE. Cells were mock-infected or infected with virus in suspension (1 × 10^7 cells/ml) at an MOI of 3, adsorbed, plated into plastic 35 mm dishes (2.5 × 10^6 cells/dish), and incubated at 33° C. Cells were pulse-labeled for 15 minutes with 100 µCi/ml ^{35}S-methionine in 0.3 ml methionine-free medium. The cells were washed twice with serum free medium and lysed with 0.1 ml B 10 [10 mM Tris-HCl (pH 7.4), 5 mM MgCl$_2$, 0.5 percent NP 40, 0.1 percent SDS, 1 percent Aprotinin, 50 µg/ml ribonuclease A, 50 µg/ml deoxyribonuclease] for 5 minutes on ice. Cellular lysates were centrifuged at 6500 × g for 1 minute at 4° C and the supernatant fluid was collected and stored at −20° C.

Immunoprecipitation of Virus-specified Protein

Cytoplasmic lysates were immunoprecipitated by a modification of the procedure of Bond et al. (5). Cytoplasmic lysates representing 1.25 × 10^6 cells were diluted 10-fold in

B 11 [50 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.02 percent sodium azide, 0.05 percent NP 40, 1 percent Aprotinin, 0.1 percent bovine serum albumin]. Twelve μl of mouse anti-mengovirus hyperimmune ascitic fluid (2) was added to the diluted lysates (0.5 ml volume) and incubated at 0° C for 60 minutes. Immune complexes were precipitated with 100 μl of 10 percent fixed *Staphylococcus aureus* (Cowan strain) (13) by incubation at 0° C for 60 minutes and pelleted by centrifugation for 1 minute at $6500 \times g$. Pellets were washed four times with B 11 buffer at 0° C and resuspended in 40 μl 20 mM dithiothreitol (DTT), 1 percent SDS. After 15 minutes of incubation at room temperature, the immunoprecipitated proteins were eluted and reduced by heating at 60° C for 5 minutes. Bacteria were removed by centrifugation and the supernatant fluids were prepared for SDS-PAGE.

Radiolabeling of Virus-specified RNA

Virus-specified intracellular RNA was labeled with [5, 6-³H]-uridine [(NEN), NET-367]. Cells were infected in suspension with virus (1×10^7 cells/ml) at an MOI of 3, plated in plastic 35 mm dishes (2.5×10^6 cells/dish) following adsorption at 33° C for 30 minutes, and incubated at 33° C. Actinomycin D (5 μg/ml final concentration) was added to each dish at various time points and the cells were incubated at 33° C for 20 minutes. The medium was aspirated, replaced with 0.3 ml DME 2 containing 10 μCi/ml ³H-uridine, and the monolayers were incubated at 33° C for 60 minutes. At the end of the labeling period, the cells were lysed at 4° C for 5 minutes with NET buffer [10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM EDTA] supplemented with 1 percent NP 40. The lysate was centrifuged at $6500 \times g$ for 1 minute. Duplicate samples containing 25 μl of the supernatant fluid were spotted onto Whatman GFC glass fiber filters and precipitated in ice cold 10 percent trichloroacetic acid (TCA). Filters were placed in vials with scintillation fluid [5 g/L 2, 5-diphenyloxazole (PPO) in xylene], and counted in a Packard LSC 460 CD liquid scintillation counter using the pre-set tritium channel (4).

To prepare virus particles containing radiolabeled RNA, cells were infected with virus at an MOI of 3 and were labeled at 4.5 HPI with 20 μCi/ml [5, 6-³H]-uridine [(NEN), NET-367] or 500 μCi/ml ³²P-orthophosphate [(NEN), NEX-054] in medium containing 12.5 mg/L monosodium phosphate. Virus-infected cells were allowed to incubate at 33° C until lysis and virions were purified as described above.

Two Dimensional Gel Electrophoresis

³⁵S-methionine labeled virions were suspended in sample buffer containing 9.95 M urea (Bio-Rad), 4 percent NP-40 (Particle Data Laboratories), 2 percent Bio-Lyte 3/10 (Bio-Rad), and 100 mM DTT as described (8) and incubated at 25° C for 30 minutes. Isoelectric focusing (IEF) was performed as described (17). Following equilibration, the tube gels were placed onto 10 percent polyacrylamide slab gels and subjected to SDS-PAGE. The pH gradients for IEF and NEPHGE were generated from 1 cm serial gel sections equilibrated in 50 mM NaCl and the pH values were lowered by 0.5 pH units to correct for measurement in the presence of urea (22). The isoelectric point (pI) value for each protein species was estimated from these gradients.

Preparation of Labeled Proteins for Peptide Analysis

Bands corresponding to the structural proteins of mengovirus were identified by exposure of dried preparative SDS-PAGE gels to x-ray film, excised, rehydrated in 10 mM ammonium bicarbonate, 0.1 percent SDS, and electroeluted as described (7, 21). Protein samples were lyophilized and SDS was removed as described (10). The proteins were dried, oxidized for 90 minutes at 4° C with 0.2 ml of fresh performic acid and lyophilized three times in distilled water. Proteins were analyzed for purity by SDS-PAGE prior to digestion. Purified, oxidized proteins were resuspended in 0.2 ml of 1 percent ammonium

bicarbonate (pH 7.8) for digestion with N-alpha-p-tosyl-L-lysine chloromethyl ketone-treated chymotrypsin (TLCK-chymotrypsin) at 37° C as described (7). Enzyme treated samples were frozen and lyophilized before peptide analysis by thin layer chromatography (TLC).

Peptide Analysis

Two-dimensional peptide mapping was performed as previously described (11). Lyophilized samples were dissolved in electrophoresis buffer [butanol-pyridine-acetic acid-water (2 : 1 : 1 : 36)] and 2–5 µl was spotted onto cellulose TLC plates (E. Merck). Separation of peptides in the first dimension was perfomed by electrophoresis for 45 minutes at 1000 V (40 to 50 mA). The TLC plates were air-dried and the peptides were separated in the second dimension by ascending chromatography in N-butanol-pyridine-acetic acid-water (393 : 304 : 61 : 243) until the solvent front reached 2 cm from the top. Dried plates were sprayed with En³Hance (NEN) and exposed to preflashed Kodak XAR-2 x-ray film at −70° C.

Adsorption of Viruses to BHK-21 Cells

The number and percentage of wild-type, mutant, and revertant virus particles adsorbing to BHK-21 cells were determined as follows. Cells were infected with purified ^{32}P-labeled virus at a multiplicity of 2000 particles/cell. The ^{32}P-labeled virus suspensions were allowed to adsorb to BHK-21 cell monolayers in plastic dishes (60 mm diameter) for 60 minutes at 33° C. The cells were lysed with 0.1 ml B 10 for 5 minutes on ice. Duplicate 100 µl samples were spotted onto Whatman glass fiber filters and precipitated in ice-cold 10 percent TCA. The filters were placed in vials with scintillation fluid and counted in a Packard 460 CD liquid scintillation counter using the pre-set ^{32}P channel (4). The fraction of cell-associated virus particles was calculated by dividing the number of cell associated counts per minute (CPM) by the CPM of the input virus. The average number of virus particles adsorbed per cell was calculated by multiplying the fraction of cell-associated virus by the multiplicity of infection (2000 particles/cell).

Results

Comparative Analysis of Mengovirus Structural Proteins

The structural proteins of purified ^{14}C-labeled wild-type and mutant mengoviruses were analyzed by SDS-PAGE on 8, 10 and 15 percent polyacrylamide slab gels. Analyses of the structural proteins resolved on 15 percent gels are shown (Fig. 1). Five structural proteins were resolved for each of the viruses: epsilon (1 AB), 40 kd; alpha (1 D), 37 kd; beta (1 B), 33 kd; gamma (1 C), 25 kd; and delta (1 A), 7.8 kd. No changes in the migration of the mutant structural proteins were observed in comparison to those of the wild-type virus. In addition, the migration of the structural proteins of several HA+ revertants of mutant 205 was also similar to that of wild-type (data not shown). Although some variation in the amount of delta (1 A) protein of the viruses was observed (Fig. 1), this was not a result found consistently throughout our analyses.

^{35}S-methionine-labeled structural proteins of wild-type and mutant viruses 205 and 280 were analyzed following digestion with TPCK-trypsin by reverse-phase HPLC to further examine the structure of the proteins by

separating peptides in a gradient on the basis of charge. However, the spectra of methionine-containing peptides of the delta (1 A), beta (1 B), gamma (1 C), and alpha (1 D) proteins from the different viruses were identical (data not shown). Although not all of the peptides generated by TPCK-trypsin digestion were likely to contain methionine, the results suggested that the peptide compositions of the four structural proteins of the wild-type and mutant viruses were remarkably similar. HPLC analyses of the tryptic peptide compositions of the four structural proteins of the wild-type and mutant viruses uniformly labeled with ^{14}C-amino acid mixture by labeled structural proteins was inconclusive because the resulting chromatograms contained many unresolvable, overlapping peaks (data not shown).

To determine whether the arrangement of the structural proteins on the surface of mutants 205 and 280 was similar to that of wild-type, purified virions were labeled with ^{125}I and analyzed by SDS-PAGE (Fig. 2). Most of the tyrosine residues on the surface of the wild-type and mutant viruses were on the alpha (1 D) protein as demonstrated by the extensive labeling of this protein. The beta (1 B) protein was labeled to a much lesser extent. There was no detectable difference in the pattern of surface protein labeling among the three viruses suggesting that the arrangement of the structural proteins on the surfaces of the virions of the wild-type and mutant viruses was similar.

To examine the structure of the surface proteins of the three viruses in greater detail, two-dimensional TLCK-chymotryptic peptide chromato-
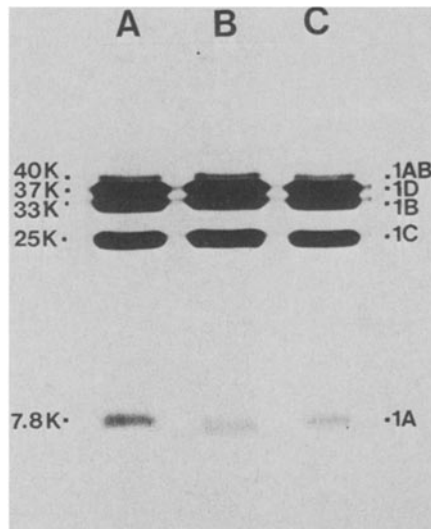

Fig. 1. SDS-PAGE of ^{14}C-labeled wild-type and mutant mengovirus structural proteins. The viruses were purified, reduced, and electrophoresed on a 15 percent SDS-polyacrylamide gel. The structural proteins of mutant 205 *(A)*, mutant 280 *(B)*, wild-type virus *(C)* are shown
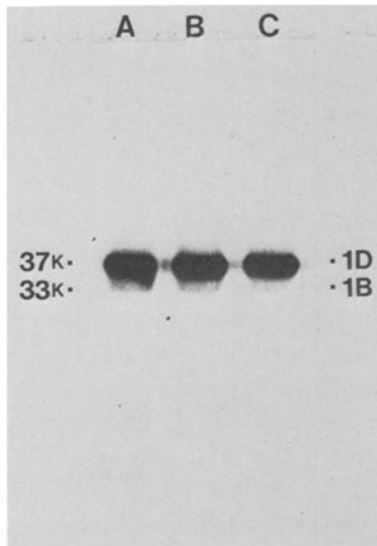
Fig. 2. SDS-PAGE of ^{125}I-labeled proteins of intact wild-type and mutant mengovirus particles. Virus particles were purified, labeled, reduced, and electrophoresed on a 12 percent SDS-polyacrylamide slab gel as described in the text. Surface-labeled proteins of the wild-type virus *(A)*, mutant 205 *(B)*, and mutant 280 *(C)* are shown

graphy was used to investigate the extent of homology among the alpha (1 D) structural proteins of the viruses (Fig. 3). The chromatograms of individual and mixed samples of surface-labeled peptides were identical. Although not all of the surface peptides generated by TLCK-chymotrypsin are likely to be labeled, numerous peptides were labeled. These data suggest that the arrangement of the structural proteins on the surface of the three viruses was remarkably similar.

The structural proteins of the wild-type, mutant, and revertant strains were analyzed by pH gradient gel electrophoresis to determine whether any changes were apparent in migration of the mutant and revertant structural proteins relative to those of the wild-type virus. The proteins were analyzed by IEF and NEPHGE two-dimensional gel electrophoresis in order to resolve both acidic and basic proteins.

The two-dimensional electrophoretic migration of the structural proteins of wild-type mengovirus is shown in Fig. 4, panel A. The epsilon (1 AB) protein had a pI of 5.32. The alpha (1 D) protein was separated into four major protein species, each with a distinct pI (5.35, 5.52, 5.61, and 5.72). The beta (1 B) protein was separated into two major protein species with pI values of 5.55 and 5.62 and a heterogenous species ranging from 6.3 to 6.6 The gamma (1 C) protein was not well resolved by this technique and appears to be slightly acidic or neutral in charge. The delta (1 A) protein does not appear in any of the two-dimensional IEF gels.

The two-dimensional electrophoretic migration of the structural proteins of mutant 205 is shown in Fig. 4, panel B. The electrophoretic migration of the proteins was identical to that of the wild-type strain with one exception. Only three alpha (1 D) protein species were resolved. One of the protein species resolved in separation of the wild-type proteins, pI = 5.72, was absent. However, this protein species was resolved in the wild-type/205 mixed sample separation (Fig. 4, panel D). These data suggest that the absence of this particular protein species represents a phenotypic change or mutation in the alpha (1 D) protein of mutant 205 relative to the wild-type virus. In addition, the two-dimensional electrophoretic patterns of the structural proteins of the revertent viruses, 205-A 7 and 205-D 2, were identical to those of mutant 205 (data not shown).
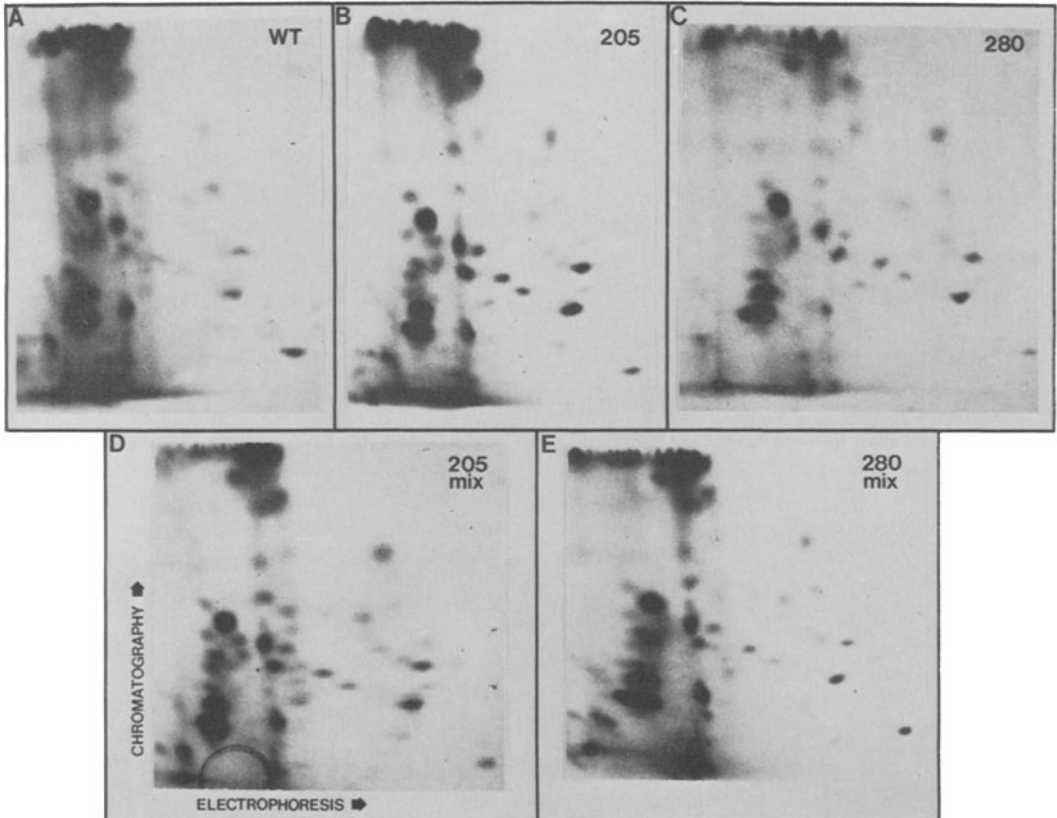

Fig. 3. Two-dimensional analysis of ^{125}I-labeled chymotryptic peptides of the alpha (1 D) proteins of wild-type and mutant mengoviruses. Peptides were prepared and analyzed as described in the text. The surface-labeled peptides of the wild-type virus *(A)*, mutant 205 *(B)*, mutant 280 *(C)* and mixed samples containing peptides of mutant 205 and wild-type virus *(D)*, and peptides of mutant 280 and wild-type virus *(E)* are shown
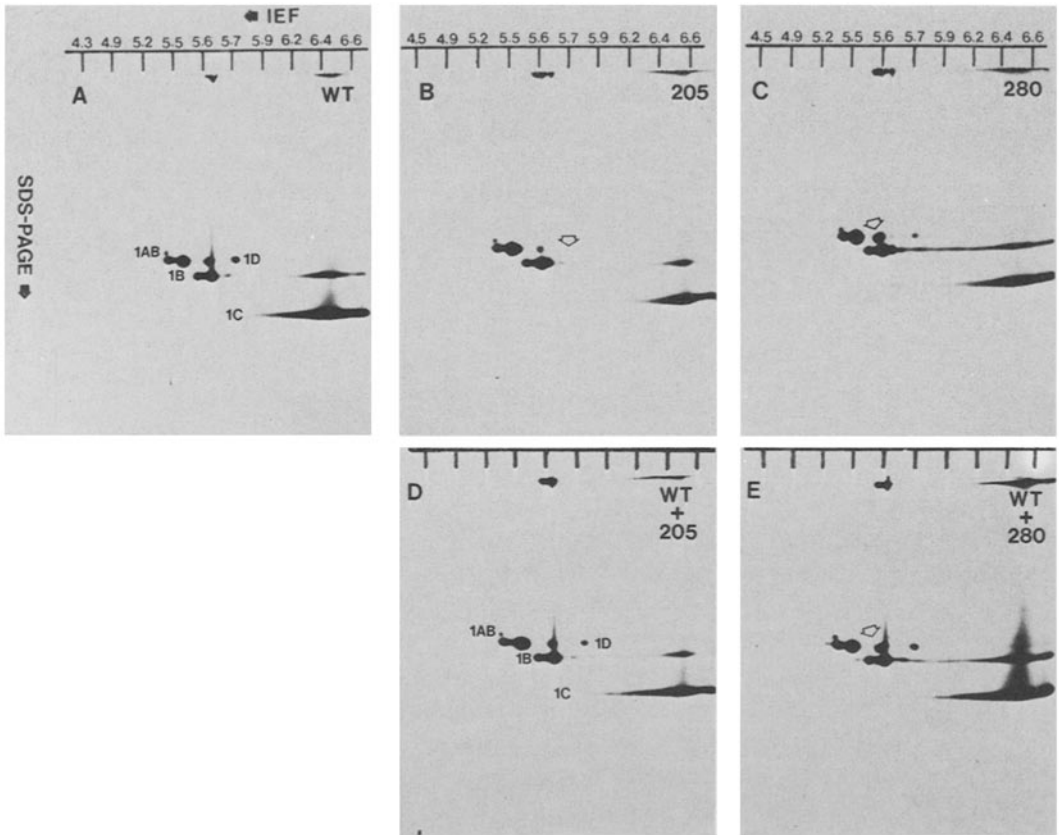
Fig. 4. Two-dimensional IEF gel electrophoresis of ^{35}S-methionine-labeled structural proteins of wild-type, mutant, and revertant mengoviruses. Samples containing purified, labeled virions were prepared and analyzed as described in the text. The pH gradients were generated as described in the text. Two dimensional analyses of the structural proteins of wild-type virus (A), mutant 205 (B), mutant 280 (C) and mixed samples of the structural proteins of wild-type virus and mutant 205 (D) and wild-type and mutant 280 (E) are shown. The pH gradients for D and E were the same as those for B and C, respectively. Arrows point to distinct differences in the protein profiles of the viruses

The two-dimensional electrophoretic migration of the structural proteins of mutant 280 is shown in Fig. 4, panel C. The electrophoretic migration of the proteins was identical to that of the wild-type strain with one exception. The pI of one of the four alpha (1 D) protein species (pI = 5.58) was slightly more acidic than the analogous wild-type isoelectric species (pI = 5.61). This species appeared broader and slightly lower in molecular weight than the corresponding wild-type protein species. The wild-type/280 mixed sample separation (Fig. 4, panel E) shows a broad protein species in the region of the gel corresponding to the apparent change in the pIs of the wild-type and 280 alpha (1 D) protein species. The altered migration of the mutant 280

isoelectric species was found consistently in two dimensional profiles of independently derived virion protein samples. These data suggest that the change in form and migration of this protein species represents a phenotypic change or mutation in the alpha (1 D) protein of mutant 280 relative to the wild-type virus.

Two-dimensional NEPHGE analyses confirmed the results obtained by IEF analysis of the wild-type, mutant, and revertant structural proteins. The delta (1 A) proteins of the wild-type and mutant viruses were resolved by this technique and migrated similarly as a single acidic protein species at pH 2.5 (data not shown).

Synthesis of Virus-specified Intracellular Protein and RNA

The specific activities (CPM/particle) of the wild-type and mutants differed when BHK-21 cells were infected and labeled with ^{35}S-methionine or ^{14}C-amino acids under similar conditions (ANDERSON and BOND, unpublished data). Therefore, to determine whether differences in the kinetics of virus-specified macromolecular synthesis exist among the viruses, virus-specified intracellular protein and RNA synthesis were examined. Virus-infected cells were pulse-labeled at 1 hour intervals from 3 to 11 HPI with ^{35}S-methionine for 15 minutes. Cytoplasmic lysates were immunoprecipitated and analyzed by SDS-PAGE (Fig. 5). Eight virus-specified intracellular proteins were resolved in wild-type and mutant strain-infected cells: A (1—2 A), B (1), C (3), D (3 CD), 1 ABC, D 2 (1 CD), alpha (1 D), and gamma (1 C). The number and molecular weights of the proteins specified by the wild-type and mutant strains were identical. However, virus-specified protein synthesis was detected initially at 5 HPI for the wild-type strain and at 6 HPI for mutant strains 205 and 280.

To determine whether changes in the kinetics of RNA synthesis could explain the delay in detectable protein synthesis observed in mutant-infected cells, virus-infected cells were treated with actinomycin D, pulse-labeled with ^{3}H-uridine, lysed, and counted. The results are shown in Fig. 6. The peak of RNA synthesis for each virus was from 10 to 11 HPI. However, the amount of RNA synthesized by the wild-type virus was 10-fold greater than that of the mutants and 2-fold greater than that of the revertants. Since the magnitude of virus-specified RNA synthesized in the mutant virus-infected cells was 10-fold less than that of wild-type virus, the beginning of virus-specified protein synthesis, as detected by immunoprecipitation (Fig. 5), would appear to be delayed due to the fact that less RNA would be available for translation.

Adsorption of Virus to BHK-21 Cells

Since the magnitude of virus-specified RNA synthesis in mutant virus-infected cells was 10-fold less than that of the wild-type virus, alterations in
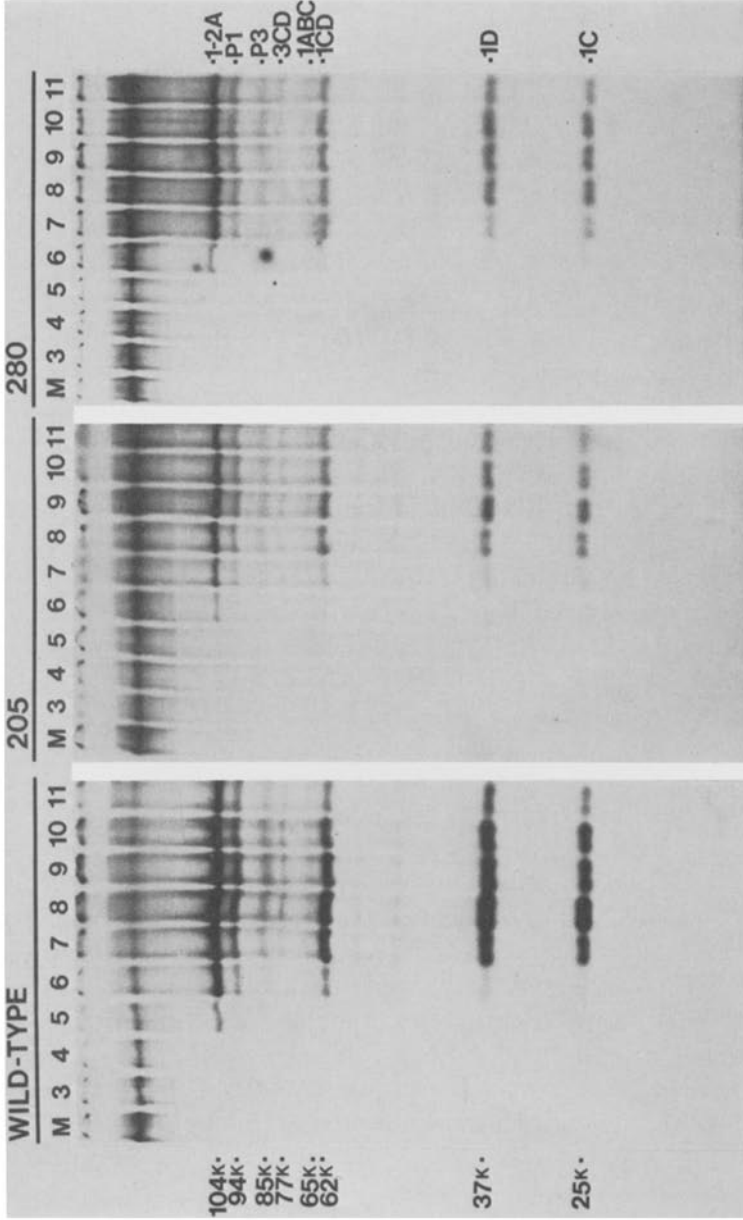
Fig. 5. Time course for mengovirus-specified protein synthesis in wild-type-, mutant 205-, and mutant 280-infected cells. Virus-infected cells were labeled with ³⁵S-methionine for 15 minutes from 3 to 11 HPI as described in the text. Cell lysates were immunoprecipitated with mengovirus-specific hyperimmune ascitic fluid and analyzed by SDS-PAGE on 10 percent slab gels. *M* shows a mock-infected immunoprecipitated sample labeled at 10 HPI. Proteins are labeled according to the nomenclature of RUECKERT and WIMMER (20)
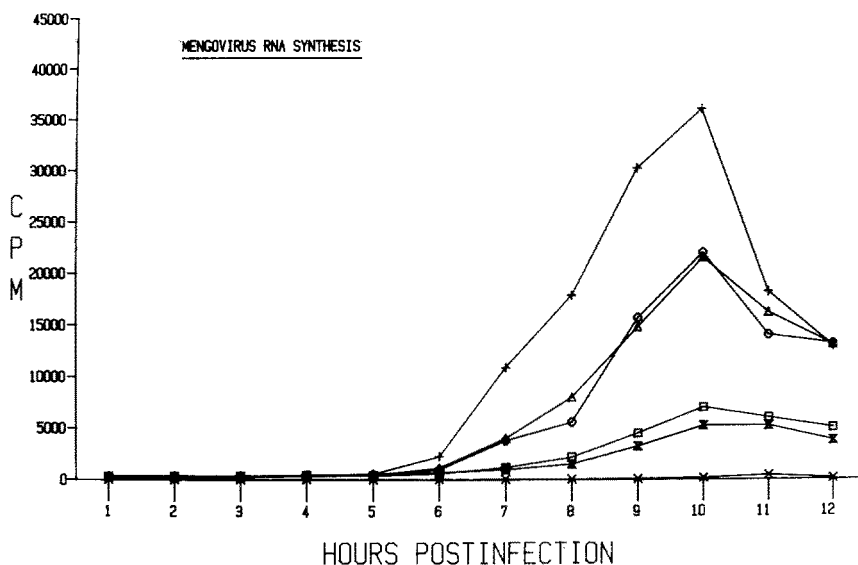
MENGOVIRUS RNA SYNTHESIS

Fig. 6. Time course for mengovirus-specified RNA synthesis in wild-type-, mutant-, and revertant-infected cells. Actinomycin D was added to virus-infected cells prior to labeling with ³H-uridine and the cells were labeled from 1 to 12 HPI as described in the text. Data were generated from scintillation counting of the acid-insoluble fraction of cell lysates. The synthesis of virus-specified RNA in mock- (×), wild-type- (+), mutant 205- (*), mutant 280- (□), revertant 205-A 7- (○), and revertant 205-D 2-infected cells (△) is shown

uncoating or adsorption of the mutant viruses to cells may account for this difference. However, surface peptide analysis suggested that the arrangement of the structural proteins of the wild-type and mutant virions was remarkably similar. Therefore, it is possible that uncoating of the virions would occur at a similar rate in infected cells due to their similarity in structure.

The average number of wild-type, mutant, and revertant virus particles adsorbing to BHK-21 cells was examined following incubation at 33° C for 60 minutes. Under these conditions, the amount of virus adsorption to cells would approximate saturation. The results are shown in Table 1. The average number of virus particles adsorbing per cell clearly differed among the wild-type, mutant, and revertant viruses. Therefore, it is apparent that the mechanisms of adsorption of the mutant and revertant viruses to cells is modified from that of wild-type. The difference in the cellular binding affinities among the wild-type, mutant, and revertant viruses would be an important factor contributing to the differences in the magnitude of viral RNA and protein synthesis observed for these viruses. These data suggest that fewer cells would be infected by the mutant and revertant viruses and therefore, fewer virus-specified macromolecules would be synthesized.

Table 1. *Adsorption of wild-type, mutant, and revertant viruses to BHK-21 cells*[a]

Virus	Particles/cell[b]	% of wild-type[c]
Wild-type	738	100
205	170	23.0
280	250	33.9
205-A 7	408	55.3
205-D 2	436	59.1

[a] Cells were infected at an MOI of 2000 particles/cell and incubated at 33° C for 60 minutes

[b] The average number of particles adsorbed/cell

[c] The percentage of adsorption of the mutants and revertants in comparison to wild-type mengovirus

Discussion

Biological and structural properties of two mengovirus mutants have been compared to those of the parental wild-type strain. These mutants, 205 and 280, exhibited alterations in agglutination of erythrocytes, virulence in mice, and plaque morphology (2). In addition, biological characterization of several HA+ revertants of mutant 205 isolated from the brains of mice infected intracranically indicated that agglutination and virulence may be linked traits and that the size of plaques produced by a particular mengovirus isolate may reflect its binding affinity to cells and virulence in mice.

Analysis of ^{35}S-methionine-labeled structural proteins of wild-type and mutant viruses by SDS-PAGE and HPLC revealed that extensive homology exists among these viruses. To further examine these viruses for structural differences, surface labeling of intact wild-type and mutant virus particles was done to compare the arrangement of the structural proteins which form the viral capsids. Previous studies have shown that surface iodination of intact poliovirus and mengovirus labels primarily the 1 D (VP 1 or alpha) protein of the respective virus capsids (3, 12) and the 1 B (VP 2 or beta) protein is also labeled, but to a much lesser extent than the 1 D protein. We obtained similar data for the surface iodination of intact wild-type and mutant virus particles. In addition, no apparent differences were evident from the analysis of chymotryptic peptides of ^{125}I-labeled wild-type and mutant alpha (1 D) proteins. These data suggest that arrangement of structural proteins on the surface of mutant capsids did not differ from that of wild-type. Therefore, the mutations may result in the expression of altered determinants that reflect differences in biological function and may not result in the masking of otherwise functional determinants due to altered arrangement of the capsid proteins.

We compared the isoelectric character of the viral capsid proteins by two-dimensional gel electrophoresis. Multiple species of the alpha (1 D) and

beta (1 B) proteins were reproducibly detected by this technique in both the presence (Fig. 4) and absence (data not shown) of SDS prior to isoelectric focusing. Previous studies have demonstrated multiple isoelectric species for the major capsid proteins of poliovirus [VP 1 (1 D), VP 2 (1 B), and VP 3 (1 C)] (9, 23) and EMC virus [alpha (1 D) and beta (1 B)] (6). VRIJSEN et al. (23) also excluded the possibility that the multiple species are derived from differential binding of SDS or the accumulation of mutants in the original virus stock. These authors suggested that the multiple species may result from heterogeneity in the cleavage of structural protein precursors; although the sequences of the amino- and carboxyl-termini of the major capsid polypeptides of mengovirus were determined without mention of sequence variations (24). We suggest that the charge heterogeneity of these molecules may reflect differences in the interaction of these proteins with the ampholines. The different protein species observed may represent different forms of the viral proteins associated with either intracellular particles or free extracellular particles or extracellular particles bound to or eluted from membranes, assuming that these particles share the same buoyant density in equilibrium gradients. Therefore, the heterogeneity observed may reflect differences in the manner of isolating virus particles, whether intracellularly or from lysed cells.

Differences were detected among the alpha (1 D) structural proteins of the wild-type and mutant viruses. Since the mutants exhibited different alterations in the alpha (1 D) protein, yet similar changes in biological activity, it is possible that alteration of this protein resulted in the observed phenotypic changes. The differences in the number and migration of the alpha (1 D) protein isoelectric species are likely to be due to changes in uncharged amino acid residues affecting the interaction of the altered species with the ampholines. Changes in charged amino acid residues would result in altered migration of all four isoelectric species.

Since the chromatograms of surface- and metabolically-labeled peptides were similar, we speculate that the alterations in the mutant alpha (1 D) proteins may be associated with a particular determinant on this protein. This determinant may serve as or be part of a functional attachment site on the surface of virus particles that determines their affinity for various cells. Atomic resolution of the structure of another picornavirus, human rhinovirus 14, revealed a large cleft on the icosahedral face that has been postulated to serve as the host cell receptor binding site (18). The large cleft separates the major part of five 1 D (alpha) subunits from the other viral protein subunits. Since the picornaviruses share similar structural characteristics, by analogy it seems reasonable to speculate that mutations in the 1 D (alpha) protein could affect the structure of the cleft, and thus, alter the binding affinities of the mutant and revertant viruses. Alteration of the mengovirus host cell receptor binding site may lead to changes in affinity for

erythrocytes as well as other cell types which may explain the lack of virulence of the mutants in mice. Previous work by MORISHIMA *et al.* (16) demonstrated that differences in binding affinities of closely related strains of EMC and mengovirus to various cells reflects their difference in pathogenicity for mice.

HA$^+$ revertants were isolated from the brains of mice infected intracranially with mutant 205 (2). In addition to regaining agglutination activity, the revertants were also virulent for mice and exhibited a slight increase in plaque size. However, revertants required 10^3- to 10^4-fold more PFU to kill mice than the wild-type virus. Since the isoelectric character of the proteins of the HA$^+$ revertants were identical to that of mutant 205, this partial phenotypic reversion may have resulted from a change in an uncharged amino acid associated with a determinant, located on the alpha (1 D) protein, which partially restores its biological activities. The partial reversion of this mutation may involve modification of the altered surface determinant, increasing the binding affinity of the virus for cells and restoring virulence, but would not result in reappearance of the isoelectric species absent in mutant 205 and revertant alpha (1 D) capsid proteins. Alternatively, mutant 205 may express more than one mutation since complete reversion to wild-type virulence and plaque size did not occur and the isoelectric profile of the alpha (1 D) protein was the same as that of mutant 205. However, revertants were not isolated from mice infected intracranially with mutant 280, which shared biological properties as well as alpha (1 D) protein alterations with mutant 205. Therefore, the mutation observed in the alpha (1 D) structural protein of mutant 280 is apparently more stable than that of mutant 205. However, unlike mutant 205, expression of the mutation of mutant 280 resulted in a more subtle, yet reproducible change in the isoelectric point of one of the four alpha (1 D) protein species.

Changes in virus-specified macromolecular synthesis in mutant and revertant virus-infected cells can be explained by a decrease in the ability of these viruses to attach to cells. These data suggest that a smaller proportion of cells were productively infected with the mutant and revertant viruses in comparison to wild-type. Therefore, the effective MOI for the mutant and revertant viruses would be less than that of wild-type. This difference can be explained by changes in the cellular binding affinities; and the higher particle : PFU ratios exhibited by the mutant and revertants (2), assuming that a greater proportion of noninfectious particles would lower the probability of these viruses to infect cells. This interference phenomenon would explain why PFU values obtained under dilute conditions could not be used to accurately predict the fraction of infected cells in high particle : cell infections. Since fewer cells would be productively infected with mutant and revertant viruses, less virus-specified RNA would be synthesized, although the peak hour of RNA synthesis would be the same as that of wild-type.

Therefore, less RNA would be available for translation; and protein synthesis, as detected by immunoprecipitation, would appear to be delayed in cells infected with the mutant viruses. Changes in the metabolic rates of mutant virus-specified RNA synthesis would predict different results than those presented here if the fraction of infected cells were similar to wild-type infections. Collectively, our data suggest that the phenotypic changes expressed by the mutants may be due to mutations located exclusively within the alpha (1 D) coding region of the genome.

Previous work by AGOL et al. (1) has indicated that the neurovirulence of poliovirus maps to the 5' end of the genome which includes the coding region of the capsid proteins. Consistent with these results, we have identified two different mutations in the alpha (1 D) capsid protein, which maps to the 3' end of this region, of two avirulent mengovirus mutants. In addition, KOHARA et al. (14) have stated that a molecular recombinant of the Sabin vaccine strain of poliovirus containing VP 1 (1 D) and most of VP 3 (1 C) of the neurovirulent Mahoney strain is virulent, but not as virulent as the Mahoney strain and retain the small plaque morphology of the Sabin strain. Our data also indicate that changes in the alpha (1 D) protein result in altered virulence of a picornavirus. Since revertants of mutant 205 shared similar characteristics with the poliovirus recombinant, the plaque-forming ability of a particular virus isolate may be a characteristic that reflects as well as contributes to its virulence. Future experiments which compare the nucleotide sequences of the mutants and revertant structural proteins to that of the parental wild-type virus will be useful in determining the genetic basis for the altered biological properties exhibited by the mutant and revertant viruses.

Acknowledgements

References

1. AGOL VL, GRACHEV VP, DROZDOV SG, KOLESNIKOVA MS, KOZLOV VG, RALPH NM, ROMANOVA LI, TOLSKAYA EA, TYUFANOV AV, VICTOROVA EG (1984) Construction and properties of intertypic poliovirus recombinants: first approximation mapping of the major determinants of neurovirulence. Virology 136: 41–55
2. ANDERSON K, BOND CW (1987) Biological properties of mengovirus: Characterization of avirulent, hemagglutination-defective mutants. Arch Virol 93: 31–49
3. BENEKE TW, HABERMEHL K-O, DIEFENTHAL W, BUCHHOLZ M (1977) Iodination of poliovirus capsid proteins. J Gen Virol 34: 387–390
4. BOND CW (1984) Liquid scintillation counting: elimination of spurious results due to static electricity. Int J Appl Radiat Isot 35: 550–551

5. BOND CW, ANDERSON K, GOSS S, SARDINIA L (1981) Relatedness of virion and intracellular proteins of the murine coronaviruses JHM and A 59. In: TER MEULEN V, SIDDELL S, WEGE H (eds) Biochemistry and biology of coronaviruses. Plenum, New York, Advances in Experimental Biology and Medicine, vol 142, pp 103—110
6. CHURCHILL MA, RADLOFF RJ (1981) Two-dimensional electrophoretic analysis of encephalomyocarditis viral proteins. J Virol 37: 1103—1106
7. DORNER AJ, DORNER LF, LARSEN GR, WIMMER E, ANDERSON CW (1982) Identification of the initiation site of poliovirus protein synthesis. J Virol 42: 1017—1028
8. GARRELLS JI (1979) Two-dimensional gel electrophoresis and computer analysis of proteins synthesized by clonal cell lines. J Biol Chem 254: 7961—7977
9. HAMANN AC, REICHEL C, WIEGERS KJ, DRZENIEK R (1978) Isoelectric points of polypeptides of standard poliovirus particles of different serological types and of empty capsids and dense particles of poliovirus type 1. J Gen Virol 38: 567—570
10. HENDERSON LE, OROSZLAN S, KONIGSBERG W (1979) A micromethod for complete removal of dodecyl sulfate from proteins by ion-pair extraction. Anal Biochem 93: 153—157
11. HUTCHINSON MA, HUNTER T, ECKHART W (1978) Characterization of T antigens in polyoma-infected and transformed cells. Cell 15: 65—72
12. LUND GA, ZIOLA BR, SALMI A, SCRABA DG (1977) Structure of the mengo virion. Distribution of the capsid polypeptides with respect to the surface of the virus particle. Virology 78: 35—44
13. KESSLER SW (1975) Rapid isolation of antigens from cells with a staphylococcal protein A-antibody absorbent: parameters of the interaction of antigen-antibody complexes with protein A. J Immunol 115: 1617—1642
14. KOHARA M, OMATA T, KAMEDA A, SEMLER BL, ITOH H, WIMMER E, NOMOTO A (1985) In vitro phenotypic markers of a poliovirus recombinant constructed from infectious cDNA clones of the neurovirulent Mahoney strain and the attenuated Sabin 1 strain. J Virol 53: 786—792
15. MILLAR WT, SMITH JFB (1983) Protein iodination using iodogen. Int J Appl Radiat Isot 34: 639—641
16. MORISHIMA T, McCLINTOCK PR, AULAKH GS, BILLUPS LC, NOTKINS AL (1982) Genomic and receptor attachment differences between mengovirus and encephalomyocarditis virus. Virology 122: 461—465
17. O'FARRELL PH, O'FARRELL PZ (1977) Two-dimensional polyacrylamide gel electrophoretic fractionation. In: STEIN G et al (eds) Methods in cell biology, vol XVI. Academic Press, New York, pp 407—420
18. ROSSMANN MG, ARNOLD E, ERICKSON JW, FRANKENBERGER EA, GRIFFITH JP, HECHT H-J, JOHNSON JE, KAMER G, LUO M, MOSSER AG, RUECKERT RR, SHERRY B, VRIEND G (1985) Structure of a human common cold virus and functional relationship to other picornaviruses. Nature 317: 145—153
19. RUECKERT RR (1976) On the structure and morphogenesis of picornaviruses. In: FRAENKEL-CONRAT H, WAGNER RR (eds) Comprehensive virology, vol 6. Plenum, New York, pp 131—212
20. RUECKERT RR, WIMMER E (1984) Systematic nomenclature for picornavirus proteins. J Virol 50: 957—959
21. SEMLER BL, ANDERSON CW, KITAMURA N, ROTHBERG PG, WISHART WL, WIMMER E (1981) Poliovirus replication proteins: RNA sequence encoding P 3—1 B and the sites of proteolytic processing. Proc Natl Acad Sci USA 78: 3464—3468
22. UI N (1971) Isoelectric points and conformations of proteins. I. Effect of urea on the behavior of some proteins in isoelectric focusing. Biochim Biophys Acta 229: 567—581

23. VRIJSEN R, WOUTERS M, BOEYE A (1978) Resolution of the major poliovirus capsid proteins into doublets. Virology 86: 546—555
24. ZIOLA BR, SCRABA DG (1976) Structure of the mengo virion. IV. Amino- and carboxyl-terminal analysis of the major capsid polypeptides. Virology 71: 111—121

Authors' address: Dr. K. ANDERSON, Department of Microbiology, School of Medicine, University of Alabama at Birmingham, University Station, Birmingham, AL 35294, U.S.A.