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Biological Properties of Mengovirus: Characterization of Avirulent, Hemagglutination-defective Mutants

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With 2 Figures

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Summary

Biological properties of two mengovirus mutants, 205 and 280, were compared to those of wild-type virus. The mutants exhibited alterations in plaque morphology, hemagglutination, and virulence in mice, but were not temperature-sensitive. Agglutination of human erythrocytes by mengovirus was dependent on the presence of sialic acid on the erythrocyte surface; however, free sialic acid failed to inhibit hemagglutination. Glycophorin, the major sialoglycoprotein of human erythrocyte membranes, exhibited receptor specificity for wild-type virus, but not for mutants 205 or 280. Cross-linking studies indicated that glycophorin exhibited binding specificity for the alpha (1 D) structural protein. The LD_{50} titers for wild-type mengovirus were 7 and 1500 plaque forming units (PFU) in mice infected intracranially (IC) and intraperitoneally (IP), respectively. However, mice infected IC or IP with 10^6 or 10^7 PFU of mutant 205 or 280 did not exhibit symptoms indicative of virus infection. Revertants were isolated from the brains of mice infected with mutant 205, but not from the brains of mice infected with mutant 280. The biological characterization of the revertants indicated that hemagglutination and virulence may be phenotypically-linked traits.

Introduction

Mengovirus has provided a particularly useful model system for the study of picornaviruses. In fact, much of our knowledge of the structure and assembly of picornaviruses has been obtained from the study of mengovirus

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(26, 30). As with other picornaviruses (28), the structure of the viral capsid is provided by the complex interaction of four major capsid proteins which are alpha (1 D), beta (1 B), gamma (1 C), and delta (1 A) (29). Approximately 60 copies of the major proteins are found per capsid (5, 28), inclusive of two copies of the epsilon (1 AB) protein, the uncleaved precursor of delta (1 A) and beta (1 B) (5, 32).

Of current interest in the study of the molecular biology of picornaviruses is the description of changes in the biological functions of related viruses and the mapping of these functions to particular viral genes. MORISHIMA *et al.* (25) and YOON *et al.* (31) have shown that closely related viruses, strains of mengovirus and encephalomyocarditis virus, have distinctly different tissue tropisms and virulence patterns. AGOL *et al.* (1) have determined from recombination experiments that the neurovirulence determinants of poliovirus map to the region of the capsid proteins in the genome, and KOHARA *et al.* (20) have examined the segregation of biological phenotypes upon the exchange of a region of the poliovirus genome encoding VP 1 (1 D) and part of VP 3 (1 C) from the Mahoney strain into the corresponding region of the attenuated Sabin strain.

In this and the adjoining publication (3) we compare the biological and structural properties of two mengovirus mutants to the parental neurovirulent mengovirus strain in an attempt to map biological functions of the virus to specific genes. Several mutants of mengovirus were originally isolated as being temperature-sensitive, but were not defective in synthesizing RNA at the restrictive temperature (M. A. GILL, personal communication). Two of these mutants lack the ability to agglutinate human erythrocytes and therefore, are likely to express altered structural proteins.

Here we compare the biological properties of the two hemagglutination-defective mutants to those of wild-type mengovirus. Revertants of one of these mutants were isolated and characterized to establish possible linkages among the altered phenotypes expressed by the mutant. In addition, we have isolated an erythrocyte membrane receptor for mengovirus and attempted to identify viral attachment protein(s) involved in the agglutination of human erythrocytes.

Materials and Methods

Viruses and Cell Culture

Wild-type mengovirus has been previously described (4, 5). Mutants of mengovirus were generated by treating virus-infected cells with acriflavin (10 μg/ml), which inhibited virus-specified RNA synthesis by 80 percent and viral yield by 99 percent (Dr. M. A. GILL, personal communication). These mutants were selected by temperature shift analysis of isolated plaques. The isolation and characterization of *ts* 25 has been previously described (4, 5). All mutants were cloned twice from plaque isolates and virus stocks were prepared by subsequent infection of BHK-21 cells at a multiplicity of infection (MOI) of 0.1

plaque forming units (PFU) per cell. Cells were maintained in Dulbecco's Modified Eagles medium (DME, Irvine Scientific) supplemented with 4.5 g/L glucose and 10 percent calf serum (DME 10) as previously described (27). Virus stocks were titered by plaque assay on BHK-21 cell monolayers.

Infection and Purification

Cells were infected with virus in suspension or as monolayers. Cells suspended in DME containing 2 percent calf serum (DME 2) or monolayers were infected with virus at a multiplicity of infection (MOI) of 3 and incubated at 33° C for 30 minutes. Following the adsorption period, virus-infected cells were incubated at 33° C until lysis, then frozen at −70° C. To purify virus, lysates were thawed, pooled and clarified by centrifugation in an HB-4 rotor at 4000 × *g* for 15 minutes at 15° C. Sodium dodecyl sulfate (SDS) was added to the supernatant fluid to 0.2 percent and reclarified as above. The resulting supernatant fluid was layered over a pad of 28 percent (w/w) sucrose in Bu 3 [10 mM Tris-HCl (pH 7.4), 10 mM EDTA, 50 mM NaCl], centrifuged in either an SW 41 rotor at 37,000 rpm for 90 minutes or an SW 27 rotor at 27,000 rpm for 150 minutes at 15° C. Pellets were resuspended in Bu 3, layered onto 15–28 percent (w/w) sucrose gradients in Bu 3, and centrifuged in an SW 41 rotor at 35,000 rpm for 105 minutes at 6° C. Gradients were fractionated and virus was detected by either absorbance at 260 nm or monitoring radioactivity by liquid scintillation counting. Peak fractions were pooled, diluted with Bu 3, and centrifuged in an SW 50.1 rotor at 43,000 rpm for 90 minutes at 6° C. Pellets were resuspended in TE buffer [10 mM Tris-HCl (pH 7.4), 10 mM EDTA], layered onto a 27–42 percent (w/w) CsCl gradient in TE buffer, and centrifuged in an SW 41 rotor at 33,000 rpm for 10 hours at 6° C. Peak fractions were pooled, dialyzed extensively against TE buffer, and the virions were pelleted by centrifugation in an SW 50.1 rotor at 43,000 rpm for 90 minutes at 6° C.

Radiolabeling of Virus-specified Proteins

Purified virions radiolabeled *in vivo* with L-[^{35}S]-methionine (New England Nuclear, NEG-009 T) were prepared as follows. At 5.5 hours postinfection (HPI) (for the wild-type strain) or 6.5 HPI (for the mutant strains), the medium was aspirated from the virus-infected cells and replaced with ^{35}S-labeling medium containing 20 percent of the normal concentration of methionine in DME 2 and 50 μCi/ml ^{35}S-methionine. The virus-infected cells were subsequently incubated at 33° C and labeled virions were purified from infected cell lysates as described above.

Hemagglutination Assays

A hemagglutination assay (24) was modified for use in a microtiter plate assay. Virus stock samples adjusted to 0.25 percent sodium deoxycholate were serially diluted 2-fold into successive wells containing Dulbecco's phosphate-buffered saline (PBS). A 0.3 percent suspension of washed erythrocytes was added to each well and incubated at 4° C for 4 hours, at which time the titers were read. Human type 0 (American Red Cross, Great Falls, Montana), sheep (Colorado Serum Co.), and rat erythrocytes (obtained from Sprague Dawley rats) were used as test cells in the hemagglutination assays. Hemagglutination inhibition assays were performed as follows. Carbohydrates were suspended in PBS to 0.2 M and diluted serially 2-fold into successive wells. Eight HA units of virus was added to each well and the microtiter plates were incubated at 4° C for 30 minutes. Erythrocytes were added to each well and the plates were incubated as described above.

Glycophorin Purification and Cross-linking

Glycophorin was purified from erythrocyte ghosts as described (22). To examine the purity of the preparation, glycophorin was subjected to SDS-polyacrylamide gel electro-

phoresis (SDS-PAGE) (21), stained with brilliant blue G or by the periodic acid-Shiff (PAS) staining method (17) and analyzed by scanning densitometry at 595 nm using a Gilson multimedia densitometer. A single protein species corresponding to the dimerized form of glycophorin (70 kd) was detected by staining with brilliant blue G. Two carbohydrate species were resolved by staining with PAS, corresponding to the dimer and monomer (35 kd) forms of glycophorin, indicating that the glycophorin preparation was acceptable for use in further experiments. The protein concentration of purified glycophorin was determined by the protein-dye binding assay (8) based on a standard curve generated by the binding of known quantities of fetuin (type III, Sigma).

The virion structural proteins involved in glycophorin binding were investigated by the use of a bifunctional cross-linking reagent, toluene-2, 4-diisocyanate (TDI, ICN Pharmaceuticals). The procedure of TDI cross-linkage was modified from a previously described method (15). Glycophorin (400 μg) was incubated with 2.2 μl TDI for 30 minutes at room temperature in 100 μl of 10 mM sodium phosphate buffer (pH 7.2). The reaction mixture was cooled to 0° C for 10 minutes and excess TDI was pelleted by two successive centrifugations at $6500 \times g$ for 5 minutes at 4° C. ^{35}S-methionine labeled virions were incubated with cross-linked glycophorin for 30 minutes at room temperature. The pH of each reaction mixture was raised to 9.6 for 10 minutes by the addition of saturated tribasic sodium phosphate to facilitate the second cross-linking reaction. The pH was then lowered to 7.2 with saturated monobasic sodium phosphate. Each mixture was dialyzed against distilled water then lyophilized. The samples were reduced and prepared for SDS-PAGE on 8, 10 or 14 percent polyacrylamide slab gels as described (6). Labeled proteins were detected by impregnating the gels with 10 percent PPO in DMSO followed by drying and exposure to preflashed Kodak XAR-2 x-ray film at −70° C (7). Autoradiograms of labeled proteins exposed to x-ray film were analyzed by scanning densitometry at 633 nm.

Infection of Mice

BALB/c mice were obtained from the Jackson Laboratories, Bar Harbor, ME, and bred in our laboratory. Four to five week old BALB/c mice were infected by the intraperitoneal (IP) route with 0.2 ml or by the intracranial (IC) route with 0.05 ml of a dilution of virus as specified in each experiment. Mice were monitored daily for morbidity and mortality due to virus infection.

Production of Hyperimmune Ascitic Fluid

Eight week old BALB/c mice were inoculated IP with 0.3 ml of wild-type mengo stock virus (1×10^8 PFU/ml) emulsified in complete Freund's adjuvant (Sigma) (1 : 1) on days 0, 7, and 14. Wild-type mengovirus was heat-inactivated at 60° C for 2 hours prior to inoculation on day 0. Live virus emusified in complete Freund's adjuvant was used as the inoculum in all subsequent inoculations. A booster inoculation of 0.3 ml stock virus without adjuvant was given IP on day 21. Ascitic fluid was induced by IP inoculation with 5×10^6 S 180 cells in 0.3 ml PBS (18). Ascitic fluid was harvested by abdominal paracentesis and adsorbed against methanol fixed BHK-21 cell monolayers at 4° C for 24 hours.

Immunofluorescence and Plaque Neutralization

Mock and virus-infected BHK-21 were monitored for the presence of mengovirus-specific antigens by immunofluorescence as described (27). Immunofluorescence was observed with an Olympus IMT inverted microscope equipped with reflected fluorescence optics. Plaque neutralization assays were performed as follows. Ascitic fluid was serially diluted in DME 2 and 0.4 ml of each dilution was mixed with 0.1 ml of a dilution of virus (approximately 600 PFU/ml), incubated at room temperature for 30 minutes, and the suspensions were titered by plaque assay on BHK-21 cell monolayers.

Determination of Particle : PFU Ratios

The number of particles/ml in suspensions of purified virus were calculated from the absorbance at 260 nm (28) assuming that one absorbance unit is equivalent to 9.4×10^{12} particles/ml; and the titers of purified suspensions were determined by plaque assay.

Results

Temperature-Sensitivity and Plaque Size

The temperature-sensitivity and plaque size of two hemagglutination-defective mutants of mengovirus, 205 and 280, were compared to those of wild-type virus. The mutants were isolated as temperature-sensitive on the basis that their plaque size on L 929 cell monolayers did not increase at 39.5° C (Dr. M. A. GILL, personal communication).

The yields of the mutant viruses incubated at restrictive (39.5° C) and permissive (33° C) temperatures were compared to those of the wild-type strain. BHK-21 cells were infected in suspension with virus at an MOI of 1 and incubated at either 33° or 39.5° C for 30 minutes. After the adsorption period, the cells were incubated at either 33° or 39.5° C. The infected cells were incubated until 70 percent of the cells were lysed, then titered by plaque assay (Table 1). The yield of virus adsorbed at 33° C and incubated at 39.5° C was less than the yield of virus adsorbed and incubated at 33° C for each virus. However, no difference in the yield of virus at 33° versus 39.5° C was observed with mutant 205 in comparison to wild-type virus. Less than a 3-fold difference in yield at 33° versus 39.5° C was observed in comparing the values obtained for mutant 280 in comparison to wild-type virus. These data suggest that mutants 205 and 280 were not significantly temperature-sensitive relative to the wild-type virus. To test whether mutants 205 and 280 were temperature-sensitive for adsorption, the yields of virus were compared from cells incubated with virus at the permissive or restrictive temperatures for 30 minutes, then incubated under permissive conditions until 70 percent of the cells were lysed. No difference in virus yield was

Table 1. *Temperature-sensitivity of mengovirus mutants as a function of yield*

Virus	$\log_{10}$ PFU/ml			
	33° C[a]	39.5° C[b]	39.5°/33° C[c]	$\log_{10}$ (33°/39.5° C)[d]
Wild-type	7.76	6.11	7.78	1.65
205	7.14	5.40	7.13	1.74
280	7.59	5.51	7.54	2.09

[a] Yield of virus after adsorption and incubation at 33° C

[b] Yield of virus after adsorption at 33° C and incubation at 39.5° C

[c] Yield of virus after adsorption at 39.5° C and incubation at 33° C. The adsorption of virus to cells at 39.5° C followed by incubation at 39.5° C was not done

[d] Log_{10} difference in yield at 33° and 39.5° C

observed under the different adsorption conditions. Thus, adsorption of the wild-type and mutant mengoviruses to BHK-21 cells was not affected by altering the incubation temperature.

The size of plaques produced on BHK-21 cell monolayers by mutants 205 and 280 was compared to the plaques made by the wild-type virus. Diameters of twenty plaques of each virus were measured and compared at 48 HPI. The mean plaque diameter produced by the wild-type virus was 3.99 ± 1.08 mm, which was significantly different than the mean plaque diameters of 1.56 ± 0.37 mm and 1.49 ± 0.44 mm produced by mutants 205 and 280, respectively ($p < 0.01$, Student t test). These data, coupled with the temperature-sensitivity data, suggest that the temperature shift method of isolating temperature-sensitive mutants may result in the isolation of mutants which appear to be temperature-sensitive due to their small plaque phenotype but are not temperature-sensitive with respect to multiplication.

Hemagglutination

Wild-type mengovirus agglutinates human type 0 erythrocytes, but mengovirus mutants 205 and 280 do not agglutinate human type 0 erythrocytes (Dr. M. A. GILL, personal communication). Hemagglutination assays were performed to confirm these results and to test whether these viruses would agglutinate sheep or rat erythrocytes (Table 2). Mutants 205 and 280 did not agglutinate human type 0 or sheep erythrocytes, but did agglutinate rat erythrocytes. However, wild-type virus and two other mutants, *ts* 25 and 237, agglutinated all three erythrocyte species. Since mutants 205 and 280 did not agglutinate human type 0 or sheep erythrocytes, attachment sites on the surface of wild-type mengovirus responsible for agglutination may be absent, altered, or masked on the surface of mutants 205 and 280. In addition, the attachment sites on wild-type capsids may function in the agglutination of both human type 0 and sheep erythrocytes. Since all of the viruses tested were able to agglutinate rat erythrocytes, perhaps a different binding site common to the surface of wild-type and mutant viruses would function in agglutination of rat erythrocytes.

Table 2. *Hemagglutination assay of wild-type and mutant mengoviruses*[a]

Virus	Human type 0	Sheep	Rat
WT	2048	128	2048
25	256	256	2048
205	0	0	1024
237	512	64	4096
280	0	0	512

[a] Titers are expressed as the reciprocal of the end point dilution

The mechanism by which wild-type mengovirus agglutinates human type 0 erythrocytes was investigated by examining the specificity of the receptors on the surface of the erythrocyte for viral capsids and viral attachment proteins. To test whether wild-type mengovirus has specificity for sialic acid, a carbohydrate residue abundant on the surface of human erythrocytes, human type 0 erythrocytes were treated with neuraminidase and used as test cells in the hemagglutination assay. Wild-type mengovirus did not agglutinate neuraminidase-treated erythrocytes. This result suggests that wild-type mengovirus may attach specifically to sialic acid residues on the erythrocyte surface, facilitating the agglutination of human type 0 erythrocytes.

To determine whether sialic acid or other carbohydrate residues present on erythrocyte surface glycoproteins could inhibit the agglutination of human type 0 erythrocytes by wild-type mengovirus, 0.2 M solutions of carbohydrates common to human erythrocyte membranes were incubated with wild-type virus prior to the addition of erythrocytes in a hemagglutination inhibition assay. A list of carbohydrates used in this experiment is shown in Table 3. None of the carbohydrates tested, including sialic acid, were able to inhibit the agglutination reaction. Since the presence of sialic acid on the erythrocytes was required for the agglutination reaction with wild-type virus, its recognition as a receptor for wild-type virus may depend upon maintaining a particular conformation or charge in association with erythrocyte surface glycoproteins or glycolipids.

Table 3. *List of carbohydrates tested as inhibitors of mengovirus hemagglutination*

alpha-D-glucose	beta-D-glucose
D-galactose	D-mannose
L-fucose	N-acetyl-D-glucosamine
N-acetyl-D-galactosamine	alpha-methyl-D-glucoside
alpha-methyl-D-galactoside	beta-methyl-D-galactoside
alpha-methyl-D-mannoside	N-acetyl neuraminic acid (sialic acid)

Glycophorin, the major sialoglycoprotein on the surface of human erythrocytes (23), is a receptor for EMC virus (16), a cardiovirus closely related to mengovirus. Purified glycophorin (10 μg) was tested for its ability to inhibit agglutination of human type 0 erythrocytes by wild-type mengovirus. Mengovirus incubated in the presence of glycophorin had an agglutination titer of 128 in comparison to the agglutination titer of 1024 exhibited by the mengovirus control. Therefore, 10 μg of glycophorin inhibited the agglutination titer of wild-type mengovirus by 8-fold. In a parallel hemagglutination inhibition experiment, fetuin and bovine serum albumin (10 μg each) failed to inhibit hemagglutination.

Purified glycophorin was tested for its ability to function as a receptor for wild-type mengovirus. Several concentrations of purified glycophorin were mixed with ^{14}C-labeled mengovirus and analyzed by rate zonal centrifugation in sucrose gradients. The number of counts per minute (CPM) associated with the pellet fractions increased relative to the amount of glycophorin mixed with wild-type mengovirus (data not shown). These results suggest that the migration of wild-type mengovirus in sucrose gradients was altered due to binding of glycophorin.

The specificity of this reaction was examined by incubating an excess quantity of glycophorin with purified, ^{14}C-labeled mutant 205 and 280

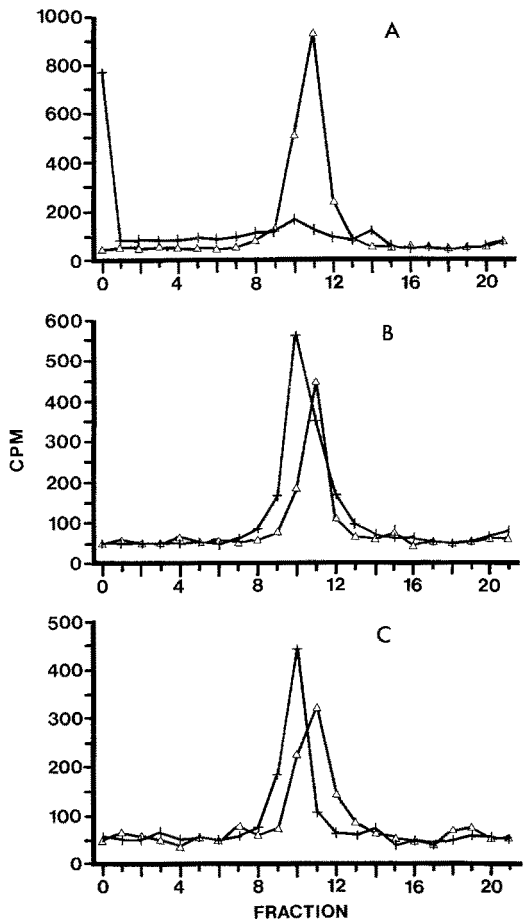

Fig. 1. Effect of glycophorin on the migration of purified wild-type and mutant mengoviruses in sucrose density gradients. Wild-type *(A)*, mutant 205 *(B)*, and mutant 280 *(C)* virions (6 μg) were preincubated in the presence *(+)* or absence *(Δ)* of glycophorin (50 μg) and analyzed as described in Materials and Methods. No differences were detected in the number or migration of the structural proteins of the pellet and peak fractions as analyzed by SDS-PAGE (data not shown). Fraction 0 represents the pellet fraction

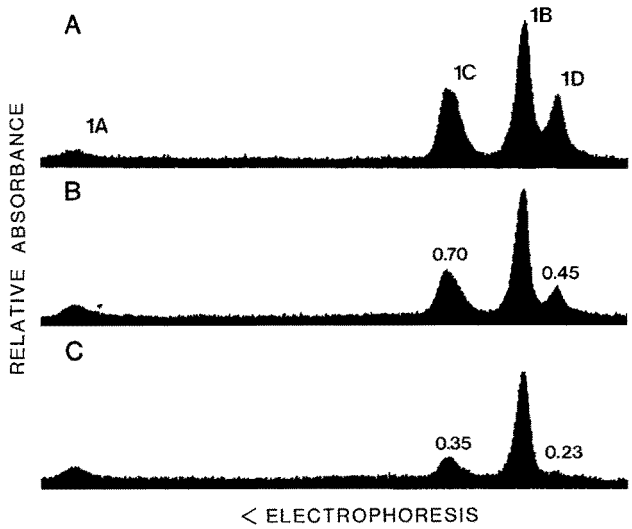

Fig. 2. Densitometer tracings of the mengovirus structural proteins cross-linked to glycophorin. Glycophorin-TDI complexes were mixed with ^{14}C-labeled mengovirus (2 μg) as described in the text. Wild-type virion proteins *(A)*, proteins of wild-type virions mixed with 2 μg of glycophorin-TDI *(B)*, and proteins of wild-type virions mixed with 4 μg of glycophorin-TDI *(C)* were analyzed on 12 percent SDS polyacrylamide gels and scanned at 633 nm using an LKB Ultroscan densitometer. The fractions of the alpha (1 D) and gamma (1 C) proteins resolved in the glycophorin treated panels *B* and *C* in comparison to the amount resolved in the untreated control panel *A* are shown numerically

virions, which did not agglutinate human type 0 erythrocytes, followed by sucrose gradient centrifugation (Fig. 1). Although labeled wild-type mengovirus was displaced into the pellet fraction in the presence of glycophorin, the single fraction difference observed for the migration of mutants 205 and 280 in the presence of glycophorin was not significant. These data suggest that wild-type mengovirus interacted with glycophorin specifically and that glycophorin may function as a receptor for mengovirus on human type 0 erythrocytes.

An attempt was made to determine which of the structural proteins of mengovirus function in the binding of glycophorin. Glycophorin was mixed with TDI, a heterobifunctional cross-linking reagent. Glycophorin-TDI complexes were mixed with purified ^{14}C-labeled wild-type mengovirus and cross-linked to virion structural proteins which were subsequently analyzed by SDS-PAGE. In this experiment any structural proteins of mengovirus cross-linked to glycophorin molecules would not enter the resolving gel. Densitometer tracings of the treated and untreated samples are shown in Fig. 2. The four major structural proteins of wild-type mengovirus were resolved (panel A); alpha (1 D), beta (1 B), gamma (1 C) and delta (1 A). A substantial decrease in the amount of the alpha (1 D) protein was observed

in the lanes of glycophorin-cross-linked virus (panels B and C). A decrease in the amount of the gamma (1 C) protein was also observed. The amounts of the beta (1 B) and delta (1 A) proteins observed decreased slightly and in the presence of increasing amounts of cross-linked glycophorin. These data indicated that the alpha (1 D) and gamma (1 C) structural proteins were cross-linked to glycophorin and that glycophorin had a greater affinity for the alpha (1 D) protein than for the gamma (1 C) protein. Since the amount of the gamma (1 C) protein cross-linked to glycophorin was not as extensive as that of the alpha (1 D) protein, it is possible that cross-linkage of the gamma (1 C) protein may occur subsequent to its binding of the alpha (1 D) protein. TDI molecules located distal to the glycophorin-mengovirus binding site(s) may be more available to bind to positively charged residues on the gamma (1 C) protein nonspecifically when increasing amounts of cross-linked glycophorin are added to the reaction.

Virulence in BALB/c Mice

To determine whether the predicted structural alterations of mutants 205 and 280 correlate with a change in virulence relative to the wild-type virus, BALB/c mice were infected IP or IC with 10-fold dilutions of virus and LD_{50} titers were calculated for each virus. Wild-type mengovirus had an LD_{50} titer of 1500 PFU in mice infected IP and an LD_{50} titer of 7 PFU in mice infected IC. All mice infected with 10^7 PFU IP or 10^6 PFU IC with either mutant survived and did not exhibit symptoms typical of mengovirus infection which suggests mutants 205 and 280 were avirulent in BALB/c mice.

The extent of virus multiplication in the brains of BALB/c mice following IP or IC inoculation was determined. Brains of mice infected with virus were removed, weighed, subjected to Dounce homogenization in PBS, and titered by plaque assay (Table 4). Wild-type virus was able to multiply in the brains of mice to titers of 10^6 PFU/g brain four days after IC infection with 10^4 PFU and to titers of 10^3 PFU/g brain five days after IP infection with 10^4 PFU. All four mice infected IC with 10^4 PFU of mutant 205 exhibited titers between 10^2 and 10^3 PFU/g brain after four days. However, infectious virus was detected in the brains of only two of four mice infected IC with mutant 280. No infectious virus was detected in mice infected IP with mutants 205 or 280. Therefore, unlike wild-type mengovirus, mutants 205 and 280 were not capable of initiating an acute infection of the brain following IP inoculation. To rule out the possibility that intracellular proteases released by Dounce homogenization would degrade virus particles and limit the number of infectious virus particles detectable, wild-type virus and mutants 205 and 280 were diluted 10-fold in 20 percent brain suspension or DME 2, incubated at 4° or 37° C for 30 minutes, and titered by plaque assay. No significant changes in titer were detectable for any of the virus strains in the presence

Table 4. *Yield of wild-type and mutant mengoviruses in BALB/c mouse brains following intracranial or intraperitoneal infection*[a]

Suspension	Route	Titer
WT-A[b]	IC	6.20
WT-B	IC	6.72
WT-C	IP	2.82
WT-D	IP	3.53
205-A	IC	2.69
205-B	IC	2.44
205-C	IC	2.47
205-D	IC	2.81
205-E	IP	<1.80
205-F	IP	<1.80
280-A	IC	2.99
280-B	IC	<1.80
280-C	IC	2.17
280-D	IC	<1.80
280-E	IP	<1.80
280-F	IP	<1.80

[a] Brains of mice infected intracranially with 10^4 PFU were harvested 4 days postinfection, weighed, and dounce homogenized in 4 ml PBS. Brains of mice infected intraperitoneally with 10^4 PFU were harvested 5 days postinfection. Titers are expressed as $\log_{10}$ PFU/g brain

[b] Suspensions are designated by the infecting virus, wild-type (WT), mutants 205 or 280, and a letter representing individual mice (A–F); i.e. WT-A represents a 20 percent brain suspension from mouse A infected with wild-type virus

or absence of 20 percent brain suspension at either temperature (data not shown).

To determine whether viruses isolated from the brains of infected mice have the same biological properties as the infecting virus, isolated plaques were harvested and virus stocks were produced by infection of BHK-21 cell monolayers using an MOI of 0.1. Virus stocks of plaque isolates were tested for their ability to agglutinate human type 0 erythrocytes (Table 5). All of the plaque isolates from IP or IC infections with wild-type mengovirus agglutinated human type 0 erythrocytes. However, several plaque isolates from three different mice infected IC with mutant 205 also agglutinated human type 0 erythrocytes. These data suggest that either phenotypic reversion of mutant 205 occurred following infection of BALB/c mice or that variants found at low levels in the mutant 205 inoculum were selected for by passage in mice. No phenotypic revertants were isolated from the brains of mice infected with mutant 280.

To determine whether the HA^+ revertants had acquired other wild-type characteristics, several plaque isolates were selected for analysis of their

Table 5. *Hemagglutination assay of plaque isolates from brain suspensions of BALB/c mice infected with wild-type or mutant mengoviruses*

Clone	Titer	Clone	Titer	Clone	Titer
WT-A 1[a]	1024	205-A 1	1024	205-C 1	0
WT-A 2	2048	205-A 2	2048	205-C 2	0
WT-A 3	1024	205-A 3	2048	205-C 3	0
WT-A 4	4096	205-A 4	1024	205-C 4	0
WT-A 5	512	205-A 5	128	205-C 5	0
WT-A 6	4096	205-A 6	512	205-C 6	0
WT-B 1	2048	205-A 7	2048	205-D 1	0
WT-B 2	4096	205-A 8	2048	205-D 2	1028
WT-B 3	2048	205-A 9	512	205-D 3	0
WT-B 4	1024	205-A 10	512	205-D 4	0
WT-B 5	4096	205-A 11	2048	205-D 5	0
WT-B 6	2048	205-A 12	4096	205-D 6	0
WT-C 1	2048	205-A 13	2048	280-A 1	0
WT-C 2	2048	205-A 14	4096	280-A 2	0
WT-C 3	1024	205-A 15	1024	280-A 3	0
WT-C 4	512	205-A 16	1024	280-A 4	0
WT-C 5	512	205-A 17	2048	280-A 5	0
WT-C 6	1024	205-A 18	4096	280-A 6	0
WT-D 1	2048	205-B 1	0	280-C 1	0
WT-D 2	1024	205-B 2	0	280-C 2	0
WT-D 3	2048	205-B 3	0	280-C 3	0
WT-D 4	512	205-B 4	1024	280-C 4	0
WT-D 5	4096	205-B 5	0	280-C 5	0
WT-D 6	1024	205-B 6	0	280-C 6	0

[a] The clones are designated by numbers, mice by letter, and the infecting virus by WT, 205 or 280; i.e. WT-A 1 represents clone 1 isolated from mouse A infected with wild-type virus (WT). The clones were isolated from the brain suspensions listed in Table 4

plaque size on BHK-21 cell monolayers, specific immunofluorescence using anti-mengovirus ascitic fluid, and pathogenicity in mice (Table 6). All of the plaque isolate-infected cells stained positive in the indirect immunofluorescence assay, suggesting that these isolates were of mengovirus origin. The plaque isolates which reverted to the HA^+ phenotype produced intermediate sized plaques on BHK-21 cell monolayers when compared to the large wild-type plaques and the small plaques typical of mutant 205. All of the plaque isolates tested that were isolated from the brains of wild-type-infected mice produced large plaques (data not shown). Therefore, these data indicate that only partial reversion to the wild-type phenotype occurred with these isolates.

To examine the pathogenicity of the plaque isolates, several mice were infected intraperitoneally with 10^4 or 10^6 PFU and monitored for 28 days post-infection. None of the mice infected with 10^4 PFU of the plaque isolates exhibited symptoms typical of mengovirus infection. However, all of the mice infected with 10^6 PFU of the plaque isolates which had reverted to the HA^+ phenotype showed symptoms typical of mengovirus infection by five days post-infection and over 80 percent of these mice died by eight days post-infection. None of the mice infected with 10^6 PFU of the HA^- plaque isolates exhibited symptoms typical of mengovirus infection. Therefore, reversion to the HA^+ phenotype coincided with a renewed capability of these isolates to cause disease in mice. These results suggest that hemagglutination and virulence may be phenotypically-linked traits. However, none of the small plaque revertants were as virulent as the wild-type virus, which indicated that complete reversion to the wild-type phenotype did not occur. Therefore, the virulence of a particular mengovirus may be linked to its average plaque diameter, since the reversion to virulence of the HA^+ revertants coincided with a slight increase in the plaque size of these viruses in comparison to the mutants.

Table 6. *Further characterization of the cloned plaque isolates from brain homogenates of mice infected intracranially with mutant 205*

Clone[a]	Plaque size[b]		Titer[c]	HA[d]	IFA[e]
	Experiment 1	Experiment 2			
205-A 1	1.83 ± 0.31		7.79	1024	+
205-A 2	1.78 ± 0.28		7.90	2048	+
205-A 5	1.87 ± 0.40		8.00	128	+
205-A 7	1.83 ± 0.27	3.8 ± 0.5	7.80	2048	+
205-A 8	1.92 ± 0.30		7.73	2048	+
205-A 12	1.86 ± 0.33		7.84	4096	+
205-A 14	1.89 ± 0.21		7.90	4096	+
205-A 15	1.86 ± 0.23		7.72	1024	+
205-A 17	1.72 ± 0.26		7.54	2048	+
205-B 4	1.85 ± 0.33	4.0 ± 0.7	7.67	1024	+
205-C 4	1.74 ± 0.33		7.67	0	+
205-D 2	1.72 ± 0.31	3.1 ± 0.4	7.86	1024	+
205-D 5	1.06 ± 0.18		7.99	0	+
Wild-type	4.00 ± 0.80	8.5 ± 0.7	8.20	2048	+
205	1.54 ± 0.34	1.8 ± 0.6	7.80	0	+

[a] Clones are designated as described in Table 5

[b] Plaque sizes were determined as described in Materials and Methods. The assays were incubated at 33° C for 48 hours in Experiment 1 and 72 hours in Experiment 2

[c] Titers are expressed as $\log_{10}$ PFU/ml

[d] Hemagglutination titers (HA)

[e] Immunofluorescence assay (IFA)

Plaque Neutralization

Mengovirus-specific ascitic fluid prepared in BALB/c mice was tested for its ability to neutralize wild-type virus, mutants 205 and 280, and revertants 205-A 7 and 205-D 2. Two-fold dilutions of ascitic fluid were mixed with a dilution of virus, incubated at room temperature for 30 minutes, and assayed for plaque production on BHK-21 cell monolayers. The reciprocal of the dilution capable of neutralizing 50 percent of the plaques produced by a dilution of virus (PN_{50} titer) was 1182 for the neutralization of the wild-type virus. The PN_{50} titers were 1970 and 2127 for neutralization of mutants 205 and 280, respectively. The PN_{50} titers were 812 and 914 for the neutralization of revertants 205-A 7 and 205-D 2, respectively. These data indicated that a significantly greater amount of antiserum was required to neutralize the revertants in comparison to the wild-type and mutant viruses ($p < 0.08$, Student *t* test). The greater resistance of the revertants to neutralization may be a result of the selective pressure generated by immune surveillance in mutant virus-infected mice or may be a measure of the frequency of stable variants produce during the passage of virus in cultured cells. In addition, the differences in neutralization titer are likely to reflect changes in the surface structure of the mutant and revertant viruses relative to the wild-type virus.

Particle: PFU Ratios

The particle to PFU ratios for the wild-type, mutant and revertant viruses were determined. Wild-type mengovirus had a particle to PFU ratio of 2200. The ratios for mutants 205 and 280 were 19,000 and 7800, respectively. The ratios for revertants 205-A 7 and 205-D 2 were 4100 and 4600, respectively. These data suggest that the phenotypic changes associated with the mutants and revertants has resulted in a decreased probability of these viruses to cause infection in comparison to wild-type.

Discussion

Two mutants of mengovirus exhibited changes in biological activity indicative of possible alterations in their structural proteins. These mutants, 205 and 280, were mutagenized during the multiplicative cycle by acriflavin and were selected as being temperature-sensitive by comparing the size of plaques produced before and after a shift in incubation temperature from 31.5° to 39.5° C. The mutants were not defective in synthesizing RNA at the restrictive temperature and lacked the ability to agglutinate human type 0 erythrocytes (M. A. GILL, personal communication). We have expanded the comparison of the biological properties of these mutants with those of the parental wild-type mengovirus to determine the extent of phenotypic variation that is likely due to the expression of altered structural proteins.

Further characterization of the mutants revealed that they were not temperature-sensitive with respect to virus yield or adsorption, but produced small plaques in cell culture. The small plaque phenotype exhibited by the mutants coincided with a slower rate of cytopathic lysis of infected cells in comparison to wild-type. Also, the yield of infectious particles was less in mutant infected-cells in comparison to wild type. However, since the mutant particle : PFU ratios were greater than that of wild-type, more virus particles would be produced in mutant-infected cells. A greater proportion of the mutant virus particles may be noninfectious and therefore, would compete for cellular receptors. This competitive interference may explain the slow spread of the mutants and consequent small plaque size.

Mutants 205 and 280 were avirulent in mice infected IP or IC. Although a small amount of virus was detected in the brains of several mice infected IC with mutants 205 and 280, these mice did not exhibit symptoms of virus infection. However, AMAKO and DALES (2) and COLTER *et al.* (13) have demonstrated that S (small plaque) variants are virulent for mice when infected IP, although to a much lesser extent than the L (large plaque) variants. COLTER *et al.* (13) have reported that the LD_{50} titers of the L, M (medium) and S plaque variants were similar for mice infected IC. Although the small plaque phenotype is shared by the S variants and mutants 205 and 280, clearly the pathogenicity of these viruses for mice is distinct. In addition, CAMPBELL and COLTER (11) have reported that the distribution of the L, M, and S plaque variants in mouse tissues are the same when lethal doses of these viruses are administered IP. Although the spleen and lymph nodes are the primary target tissues for mengovirus, the greatest concentration of virus is found in the brain and spinal cord. We demonstrated high titers of virus in the brains of mice infected IP with wild-type virus. However, no virus was detected in the brains of mice infected IP with either mutant suggesting that the mutants were unable to infect the nervous system.

HA^+ revertants were isolated from the brains of three different mice infected IC with mutant 205. In addition to regaining agglutination activity, the revertants were also virulent for mice, suggesting that hemagglutination and virulence may be phenotypically-linked traits. However, the revertants required 10^3- to 10^4-fold more PFU to kill mice than wild-type virus and the relative plaque size of the revertants was intermediate between wild-type and the mutants, suggesting that the degree of virulence of a particular mengovirus may be linked to its relative plaque size.

Of the three plaque variants isolated by ELLEM and COLTER (14) only the M variant agglutinates human erythrocytes. The infectivity of the M variant in mice infected IP was approximately 100-fold less than that of the L variant (13). The L variant shares a similar degree of virulence with the wild-type mengovirus used in our experiments, but does not agglutinate human erythrocytes. These data suggest that the patterns of virulence and

agglutination of the plaque morphology variants of ELLEM and COLTER (14) are distinct from mutants 205 and 280.

Unlike wild-type virus, mutants 205 and 280 did not agglutinate human type 0 or sheep erythrocytes, however, all three viruses were able to agglutinate rat erythrocytes. The differences in cellular receptor specificity likely reflect the expression of altered structural proteins by the mutants. These data also suggest that different erythrocyte species present different surface molecules which differ in their ability to function as virus receptors. Viral attachment proteins involved in the agglutination of rat erythrocytes may represent determinants distinct from those involved in human or sheep hemagglutination or alternatively, alteration of multifunctional viral attachment proteins may result in loss of human and sheep hemagglutination activity, but not rat hemagglutination activity. Although the mutants are likely to share extensive structural and antigenic homology with wild-type mengovirus, they exhibited different cellular receptor specificity. Similar results have been obtained in comparing the cellular receptor specificities among antigenically similar influenza viruses (12).

A mechanism for the agglutination of human erythrocytes by mengovirus can be proposed based on the data presented here. Since mengovirus did not agglutinate neuraminidase-treated erythrocytes, sialic acid residues on the surface of human erythrocytes may serve as receptor molecules for mengovirus. However, sialic acid failed to inhibit the agglutination reaction. Therefore, sialic acid must be recognized by the virus in a particular conformation or charge and/or in conjunction with other molecules (proteins, lipids, or other carbohydrates). BURNESS and PARDOE (9) reported similar results for the agglutination of human erythrocytes by EMC virus. Loss of hemagglutination activity was associated with treatment of erythrocytes with neuraminidase. This suggests that the mechanisms for mengovirus and EMC virus hemagglutination may be similar.

Glycophorin, the major sialoglycoprotein on human erythrocyte membranes, was shown to be a receptor for mengovirus by specifically altering the migration of wild-type mengovirus in sucrose gradients. BURNESS and PARDOE (10) have demonstrated that a particular chymotryptic peptide of glycophorin serves as a receptor for EMC virus and that sialic acid residues are necessary for its receptor activity. Since this peptide forms aggregates in solution, it is postulated that the receptor activity of the glycopeptide is due to its multivalence in the aggregated form. These data are consistent with ours showing that large amounts of glycophorin (5 to 50 μg) were necessary to alter the migration of wild-type virus in sucrose gradients. Since glycophorin aggregates in the presence or absence of SDS, a large number of the aggregates may be necessary to displace the virus into the pellet fractions.

The specificity of the glycophorin receptor activity for viral proteins of intact virions was examined by cross-linking studies. Glycophorin exhibited

binding affinity for the alpha (1 D) and gamma (1 C) proteins, however, the amount of the gamma (1 C) protein cross-linked to glycophorin was not as extensive as that of the alpha (1 D) protein. Therefore, cross-linkage of this protein may occur subsequent to the binding of the alpha (1 D) protein. Once glycophorin is bound to the alpha (1 D) protein, TDI molecules located distal to the glycophorin-mengovirus binding site(s) on the glycophorin molecule may bind to the gamma (1 C) protein, forming alpha (1 D)-glycophorin-gamma (1 C) multimers. HORDERN *et al.* (19) have shown that cross-linking reagents such as TDI, which act by binding positively charged amino acids, may cause the formation of alpha (1 D)-gamma (1 C) dimers. This supports our contention that the alpha (1 D) protein serves as the primary receptor for glycophorin and the agglutination of human erythrocytes.

In comparing the biological properties of two mengovirus mutants and revertants with those of the parental wild-type strain, we have gathered evidence suggestive of changes in the structural proteins of these viruses which may account for the expression of altered phenotypes. In the adjoining communication (3) we investigated the nature and the extent of the structural differences exhibited by these viruses. In addition, we compared physiological changes associated with virus-specified macromolecular synthesis in wild-type, mutant and revertant cells and attempted to determine whether these differences are related to the structural alterations exhibited by these viruses.

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