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Hemagglutinin-Neuraminidase Glycoprotein as a Determinant of Pathogenicity in Mumps Virus Hamster Encephalitis: Analysis of Mutants Selected with Monoclonal Antibodies

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With the aid of monoclonal antibodies directed against a specific site on the hemagglutinin-neuraminidase surface glycoprotein, four mutants of the Kilham neurotropic strain of mumps virus were isolated. All four mutants had increased neuraminidase activity. Two mutants (M10 and M12) lost their hemagglutination capacity with human O erythrocytes but retained their ability to agglutinate guinea pig erythrocytes at 4°C. A third mutant (M11) showed a change in the molecular weight of the hemagglutinin-neuraminidase glycoprotein. These three mutants (M10, M11, and M12) showed unaltered capacity to infect tissue cultures and to cause encephalitis in newborn hamsters. A fourth mutant (M13) retained its hemagglutination activity and capacity to infect Vero cell cultures but showed significantly lower neurovirulence in the suckling hamster brain than did the parental Kilham strain and the other three mutants. Both the number of infected neurons and the amount of infectious virus in the brain was reduced. On the other hand, there were no apparent differences in the occurrence of viral antigen in ependymal cells, indicating a selective change in affinity for neurons in the brain. These results suggest that certain changes in the hemagglutinin-neuraminidase glycoprotein may lead to an alteration of the neuropathogenicity of the Kilham strain of mumps virus.

Encephalitis in newborn hamsters caused by the Kilham strain of mumps has been a subject of extensive studies from the time it was first described (11, 13, 17, 34, 49–51). The characteristics of the virus which lead to its capacity to attack the nervous system are not well understood. It has been suggested, however, that the envelope glycoproteins play an important role (19, 21, 22) as has been shown in various virus host systems, e.g., other paramyxoviruses (9, 24, 37, 41), influenza viruses (14, 48), and reoviruses (6, 46). The envelope proteins of mumps virus which may be important for pathogenicity in encephalitis are the hemagglutinin-neuraminidase (HN) and the fusion proteins (10, 18, 29, 30). These two proteins have been implicated in the virulence of many paramyxoviruses. In Sendai (9, 41) and Newcastle disease virus (24) infections, cleavage of the fusion protein precursor is necessary for effective infection. The HN protein has two functions, hemagglutination (HA) activity which has been considered to be responsible for the adsorption of virus to cells (3) and neuraminidase activity which probably promotes the efficient spread of virus particles from infected cells (3). Antibodies to specific sites of measles virus hemagglutinin have been shown to alter the clinical course of encephalitis in mice (39), and antisera to simian virus 5 HN and fusion proteins prevent the spread of infection and cell fusion, respectively (20). Monoclonal antibodies directed against specific epitopes of viral proteins can now be produced. These can, in turn, be used to impose selective pressure on the virus replication and isolate variants resistant to the monoclonal antibodies used (16, 36, 38, 47). For instance, this method has been used in determining the neutralizing antigen site of poliovirus (5, 23).

In this study, a monoclonal antibody against a hemagglutinating site of the Kilham strain of mumps virus was utilized for the selection of mutants. The analyses of the mutants isolated led to the discovery of one which has a much lower capacity to induce encephalitis in newborn hamsters. This method seems to be promising in determining the importance of individual surface proteins in pathogenesis, as only one protein and primarily one of its epitopes is affected at a time. This is in contrast with the situation of various wild, vaccine, and laboratory strains which may show differences in many, if not the majority of proteins, thus making it difficult to determine which protein or epitope is partially or wholly responsible for certain pathogenic properties.

MATERIALS AND METHODS

Cell cultures. In all experiments, Vero cells were used. They were grown in Eagle minimal essential medium (Flow Laboratories, Irvine, Ayrshire, Scotland) supplemented with penicillin (60 μg/liter), streptomycin (50 μg/liter), and 5% inactivated newborn bovine serum (Flow Laboratories). Culture flasks (volume, 50 ml) and petri dishes (diameter, 60 mm) were purchased from NUNC, Roskilde, Denmark.

Virus and method of assay. The Kilham strain of mumps virus (13) was initially obtained from Jerry Wolinsky (University of Texas Health Science Center at Houston, Houston, Tex.) and was propagated in Vero cells. Plaque assays and purifications were performed in 34-mm petri dishes with a 3-ml overlay of 0.5% agar in HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (32) medium. For titrations of virus infectivity, 10-fold dilutions of the material were made, and 0.1-ml samples were inoculated per dish and adsorbed for 1 h. The plaques were counted 4 to 5 days after inoculation.

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Monoclonal antibodies. A large number of monoclonal antibodies against mumps virus has been extensively described in a separate publication (31). One monoclonal antibody designated as 5500 was used for mutant selection. This antibody gave effective hemagglutination inhibition (HI) and neutralization of the Kilham mumps virus strain but did not react with the RW strain. Monoclonal antibodies against other epitopes of the HN glycoprotein were used for characterization of the properties of mutants obtained.

Ascites was prepared by injecting hybridoma cells into the peritoneal cavity of mice as previously described (32). The antibody titer of the 5500 ascites used was $>10^6$ as measured with the enzyme-linked, immunosorbent assay microtiter technique (32). The protein content of the ascites of clone 5500 was 2.0 mg/ml (12), and the plaque reduction capacity with Kilham virus was 10^5; the endpoint was defined as an 80% reduction of plaques (32).

Mutant isolation. The mutant screening was done in 50-ml plastic flasks. Vero cells were infected with 100 μl of Kilham stock virus at a multiplicity of infection of 3×10^{-4} PFU per cell. After adsorption during gentle rocking for 60 min at 37°C, 10 ml of minimal essential medium containing 0.4 ml of ascites fluid was added. About 40 flasks were infected in each experiment and monitored daily for cytopathic effects in an inverted phase-contrast microscope.

HA and hemadsorption tests. HA tests were done in microtiter plates as previously described (28). The virus used was harvested from infected Vero cell cultures showing advanced cytopathic effects. The cell remnants and fluid were first sonicated and then spun for 10 min at 3,000 rpm and subsequently for 20 min at 6,000 rpm in a Sorvall GSA high-speed centrifuge. The pellet was then suspended in 1 ml of phosphate-buffered saline (PBS), sonicated, and diluted to ca. 3 ml in PBS. Subsequently, the material was Tween 80 and ether treated (25) and used for the test. The total amount of protein was determined (12), and the protein concentration of each virus preparation was adjusted to the same level. Tests were performed with human O and guinea pig erythrocytes at incubation temperatures of 4 and 22°C.

In the hemadsorption test, Vero cells in 25-ml flasks were infected with a multiplicity of infection of 1 to 3 PFU per cell of each mutant (M10, M11, M12, and M13) and the parent Kilham strain. After 2 days, syncytia were widespread and approximately equal in all flasks. Then, 1 ml of 0.5% human type O blood was added to each flask, distributed evenly, and allowed to adsorb to the cell monolayer for 0.5 h. The test was performed both at 4 and 22°C. Thereafter, the cell monolayer was washed twice with PBS and examined under an inverted microscope.

Neuraminidase test. The neuraminidase test was done as previously described (28, 32), with fetuin as a substrate and a pH of 5.0 for the reagent mixture. The virus material used was extracellular virus from the supernatant of infected cells showing extensive cytopathic effects. The virus was pelleted at 13,000 rpm for 90 min in a Sorvall GSA rotor; after being suspended in PBS, the pellet was run through a 30% sucrose gradient in Tris-hydrochloride buffer (pH 7.4) at 36,000 rpm for 90 min in an SW40 Sorvall ultracentrifuge rotor. Subsequently, the pellet was resuspended in 1 ml of sodium acetate buffer at pH 4.5 and sonicated thoroughly. Test samples were adjusted to contain equal amounts of protein.

Adsorption test to Vero cells. Confluent Vero cell cultures in 60-mm petri dishes were used (35). Two hours before starting the test, a medium change was performed. Each petri dish was inoculated with an equal amount of virus, ca. 100 PFU/0.1 ml in each. After regular time intervals, from 5 to 60 min, four plates were taken and washed with 10 ml of minimal essential medium; residual fluid was then removed. Subsequently, an overlay of 0.5% agar in HEPES medium with 5% newborn bovine serum was added. Five days later, the plaques were counted.

Characterization of antigen epitopes. RIPA. The radioimmunoprecipitation assay (RIPA) was done as previously described (33, 42). Each virus mutant and the Kilham strain were inoculated onto Vero cells in 50-ml plastic flasks at a multiplicity of infection of 1 to 3 PFU per cell. After 10 to 20% of the cell layer had begun to show cytopathic effects, 60 μCi of [^{35}S]methionine in 3 ml of minimal essential medium containing only 25% of the usual amount of methionine was added. Subsequent steps have been described in previous publications (33, 42).

HI test. The HI test used here has been described previously (28, 32). Four HA units of antigen were used in each ascites dilution in the test. The characteristics of the monoclonal antibodies used (2015, 5342, 5374, and 5500) are described in a separate publication (31). The various antibody clones are directed against various epitopes of the HN glycoprotein. The test was performed at 4°C with guinea pig blood.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Gel electrophoresis was done as previously described (30). The radioactively labeled extracellular virus (as in RIPA procedure) in the supernatant fluid of an extensively infected cell culture was spun on a 65%-30% sucrose gradient in Tris-hydrochloride buffer at pH 7.4 for 90 min at 24,000 rpm in a Sorvall ultracentrifuge. The interphase was then collected and diluted in Tris-hydrochloride buffer. Subsequently, the sample was pelleted in an SW40 Sorvall ultracentrifuge rotor at 25,000 rpm for 60 min. Finally, the pellet was collected in RIPA sample buffer (42); the radioactivity was then counted in a Beckman scintillator and adjusted to correct sample volumes before being mounted on a polyacryamide gel (30).

Virus infection of animals. Locally bred newborn (<20 h old) Syrian golden hamsters were used in this experiment. The inoculation titer of the Kilham strain and the mutant variants was 2×10^7 PFU/ml. The animals were injected intracerebrally in the midline with a 27-gauge needle. The inoculum consisted of 0.03 ml of virus material.

For infectivity titrations of brains, several hamster litters were infected. Generally, four hamsters were picked randomly and sacrificed at each time point. Between days 8 and 12, most Kilham-infected animals died. Thus, brains taken from these animals on and after day 8 cannot be considered picked at random. The brains from sacrificed animals were homogenized in a mortar, mixed to a 10% solution in PBS, and frozen at −70°C until titers were determined by the plaque assay method (51).

For immunofluorescence, the brains were rapidly frozen in dry ice (15), mixed with isopentane, and stored at −70°C until used. Coronar sections, 3 μm thick, were cut through the brains with a cryostat, fixed in cold acetone (−20°C), and stored frozen (−70°C) until stained. For the indirect immunofluorescent stainings, monoclonal antibodies against the nucleoprotein (designated as 781) were used. A dilution of 1:25 proved to be optimal. Controls with noninfected brain slices and mumps-infected Vero cells were included. The staining was performed as previously described (15).

For histological analysis, infected animals were perfused through the heart with 5% glutaraldehyde in Sörensen phosphate buffer. The brains were postfixed in Formalin and embedded in paraffin; sections were then cut and stained

TABLE 1. HA and hemadsorption patterns of the Kilham strain and four mutants[a]

Virus sample	HA titers in[b]:				Hemadsorption (with human O blood)	
	Human O blood		Guinea pig blood			
	at 4°C	at 22°C	at 4°C	at 22°C	at 4°C	at 22°C
Kilham	64	16	128	256	+4	+4
M10	0	0	512	0	+2	0
M11	512	256	2,048	1,024	+4	+4
M12	0	0	1,024	0	+2	0
M13	512	512	8,192	2,048	+4	+4

[a] The protein content of samples was 3.7 mg/ml.
[b] Reciprocal of dilution.

with hematoxylin and eosin and Luxol fast blue stain with cresyl violet acetic acid.

RESULTS

Isolation of HN mutants. Several mutants which grew in the presence of the anti-HN (5500) antibodies were isolated. Four effectively growing mutants were selected for purification and further analysis. These were designated as M10, M11, M12, and M13. The mutant strains were passaged three times in Vero cells in the antibody-containing medium used for their isolation. After passaging, they were plaque purified twice. In between the plaque isolations, the mutant material was mixed with the monoclonal antibodies and grown in their presence. After the second plaque purification, a stock of each mutant was prepared and then subjected to further analysis. The homogenicity of the virus population of the stock material was controlled by a plaque reduction assay (32). Identical numbers of plaques were obtained in the absence and presence of the anti-HN 5500 antibodies.

Characterization of functional properties of the four mutants. The hemagglutinating and hemadsorption patterns of the Kilham strain and the four mutants are shown in Table 1. Mutants M11 and M13 showed increased HA with human O and guinea pig erythrocytes compared with the parent Kilham strain. On the other hand, mutants M10 and M12 had no hemagglutinating activity with human O blood at 4 and 22°C or guinea pig blood at 22°C. However, at 4°C, both of these mutants (M10 and M12) demonstrated increased hemagglutinating activity compared with the parent strain.

The neuraminidase activity of the four mutants was found to have increased compared with the parental Kilham strain (Table 2). This indicates changes in the HN protein structure distant to the attachment site of clone 5500.

The adsorption test to Vero cells (data not shown) revealed no significant differences in the adsorption kinetics between the Kilham strain and the four mutants. Similar amounts of infectious virus particles had adsorbed after 60 min in all cases.

TABLE 2. Neuraminidase activity of the Kilham strain and four mutants

Virus sample	Absorption at 549 nm[a]
Kilham	0.14
M10	0.39
M11	0.67
M12	0.34
M13	0.29

[a] The absorption at 549 nm was measured after 30 min of reaction. The protein content of each sample was 4.2 mg/ml.

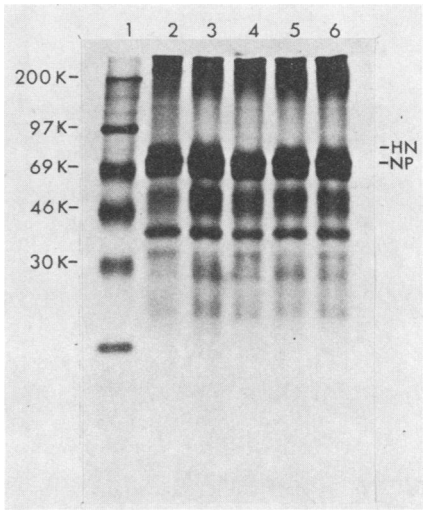

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the Kilham strain and four mutants. Shown from left are the molecular weight marker (lane 1), Kilham strain (lane 2), M10 (lane 3), M11 (lane 4), M12 (lane 5), and M13 (lane 6). The electrophoretic mobility of the HN glycoprotein of the M11 is slightly altered. The nucleoprotein (NP) closely precedes the HN.

Physicochemical and immunological characterization of the HA glycoprotein of the virus mutants. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis and RIPA pictures are shown in Fig. 1 and 2, respectively. Both pictures show a reduction of the molecular weight of the HN protein of the M11 mutant. The other mutants, M10, M12, and M13, showed no differences in the molecular weight of the HN molecule compared with the parental Kilham strain. As expected, no bands are seen on the RIPA picture in lanes 8 to 11 in which the mutants were mixed with the 5500 antibody. On the other hand, the HN protein is very distinct in lanes 12 to 21 in which antibodies to other epitopes on the HN molecule were used (5342 and 5374).

Two-dimensional gel electrophoresis (26, 27) of the four mutants and Kilham strain confirmed the lower molecular weight of the HN protein in M11 but did not reveal other changes in the viral proteins (data not shown).

The results from the HI test are shown in Table 3. The HI titers of the Kilham strain and mutants M11 and M13 show a similar pattern. In contrast, mutants M10 and M12 demonstrate a somewhat altered reactivity, suggesting a conformational change of the HN molecule. As expected, no HI activity was detected when the four mutants were reacted with the 5500 antibodies.

Encephalitogenic activities of the virus strains. Animals infected with the Kilham strain usually started to show signs of disease 5 to 7 days after inoculation. They developed lethargy, wasting, incoordination of limbs, impaired ability to suck, and arched backs. No seizures or signs of pareses were noted. Most animals died within 48 h after the onset of disease, and only a few hamsters survived. The animals infected with M10, M11, and M12 showed similar symptoms as did the Kilham-infected ones. In contrast, the M13-infected animals showed strikingly less signs of disease. Only somewhat reduced activity and slight incoordination of movements was occasionally noted ca. 4 to 6 days after injection. Most animals survived beyond 2 weeks. In the

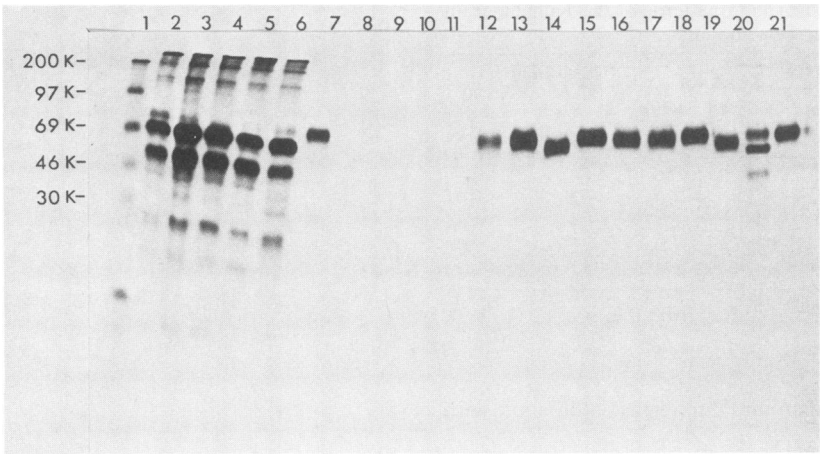

FIG. 2. RIPA of the parental Kilham strain (lanes 2, 7, 12, and 17) and four mutants M10 (lanes 3, 8, 13, and 18), M11 (lanes 4, 9, 14, and 19), M12 (lanes 5, 10, 15, and 20), and M13 (lanes 6, 11, 16, and 21). The precipitations were made against rabbit anti-mumps whole serum (RS, lanes 2 to 6), anti-HN antibody clone 5500 (lanes 7 to 11), anti-HN antibody 5342, and anti-HN antibody clone 5374. Lane 1, Molecular weight marker. There are no precipitations in lanes 8, 9, 10, and 11 in which the four mutants were reacted with antibody 5500, i.e., the one they were selected with. Antibodies 5342 and 5374 are directed against other epitopes of the HN glycoprotein. The electrophoretic mobility of the HN molecule of M11 (lanes 14 and 19) has slightly increased. The three bands in lane 20 probably only signify a breakdown of the HN molecule during the RIPA processing.

second and third weeks of life, signs of hydrocephalus appeared in all observed cases.

Initial screening with immunofluorescence revealed no difference in viral infection of brains infected with the M10, M11, and M12 mutants compared with the Kilham strain. On the other hand, a marked difference was found between the M13 mutant and the Kilham strain. The M13 and Kilham strains were therefore subjected to a closer evaluation and comparison. Several sections from four to six brains, sampled at three time points (5, 7, and 11 days) postinfection were examined. Approximate grading of the infection was done and performed blindly under coded numbers; infection was much more extensive in the Kilham virus-infected animals (Fig. 3). In virtually all animals infected with this strain, a widely distributed and general viral infection was seen (Fig. 4a), but in M13-infected brains only single neurons (Fig. 4b) or small clusters of foci (Fig. 4c) of neurons were infected. The antigen had a granular distribution in the neurons and extended into dendritic processes. The ependymal cells were involved both with Kilham and M13 strains (Fig. 4d), and there was no apparent quantitative difference between the two.

Histologically, hamsters sacrificed 5 and 7 days after infection with the Kilham strain showed a marked infiltration of mononuclear inflammatory cells in the leptomeninges and around intracerebral vessels. There was also marked inflammation at the ependyma; many ependymal cells were degenerated and sloughed. The choroid plexus was better preserved. There was widespread cellular necrosis in both the cerebrum and brain stem, most striking 7 days after infection. After infection with the M13 strain, the inflammatory cells infiltration was less pronounced, but cells were present both in the leptomeninges and around intracerebral vessels. Degeneration of ependymal cells and occlusion of the aqueduct was present. Cellular necrosis in the brain parenchyma was not evident. All hamsters showed marked dilatation of the ventricular system.

TABLE 3. HI pattern of four monoclonal antibodies reacted with the Kilham strain and four mutants[a]

Monoclonal antibody	HI titer (reciprocal of dilution) for:				
	Kilham	M10	M11	M12	M13
5500	16,400	<2	<2	<2	<2
5342	128	8	32	8	64
5374	64	32	32	32	32
2015	8	8	8	16	16

[a] The test was performed with guinea pig blood at 4°C. Tests were read after incubation overnight.

Replication of infectious virus in animal brains. There proved to be a much greater multiplication in vivo of the Kilham strain than the M13 mutant. Titration curves (Fig. 5) indicate an initial proliferation of the M13 which had already reached its highest levels at day 2. After this, the M13 titers were stable past day 6 and then sharply decreased but remained detectable through day 16. The Kilham strain titers, on the other hand, increased through day 6, after which most animals died. Remaining survivors at days 8 and 10 showed a lowering of viral titers. The maximal virus titers present in the Kilham-infected brains were ca. 35 times greater than those infected with the M13 mutant.

DISCUSSION

The four mutants characterized in this study were selected by the same monoclonal antibody and thus were altered in the same epitope on the HN glycoprotein. Possibly, other slight changes in antigenic properties were detectable by monoclonal antibodies against three other epitopes. Despite the same mode of selection of the four mutants, markedly different biological properties were observed. One of the mutants (M13) altered neurovirulence, but this did not correlate with changes in other biological activities. The other three mutants showed the same capacity to cause encephalitis in newborn hamsters as did the parental strain. However, two of them (M10 and M12) lost their hemagglutinating activity for human O cells, and the third mutant (M11) showed a reduction in the molecular weight of the HN glycoprotein without any apparent consequences for the neurovirulence of the virus.

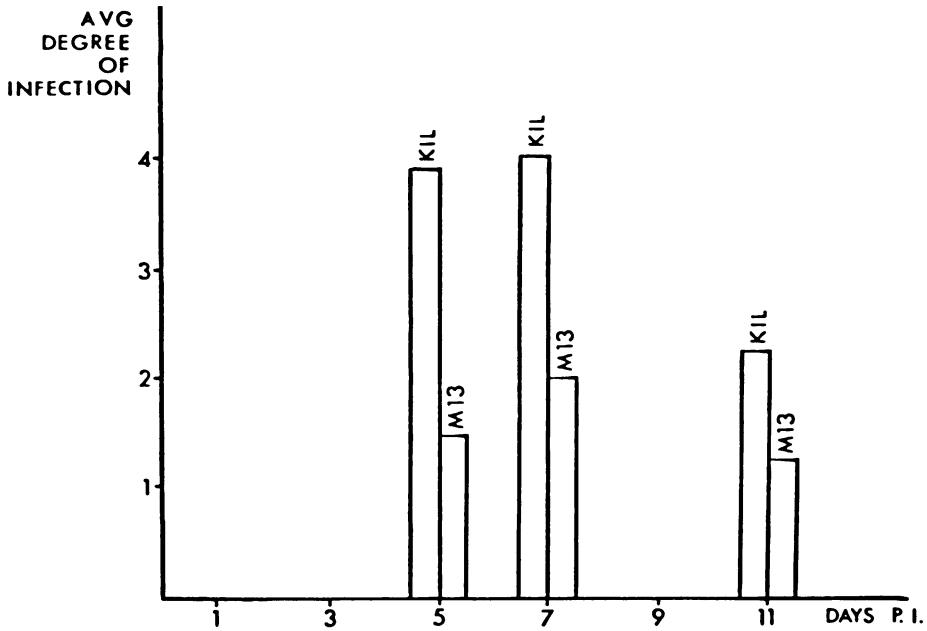

FIG. 3. Immunofluorescence study of the Kilham strain (KIL)- and M13-infected brains. The severity of infection (number of infected neurons in a coronal section from the brain) was graded as follows: 1, 0 to 100; 2, 100 to 500; 3, 500 to 1,000; and 4, ≥1,000. Each column represents an average of four to six animals. From each animal, three to five sections were examined. P.I., Postinfection.

The altered hemagglutinating characteristics of mutants M10 and M12 can be interpreted as a result of a combined effect of increased neuraminidase activity and a less efficient interaction between the hemagglutinin and its receptor. It is of interest that all four mutants showed increased neuraminidase activity. Since the anti-HN 5500 antibodies interfere with multiple functions of the HN molecule (31), the primary change could be either at the hemagglutinin, neuraminidase, or another site, with secondary allosteric changes in one or both of these two. Thus, the role of neuraminidase in neuropathogenicity still needs to be defined (22).

The hemagglutinating activity of various mumps strains differs slightly (2). Most strains agglutinate fowl, guinea pig, and human erythrocytes at any temperature between 4 and 37°C. The hemagglutinating capacity of the Habel (2) mumps strain differs from others, as it only agglutinates guinea pig erythrocytes. On the other hand, marked differences in hemagglutinating activity between various strains and mu-

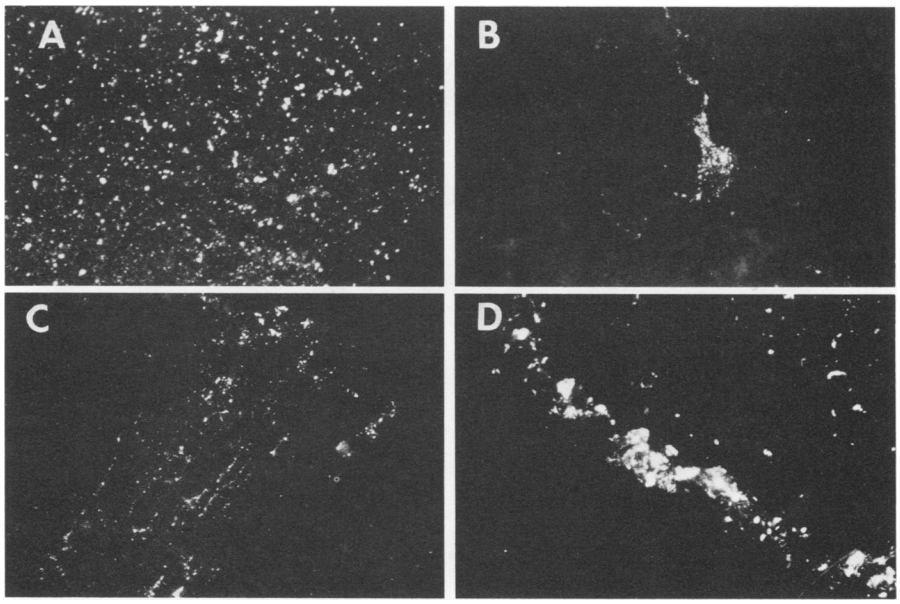

FIG. 4. Immunofluorescence picture of Kilham virus-infected brain showing extensive viral infection (A). Magnification, ×225. M13-infected brain exhibiting a single infected neuron (B). Magnification, ×375. M13-infected brain demonstrating a cluster of infected neurons (C). Magnification, ×225. Infected ependymal cells (D). Both the Kilham strain and M13 showed similar magnitude of infection. Magnification, ×375.

tants have been described in other viral systems, e.g., measles (1, 45) and vaccinia (43, 44).

The carbohydrate structure and content of the glycoproteins presumably has an effect on their function (3, 7, 40). Achange in glycosylation as a result of a mutation could therefore contribute to changes in biological activities, such as HA, adsorption, and neurovirulence. In addition, physicochemical properties such as molecular weight could change with altered glycosylations. This is a possible reason for the increased electrophoretic mobility of the HN protein of the M11 mutant.

The observed difference in neurovirulence between the original Kilham strain and its derivative M13 indicates that the HN glycoprotein plays an important role in neurovirulence. Thus, a certain conformation of the molecule may be necessary for neurovirulence, a property retained in the mutants M10, M11, and M12, but partially lost or destroyed in the M13 variant. It is of interest that the 5500 monoclonal antibodies which were used for selection of the mutants do not react with the RW strain of mumps; RW is known to be significantly less neurovirulent than the Kilham strain (19). The observation that viral infection was similar in ependymal cells of both Kilham- and M13-infected brains may imply that different receptors or modes of replication are involved in the two cell types, i.e., ependyma and neurons. The ependymal infection may cause the high incidence of hydrocephalus observed in hamsters infected with the M13 mutant. Previous studies of mumps strains (19) have also shown that RW and Kilham strains have variable affinity for ependymal cells and neurons.

The possibility that more than one change occurred in one or more of the mutants must be borne in mind. The probability of such additional changes in the four mutants is relatively low (8). Also, a screening of the mutants against a series of monoclonal antibodies to the fusion, nucleoprotein, polymerase, and matrix proteins revealed no additional differences between the parental Kilham strain and the four mutants. This strengthens the view that the change in virulence in the M13 mutant is a result of a modification in the HN molecule only. Still, it remains possible that factors other than the HN protein are necessary for neurovirulence. Previous studies of pathogenic determinants have mainly been done in the reovirus system (6, 46). The three outer capsid proteins of these viruses have highly distinct and specialized roles. One, the σ1 (hemagglutinin), is responsible for binding to cell surface receptors; another, the μ1C, determines the capacity for viral growth in intestinal tissues and its spread in the host; the third, σ3, inhibits protein and RNA synthesis. Presumably, similar determinants of pathogenicity exist in other viral systems. Such information has begun to emerge from studies on rabies virus (4) which can be rendered nonpathogenic after treatment with neutralizing monoclonal antibodies to a surface glycoprotein.

The ease with which antibody-resistant mutants appear raises several important questions about the pathogenesis of disease. First, can antibody-resistant mutants appear in vivo? Preliminary results (R. Rydbeck and A. Löve, unpublished data) indicate a similar frequency of antibody-resistant mutants appearing in vivo and in vitro. This has also been found in rabies (4, 16). Second, can the mutants compete successfully with the parent virus? Should the mutants be in some way defective or noncompetitive, their significance in pathogenesis would evidently be minimal. Third, are the mutants stable? Some mutations are unstable (4) (presumably point mutations), thus rendering the mutant biologically insignificant. Fourth, could altered tissue tro-

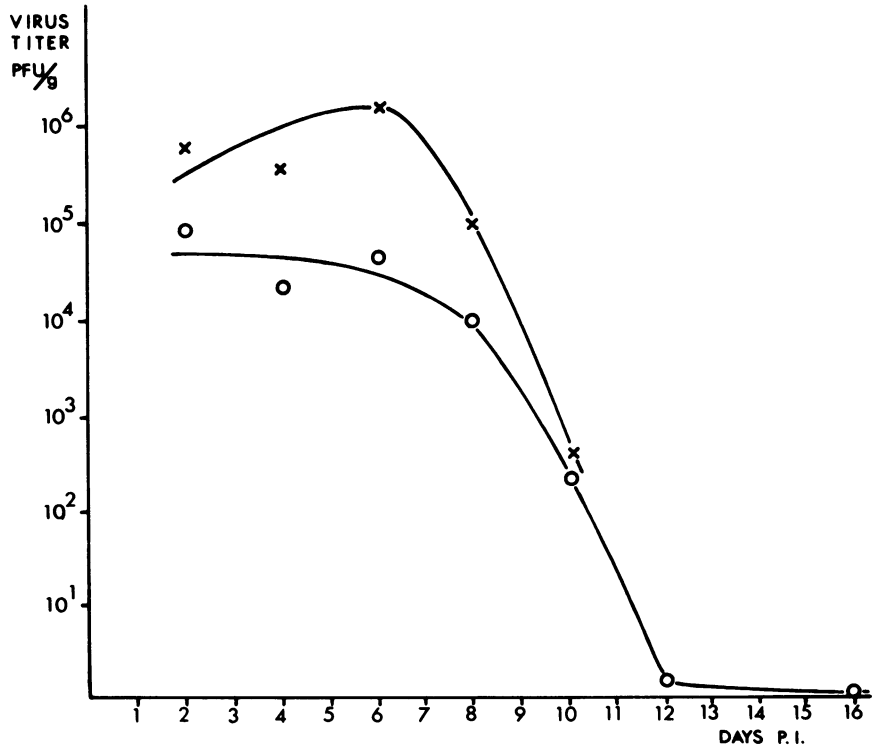

FIG. 5. Titration of viral infectivity in brains infected with Kilham strain (X) and M13 (O). Each point represents an average titer from four infected brains.

pism give mutants a selective advantage (6)? Another point would be previous exposure of the host to related viruses and thus its antibody profile. A host having antibodies against one or several epitopes of a new challenging strain would provide a fertile environment for the rise of mutants. Consecutive mutations in this manner could be an evolutionary explanation of the diversity of viruses. Active immunization with whole virus or intact structural components would pose none of these problems, because of the polyclonal immune response involved, unless the challenging strain shared only few epitopes with the vaccine strain. Lastly, mutants could be important in passive immunization with monoclonal antibodies. Antibodies to a single site could promote resistant mutants; therefore, the consequences of such immunizations should be carefully evaluated.

This approach in elucidating determinants of pathogenicity in vivo with mutants selected with site-specific monoclonal antibodies could prove to be very useful in determining which components, particularly surface components, are the main contributors of pathogenicity and virulence. This, in turn, can provide useful information in evaluating the efficiency and dangers of vaccines or other methods which are used in the prevention and treatment of infectious disease.

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