Role of Hemagglutinin Cleavage and Expression of M1 Protein in Replication of A/WS/33, A/PR/8/34, and WSN Influenza Viruses in Mouse Brain

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The combined presence of WSN gene segments 6 (neuraminidase), 7 (M1 and M2), and 8 (NS1 and NS2) in reassortants of WSN with A/Aichi/2/68 (H3N2) has been found by others to be necessary for full expression of neurovirulence in mice. We are examining the expression of the analogous three gene segments in brains of mice after intracerebral infection with non-neuroadapted strains A/WS/33 (WS) (from which WSN was derived) and A/PR/8/34 (PR8). Our aim is to determine possible mechanisms by which one or more of the five gene products may restrict replication of these strains in mouse brain cells to a single cycle, yielding noninfectious hemagglutinating particles (incomplete growth cycle). We found that minority subsets of such particles did produce plaques, provided they were activated by trypsin (analogous to other abortive systems producing virions with uncleaved HA), a step obviated for some WSN virions by indirect promotion of hemagglutinin cleavage by the neuraminidase of that strain. The percentage of such potentially infectious virions, relative to total hemagglutinating particles, was significantly lower in WS- or PR8-infected than in WSN-infected brains, suggesting possible defects in synthesis or function of M1 protein in the former. Cells in immunostained sections and appropriate bands in Western blots (immunoblots) of viral proteins electrophoretically separated from lysates of PR8-infected brains reacted with antibody to nucleoprotein but not to M1 protein. Either method revealed the presence of both proteins in WSN-infected brains. In contrast, Western blot analyses of particles concentrated from PR8-, WS-, or WSN-infected brains by hemadsorption, elution, and pelleting did reveal NP and M1 bands with comparable relative peroxidase-antiperoxidase staining intensities. The findings suggest that availability of M1 protein is a factor influencing the extent or rate of assembly of potentially infectious (i.e., trypsin-activated) progeny virions in mouse brains and that in this respect the two non-neurovirulent strains differ from WSN quantitatively rather than qualitatively.

Human infections with representative members of most families of animal viruses are associated with occasional, sometimes pathognomonic, neurological illnesses (24). An exception seems to be human influenza, in which neurological complications or sequelae are rarely reported and have been even less frequently proved by rigorous etiological evidence (for reviews, see references 13, 26, and 52). The intriguing question of a possible causal relationship between the pandemic of 1918 to 1919 and the overwhelming worldwide occurrence of von Economo's encephalitis lethargica, with its common sequel of postencephalitic Parkinsonism, has remained unresolved for 70 years (3, 14, 34, 42). During the destructive epizootic in seals caused by an avian H7N7 strain (A/seal/Mass/80), virus was isolated from lungs and brains of dead seals (60). It caused severe conjunctivitis in accidentally infected laboratory workers (59) and was capable of replicating in brains of mice (60). This episode in a mammalian host led Webster et al. (61) to ask "what would happen if such an event occurred in man instead of seals? Would the resulting pandemic be similar to that of 1918-19?" We believe that this question ought to include concern about possible neuropathological effects in such a pandemic.

Experimental evidence linking human influenza viruses to potential neurovirulence is the adaptation of A/WS/33 (WS) to mouse brain, yielding the variants NWS (51) and WSN (15). These strains are able to undergo multicycle replication and induce brain lesions, resulting in acute fatal encephalitis in adult mice after intracerebral (i.c.) inoculation or in newborn mice as part of generalized pantropic spread after intranasal infection (58).

The seemingly unique properties of these variants have been examined repeatedly by genetic analysis of recombinants (extensively reviewed in references 26 and 53). It has been reported that among cloned reassortants derived from crosses of WSN and A/Aichi/2/68 (H3N2), only those possessing WSN gene segments 6 (neuraminidase [NA]), 7 (M proteins), and 8 (NS proteins) express full neurovirulence in adult mice (54). Moreover, among reassortants between non-neurovirulent avian A/fowl plague/Rostock/34 (H7N1) and human H1N1, H2N2, or H3N2 strains, only those carrying at least one of the RNA polymerase genes (usually segment 2 [PB1]) of the human parental strain, in addition to avian H7N1 segment 4 (hemagglutinin [HA]), are reported to be neurovirulent in newborn mice infected intranasally (2). Such analyses show that human strains or their derivatives can contribute to at least part of the genetic information that determines the neurovirulent phenotype.

It has been known for a long time that non-neurotropic, egg-adapted human strains such as WS, A/PR/8/34 (PR8), and B/Lee/40 (Lee), when inoculated i.c., induce in mice (i) toxic convulsions and often death within 12 to 72 h after large doses of infectious, but not of inactivated, virus (18, 19,
in Eagle minimal essential medium plus 10% heat-inactivated calf serum at 37°C, 5% CO₂. Primary chicken embryo fibroblasts were grown in the same medium. Confluent MDCK or, rarely, chicken embryo fibroblast monolayers in 50-mm plastic petri dishes were washed with phosphate-buffered saline (PBS) and then infected with 0.5 ml of sample diluted in PBS. After adsorption for 1 h at 37°C, the inoculum was replaced by 5 ml of serum-free overlay medium (Earle balanced salt solution, Eagle essential amino acids, minimal essential medium nonessential amino acids, minimal essential medium vitamins and L-glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 0.9% Noble agar). Plaque titrations were usually done in parallel in three different ways, designated TR— (sample not pretreated with trypsin, overlay contains no trypsin), TR+ (sample not pretreated, overlay contains p-tosyl-L-phenylalanine chloromethyl ketone hydrochloride-treated trypsin [TPCK-TR] at 2 μg/ml), and TR++ (sample pretreated with TPCK-TR [10 μg/ml] for 20 min at 37°C and overlay contains TPCK-TR). TR— and TR++ monolayers were stained after 3 days at 37°C with 0.025% neutral red and counted; TR+ plates were overlaid on day 3 with 2 ml of nutrient agar containing 5% fetal calf serum and then stained and counted the next day.

(ii) EID₅₀. For determination of 50% egg infective dose (EID₅₀), dilutions of trypsin-treated or untreated samples were inoculated intraallantoically into 11-day embryonated eggs. EID₅₀ titers were based on HA monitoring at 48 h p.i.

(iii) HA particles. The estimated number of hemagglutinating particles (HA particles) is given by (reciprocal of HA titer) × (number of erythrocytes [RBC]) and expressed per unit of wet weight or volume of the starting material (12, 62).

Infection of mice. Outbred Swiss mice, 4 weeks old or pregnant, were purchased from a commercial dealer. Volumes of i.c. inocula were 20 μl for mice up to 10 days old and 30 μl for weanlings; all inoculations were done under ether anesthesia. For harvest of tissues, mice were deeply anesthetized with ether or chloroform; usually, the chest cavity was opened aseptically and the right atrium was punctured to allow partial exsanguination, occasionally followed by partial perfusion through a 26-gauge needle inserted into the aorta of the heart.

Preparation of brain extracts. Brain extracts were prepared as previously described (45, 62) with modifications: a 10 to 20% (wt/vol) homogenate in PBS or Tris-buffered saline (TBS) containing 3% crystalline Vibrio cholerae NA (Calbiochem-Behring, La Jolla, Calif.) was incubated for 1 h at 37°C and then centrifuged for 15 min at 1,500 × g. A small portion of the supernatant was set aside for infectivity titrations; the remainder was mixed with 1/3 volume of a 4.5% packed fowl RBC suspension in 0.85% NaCl containing 3% sodium citrate. After hemadsorption (HAds) for 1 h on ice with occasional shaking, the RBC were sedimented, washed three times with ice-cold PBS, and then suspended in 1/5 to 1/10 volume (relative to original mouse brain homogenate) of 1% NA in prewarmed PBS or 0.85% NaCl. After 1 h at 37°C, the RBC were sedimented. The supernatant eluate was titrated for HA and then either made 0.2% with bovine serum albumin (BSA) and stored frozen or centrifuged at 55,000 × g for 1 h at 4°C; the usually pink pellet was suspended in 1/10 to 1/100 volume (relative to eluate) of PBS, resulting in a final 100-fold concentration (in terms of brain wet weight) of HAd material. This final preparation was used for HA titrations, total protein determination (33), sodium dodecyl sulfate-polyacrylamide
gel electrophoresis (SDS-PAGE) and Western blot (immunoblot) analyses, as described below.

SDS-PAGE. Two procedures were used for SDS-PAGE. In procedure 1, trichloroacetic acid-precipitated samples, dissolved in buffer containing 1.25% SDS, 0.156 M Tris hydrochloride (pH 6.8), 12.5% glycerol, 1.25% β-mercaptoethanol, and 0.001% bromophenol blue, were boiled for 2 min. Polyacrylamide stacking and separating gels were prepared according to the method of Laemmli (29), except that N,N,N′,N′-tetramethylenediamine and ammonium persulfate were present at concentrations of 0.04% (vol/vol) and 0.04% (wt/vol), respectively. Electrophoresis was done in slab gels (16 by 14 by 0.15 cm; dual vertical slab gel apparatus; Bio-Rad Laboratories, Richmond, Calif.) with a constant current of 200 mA in transfer buffer adjusted according to the method of Towbin et al. (55) except that the pH 8.6 with 4 N NaOH. Nitrocellulose strips transfers were done overnight in a Bio-Rad Trans-Blot cell with shaking for 2 h at room temperature with the antibody diluent, and washed again as before.

Antibodies for Western blots. Rabbit antisera to type A NP, prepared against reassortant X-73 (NP from H3N2), was a generous gift from Kathleen van Wyke-Coelingh, National Institute of Allergy and Infectious Diseases (Bethesda, Md.); when used in Western blot analyses of concentrated stock virus preparations, this serum cross-reacted with other viral proteins. Antiserum to type A M1 protein, designated rabbit 3735, final HPLC (high-pressure liquid chromatography), was a gift from Doris Bucher, then at Mount Sinai School of Medicine (New York, N.Y.) who also supplied monoclonal anti-M1 ascitic fluids X-73 (M1 from PR8) and 28-BioF1 10/84.

Immunohistological examination of mouse brains. Immunohistological experiments were done with PR8 and WSN virus stocks in use at the Institut für Virologie, Justus-Liebig-Universität, Giessen, Federal Republic of Germany, which have hound histologic sections different from that of the stock viruses described above. Mouse brains were fixed, processed, sectioned, and immunostained with rabbit antiserum reacting monospecifically with NP or M1 protein as described by Reinacher et al. (43).

RESULTS

The work leading to the initial report on incomplete replication of WS, PR8, and Lee strains in MB after i.c. inoculation (45) preceded the discovery that infectivity of progeny virions depends on cleavage of HA by cellular proteases (and that fully assembled virions produced in host systems lacking the appropriate cleavage enzyme can be made infectious by treatment with trypsin, plasmin, or other suitable proteolytic enzymes (27, 30). Therefore, we first examined the effect of trypsin on PFU and EID₅₀ titers of brain extracts prepared at various intervals after i.c. inoculation of PR8 or WSN.

In the experiment shown in Fig. 1, mice aged 4 to 6 days were inoculated with fivefold serial dilutions of either WSN-infected mouse brain homogenate or PR8-infected allantoic fluid, ranging from 1 × 10⁵ to 8 × 10⁶ PFU/20 μl for WSN and from 4 × 10⁶ to 6.4 × 10³ PFU/20 μl for PR8. Brains were harvested at the times indicated from partially exsanguinated mice and processed as described in Materials and Methods. Plaque assays were done on low-speed supernatants of 10% (wt/vol) tissue homogenates; HA titrations were done on 20-fold concentrated eluates of HAds particles. For WSN, the yields of HA particles as well as of PFU, measured with or without trypsin activation, were independent of the infecting dose (Fig. 1A), as expected of a virus capable of undergoing multiple cycles of replication. In contrast, for PR8, the yields in terms of PFU(TR+) as well as of HA particles (where measurable) followed reasonably well the regression line plotted for the fivefold dilution steps of the inocula (Fig. 1B). This type of response is as expected for a system in which viral replication is restricted to the initially infected cells. In the case of PR8, no plaques were obtained without trypsin activation. However, the percentages of HA particles registering as PFU(TR++), though lower than for WSN, were significantly higher than those obtained previously by intraallantoic titrations in embryonated eggs without trypsin activation (45, 46, 62). Therefore, brains harvested at 48 (WSN) or 72 (PR8) h were also titrated in eggs with and without trypsin pretreatment (see Materials and Methods).

The results, shown in Fig. 1C along with those of the corresponding plaque and HA assays from Fig. 1A and B, were virtually identical by the two assay methods. However, whereas no plaques were produced by PR8-infected brain homogenates without trypsin activation, a small fraction of the virus was able to grow in eggs without such treatment, presumably because occasional particles gained access to protease produced by allantoic cells. The resulting titers were comparable to those reported in 1950 (45).

In a similar experiment, strain WSN was compared with its progenitor, WS, by i.c. inoculation of 10⁻³ PFU into mice ranging in age from 5 to 10 days (all groups were matched for age distribution). Brains harvested at 48 h p.i. contained...
10^{6.6} HA particles of either strain and 10^{7.7} or 10^{6.5} PFU/(TR− +), respectively, of WSN or WS.

Since all previously published experiments had been done in adult rather than suckling mice, it seemed possible that age might be a factor determining the yields of trypsin-activable infectious virions. Accordingly, 4-week-old mice were inoculated i.c. with comparable amounts of WSN, WS, or PR8. All infectivity assays were done on brains harvested at 24 h.p.i. All three strains yielded comparable TR++ and TR− plaque titers on MDCK cells or chicken embryo fibroblasts.

### TABLE 1. Yields of WSN, WS, or PR8 progeny recovered from brains of 4-week-old mice 24 h after i.c. inoculation

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Titer (log_{10})/brain*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PFU/30 μl (log_{10})</td>
</tr>
<tr>
<td>WSN</td>
<td>5.0</td>
</tr>
<tr>
<td>WS</td>
<td>5.5</td>
</tr>
<tr>
<td>PR8</td>
<td>5.4</td>
</tr>
</tbody>
</table>

* For explanations, see legend to Fig. 1 and Materials and Methods. CEF, Chicken embryo fibroblasts.
TABLE 2. Proportion of PFU among HA particles recovered by HAds-elution from WSN- and PR8-infected mouse brains (from Fig. 1 and Table 1) and in four allantoic fluid stock preparations of each virus

<table>
<thead>
<tr>
<th>Virus</th>
<th>Source*</th>
<th>No. of samples</th>
<th>Mean % of HA particles scoring as PFU on MDCK cells*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>TR++</td>
</tr>
<tr>
<td>WSN</td>
<td>MB</td>
<td>12</td>
<td>6.99 (3.6–12.0)</td>
</tr>
<tr>
<td></td>
<td>AF</td>
<td>4</td>
<td>ND</td>
</tr>
<tr>
<td>PR8</td>
<td>MB</td>
<td>8</td>
<td>1.05 (0.08–1.76)</td>
</tr>
<tr>
<td></td>
<td>AF</td>
<td>4</td>
<td>ND</td>
</tr>
</tbody>
</table>

* MB, Mouse brain; AF, allantoic fluid.
* Figures in parentheses indicate range of values. ND, Not done; —, no plaques observed.
* Only six suitable samples.

fibroblasts (Table 1). Without activation, PR8 failed to produce plaques, whereas WS occasionally caused some minute areas of apparent cell death which were so ill defined that counting was not possible.

A conclusion from these experiments is that brain cells are capable of producing fully assembled, trypsin-activable progeny virions of PR8 and WS, both non-neurotropic strains. For PR8, the average yields of such virions, relative to the number of HA particles, were lower than those for WSN. For the WS parental strain, we do not have enough in vivo data (Table 2). In experiments in mouse embryo brain cell cultures, various parameters of the replication of this strain are intermediate between those of the other two strains (5, 6; G. L. Bradshaw and R. W. Schlesinger, manuscript in preparation).

Previous electron microscopic evidence (62) has suggested that the majority of HA particles recovered from infected tissues or cell cultures resemble cytoplasmic membrane-associated precursor particles ("viromicrosomes" as defined by Rott and Schäfer [44]; see Discussion). It seemed reasonable to ask whether strain-specific variations in availability of M1 protein might explain differences in the percentage of such particles progressing to maturation.

The first indication that expression of M1 protein might be restricted in PR8-infected mouse brain came from immunohistological examination of WSN- and PR8-infected brain sections reacted with monospecific anti-NP and anti-M1 antibodies. Examples of parallel sections of PR8-infected specimens with the two antibodies are shown in Fig. 2. In each pair, leptomeningeal (Fig. 2A and B) and/or parenchymal (Fig. 2C to F) and ependymal (not shown) cells displayed NP reactivity but no reactivity for M1 protein. Of special interest is the unequivocal reaction of putative neurons with anti-NP antibody (Fig. 2G). In contrast, comparable staining intensity was observed with both antisera in ependymal cells of WSN-infected brains (not shown).

Several methods were used in attempts to demonstrate and quantitate NP and M1 protein in brain extracts by

FIG. 2. Peroxidase-antiperoxidase-stained sections of PR8-infected mouse brains harvested 48 h after i.c. inoculation. Panels A and B, C and D, and E and F are pairs of parallel sections from the same acetone-fixed blocks reacted with monospecific anti-NP (A, C, and E) and anti-M1 rabbit serum (B, D, and F). (A and B) Leptomeninges and cortical parenchyma; (C and D) cross section of needle track inadvertently containing a mouse hair; (E and F) midbrain nucleus; (G) two putative neurons showing extension of anti-NP reaction into cytoplasmic processes. Markers, 30 μm.
Western blot analysis. The best results were obtained when frozen WSN- and PR8-infected brains from the experiment shown in Table 1 were extracted according to procedure 2 (see Materials and Methods). As described, centrifugation of such extracts yielded a clear supernatant, a turbid intermediate layer, and a pellet. Equivalent amounts of total protein of each fraction were subjected to SDS-PAGE. Each of the silver-stained gels contained some 70 bands, none of which were identifiable as viral proteins.

Western blots of parallel gels are presented in Fig. 3. The WSN samples (Fig. 3A) revealed approximately equivalent anti-NP- and anti-M1-reactive bands in the pellet fraction (lane d); in the interphase (lane c), the M1 band was most prominent, with NP greatly diminished relative to the pellet fraction; the supernatant (lane b) displayed reduced amounts of both proteins. The results suggest that in WSN-infected mouse brain extract, the two proteins form complexes which are enriched in the pellet; a relative excess of M1 protein, not similarly complexed, remained concentrated in the intermediate fraction. The partitioning of the PR8 sample (Fig. 3B) was strikingly different in that strong reactions with anti-NP were evident in each of the three fractions, none of which contained a detectable M1 protein band.

Since complete absence of M1 protein seemed incompatible with the presence of fully assembled virions in PR8-infected brains, hemadsorbed-eluted particles were further concentrated by pelleting. Figure 4 illustrates Western blot analyses of such particles with anti-NP and anti-M1. The M1/NP ratios calculated from densitometric tracings were similar within each of the two experiments shown in Table 3, which confirmed the biological evidence indicating that HA particles isolated from brains infected with either of the two non-neuroviral H1N1 strains, like those from WSN-infected brains, include subsets of fully assembled virions.

**DISCUSSION**

This study has had as its limited aim a reexamination of some features of the incomplete replication cycle of certain non-neuroadapted strains of human type A influenza virus in mouse brains, first described almost four decades ago (45). Knowledge gained in the intervening years has confirmed that expression of viral information may have pathological consequences even in the absence of infectious progeny virus detectable by conventional isolation methods. Moreover, the more recent identification of the specific variant genes that apparently enable the WSN strain and its reassortants to express the neurovirulent phenotype (54) permits us to focus on possible defects in the expression of the corresponding genes in brain cells infected with non-neurotropic H1N1 strains closely related to the WSN variant.

Immunofluorescence evidence for the presence of viral antigen(s) in WSN-infected adult mouse brains at 24 h after i.c. inoculation seems to be pretty much restricted to the ependymal lining of the ventricles and to the meninges (1, 16, 36, 38, 56). In the case of WSN, it seems that general spread of virus to brain parenchymatous cells occurs later, either by extension from ependyma and meninges or secondarily via hematogenous seeding. This process may possibly be slowed by natural killer cell and interferon activity in cerebrospinal and interstitial fluid during the first 24 h p.i., recently demonstrated in PR8-infected mice (57). We have shown in this paper that use of appropriate antibodies (anti-NP) can reveal limited involvement of parenchymatous cells in PR8-infected adult brains as well. Several other observations deserve emphasis, as discussed below.

Although WSN virus can undergo multiple growth cycles in mouse brains, even with this strain only a fraction of the progeny virions register as plaque formers on MDCK cells; addition of trypsin before and during assay can lead to significant amplification of infectious titers (e.g., 10-fold in Table 2). This finding is in accord with those reported for the same strain in different host cell systems (30). For example, Lazarowitz et al. (31) found that more than 85% of the HA of WSN virions grown in Madin-Darby bovine kidney cells in the absence of serum was uncleaved, whereas the HA of WSN grown under medium with 2% calf serum consisted of more than 90% HA1 plus HA2. This effect was attributed to the presence of plasminogen in calf serum, which was assumed to be converted to plasmin by host cell enzymes.

Such low efficiency of the cleavage mechanism would be consistent with the genetic evidence suggesting a decisive role for the WSN-specific NA (gene segment 6) rather than the HA itself (segment 4). If strain-specific structural features of the latter were the sole determinant of successful cleavage by cellular protease(s) in a permissive host system, then all or most HA molecules of maturing virions should be accessible to cleavage. On the other hand, if cleavage of only some small, critical number of HA molecules is needed to render a virion infectious (as we have suggested [5; G. L. Bradshaw and R. W. Schlesinger, manuscript in preparation]), then the proposed indirect facilitation by a specific NA would make sense: Murti and Webster (39) have shown that on influenza virions the NA spikes occur in one or a few discrete patches, whereas HA spikes are uniformly distributed over the entire surface. Such an arrangement could limit cleavage to HA molecules situated close enough to NA spikes to permit some required physical interaction, whatever its nature may be. Similar spatial restrictions might govern the effects of activation or inactivation of host cell proteases by different NAs, proposed by Schulman (47) as a
FIG. 4. (A) Western blot analysis of particles purified by HAds-elution and pelleting from WSN-infected (lanes 1), WS-infected (lanes 2), or mock-infected (lanes 3) mouse brain (MB) homogenates and of WSN stock virus (lanes 4), as described in Materials and Methods. The final suspended pellets of the brain samples were equivalent to 100-fold concentrates (wet weight/vol) of brain tissue. Triplicate gels and transblots were reacted separately with normal rabbit serum (left), polyclonal anti-NP which was broadly cross-reactive at high antigen concentration in purified WSN stock virus (middle) and monoclonal anti-M1 (right), as in Fig. 3. (B) Densitometric tracings of lanes 1 and 2 in middle (anti-NP) and right (anti-M1) portions of panel A. Weights of peaks and ratios are presented in Table 3. MW std, Molecular weight standard.

TABLE 3. M1/NP protein ratios calculated from weights of peaks in densitometric tracings of Western blots with purified and concentrated HAds particles from mouse brains

<table>
<thead>
<tr>
<th>Expt no.</th>
<th>Virus</th>
<th>Time (h) p.i.</th>
<th>Wt (mg) of peak</th>
<th>M1/NP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>M1</td>
<td>NP</td>
</tr>
<tr>
<td>1*</td>
<td>WSN</td>
<td>24</td>
<td>1.8</td>
<td>13.3</td>
</tr>
<tr>
<td></td>
<td>WS</td>
<td>24</td>
<td>2.0</td>
<td>13.7</td>
</tr>
<tr>
<td></td>
<td>PR8</td>
<td>24</td>
<td>1.1</td>
<td>13.4</td>
</tr>
<tr>
<td>2*</td>
<td>WSN</td>
<td>48</td>
<td>7.3</td>
<td>13.8</td>
</tr>
<tr>
<td></td>
<td>WS</td>
<td>48</td>
<td>2.4</td>
<td>6.6</td>
</tr>
</tbody>
</table>

* Shown in Table 1.
* Shown in Fig. 4.

The finding of relatively high yields of trypsin-activable infectious virions in PR8- or WS-infected mouse brain may explain results reported by Cairns (8, 9) to the effect that these strains, after i.c. inoculation of "small" doses, undergo single-cycle increases in EID<sub>50</sub> paralleling those of HA titers, even without trypsin treatment. In retrospect, this finding may have been caused by the use of 10% horse serum in a diluent for titrations in eggs. The presence of serum containing plasminogen would permit activation of plasmin
by allantoic cell enzymes, leading to cleavage of HA (31); conversion of uncleaved progeny particles to their infectious form would have occurred in the eggs, not in mouse brain. Moreover, no rise of infectious titer was demonstrable in Lee-infected brains: the NH2-terminal sequence of the HA2 subunit of this strain differs in two amino acids from that of type A strains (28, 49). Another type B strain (B/1760) has been shown to be resistant to activation by plasmin (30), and it seems likely that this property may be shared by the Lee strain.

We have failed to visualize immunoreactive M1 protein in sections or in Western blots of lyastes prepared from PR8-infected brains that do react with anti-NP antibody. In contrast, concentrated HAds particles extracted from brains infected with PR8, WS, or WSN seem to react in Western blots with antibodies to M1 and NP at comparable ratios of peroxidase-antiperoxidase staining intensity (Table 3 and Fig. 4). We assume that the M1 in such preparations is contributed, at least in part, by the minority subsets that can be made infectious by exposure to trypsin under ++ or + conditions (Table 2). In contrast to M1, NP appears to be present in great excess in immunostained sections as well as in lysates of whole PR8-infected brains (Fig. 2 and 3). Intranuclear accumulation of NP is characteristic of PR8-infected mature astrocytes in mouse embryo brain cell cultures (5, 6).

It is the circumstantial evidence discussed above that leads to the assumption that most of the HAds material recovered from brain tissue consists of cytoplasmic membrane fragments modified by insertion of viral glycoproteins. Such viromicsomes (44) should be deficient in M1 protein and NP but would contain ribosomes and enzymes associated with the microsomal fraction of normal cells, e.g., glucose-6-phosphatase (44). This interpretation would be in agreement with the predominant morphological features of the HAds particles described earlier (62).

The experiments described above have not permitted selective identification of those types of brain cells that support replication of PR8 or WS to the endpoint of completely assembled virions. Although some of the latter are bound to be genetically defective (21), those that are converted to plaque formers by trypsin treatment clearly are not, by definition, defective-interfering particles. We propose that there may be a direct relationship between the amount of M1 protein available and the efficiency with which such progeny virions are produced. These in vivo experiments do not permit, however, separation of released virus from cell-associated viral material, strict quantitation by radiochemical analyses of strain-specific differences, assignment of proper roles to cell-specific factors, correlation of synthesis or steady-state amounts of M1 with those of M2 protein, or assessment of possible consequences resulting from the absence of WSN gene segment 8; the presence of this gene segment has also been reported as essential for full expression of neurovirulence (54). All of these subjects will be dealt with in forthcoming publications describing studies in mouse embryo brain cell cultures at different stages of differentiation (5, 6; G. L. Bradshaw, C. D. Schwartz, P. J. Husak, and R. W. Schlesinger, submitted for publication), in cerebral capillary endothelial cells, and in other cell types suitable as controls. Ultimately, we hope to understand implications of the findings in these systems for possible neuropathology in the intact natural host.

LITERATURE CITED


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